Mechanism of Calf Thymus DNA Primase: Slow Initiation, Rapid Polymerization, and Intelligent Termination†

Robert J. Sheaff and Robert D. Kuchta*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received August 20, 1992; Revised Manuscript Received January 11, 1993

ABSTRACT: The mechanism by which calf thymus DNA primase synthesizes RNA primers was examined. Primase first binds a single-stranded DNA template ($K_D \ll 100$ nM) and can then slide along the DNA in order to find a start site for initiating primer synthesis. NTP binding appears ordered, such that the NTP which eventually becomes the second nucleotide of the primer binds the E·DNA complex first. The NTP that becomes the second nucleotide of the primer thereby influences where primase initiates. Primer synthesis is remarkably slow (0.0027 s^{-1} at 20 μM NTP). The rate-limiting step is after formation of the E·DNA·NTP·NTP complex and before or during dinucleotide synthesis. After synthesis of the dinucleotide, additional NTPs are rapidly polymerized. Primase products are 2–10 nucleotides long. If the enzyme fails to synthesize a primer at least 7 nucleotides long, it reinitiates rather than dissociating from the template. Once a primer at least 7 nucleotides long has been generated, however, subsequent primase activity is inhibited. This inhibition is due to the generation of a stable primer·template complex, which likely remains associated with pol α·primase. The role of primase is to synthesize primers that pol α can elongate. The ability of primase to distinguish between primers at least 7 nucleotides long and shorter products therefore likely reflects the fact that pol α only utilizes primers at least 7 nucleotides long.

DNA polymerase α·primase (pol α·primase)[1] is an essential component of the chromosomal DNA replication machinery (Huberman, 1981, Lehman & Kaguni, 1989). It contains four subunits of ca. 180, 70, 60, and 50 kDa in a tightly bound complex (Tseng & Ahlem, 1983; Kaguni et al., 1983; Grosse & Nasheuer, 1988). Polymerase activity resides in the 180-kDa subunit, while primase activity requires both the 60- and 50-kDa polypeptides. The function of the 70-kDa subunit is currently unknown. In the presence of single-stranded DNA, primase synthesizes RNA primers which are then elongated by the polymerase, thus making the enzyme complex a prime candidate for performing lagging-strand synthesis of Okazaki fragments (Fry & Loeb, 1986).

Primase is a rather slow enzyme, with the rate of NTP polymerization by primase typically 2 orders of magnitude less than the rate of dNTP polymerization by pol α (Grosse & Krauss, 1988). Primers are generally 2–10 nucleotides long, but only those primers 7–10 nucleotides long (unit-length primers) are elongated by pol α (Chang et al., 1984; Kuchta et al., 1990; Podust et al., 1991). Products 2–6 nucleotides long are not substrates for polymerase and hence are termed abortive primers. On poly(dT), the majority of ATP is incorporated into the abortive 2mer, although if polymerization proceeds beyond the 2- and 3mer, most of the products become unit-length primers. The size distribution of products does not change over time, indicating that primase incorporates multiple NTPs per template binding event (i.e., primase is a processive enzyme) (Kuchta et al., 1990).

The kinetic pathway leading to product formation for primase must be quite different from that of pol α. All known DNA polymerases require an oligonucleotide primer in order to polymerize dNTPs (Kornberg, 1992). After binding a primer·template, polymerization by pol α simply requires that the next correct dNTP bind the pol α·DNA complex and be incorporated into the growing strand. In contrast, primase synthesizes RNA primers de novo and hence binds single-stranded DNA. Potentially any location along the template is a possible start site for primer initiation. It is unclear how primase chooses a primer synthesis site, although previous work has shown that primase prefers to utilize a purine as the 5′-terminal NTP of the primer (Tseng & Ahlem, 1984; Yamaguchi et al., 1985; Grosse & Krauss, 1988; Davey & Faust, 1990).

In order to initiate the template-directed synthesis of an RNA primer, primase must bind two NTPs in addition to the template, thereby forming a primase·ssDNA·NTP·NTP quaternary complex. This complex can then undergo catalysis to generate the dinucleotide. Further NTP binding events, followed by phosphodiester bond formation, ultimately result in a unit-length primer. Primase must contend with a number of difficulties in order to efficiently provide substrates for pol α: (i) The enzyme must stabilize the short, initial products on the template in order to allow subsequent rounds of NTP incorporation rather than dissociation of abortive products, (ii) once a primer has been initiated, primase must know when to terminate synthesis, and (iii) the 3′ OH of the primer must be relocated into the pol α active site for further elongation by pol α. Earlier work has suggested that completion of a unit-length primer acts as a signal for the activity switch from primase to pol α (Kuchta et al., 1990). Thus it seems likely that primase "knows" when a unit-length primer has been synthesized.

Using pre-steady-state and steady-state techniques, we have examined the mechanism whereby primase synthesizes RNA primers. A central feature of our approach has been the use of mixed-base templates of defined sequence. Some of the critical steps in the initiation, polymerization, and termination of primer synthesis have been identified.

† This work was supported by grants from the Council for Tobacco Research (2612) and the National Science Foundation (DMB-9105808). R.D.K. was partially supported by a Junior Faculty Research Award from the American Cancer Society (337).

* Address correspondence to this author.

[1] Abbreviations: pol α·primase, DNA polymerase α·primase; EDTA, ethylenediaminetetraacetic acid, sodium salt; Tris, tris(hydroxymethyl)-aminomethane hydrochloride: ssDNA, single-stranded DNA.

0006-2960/93/0432-3027$04.00/0

Table I: Templates

$d(TC)_{30}$	5′-TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$d(TCC)_{20}$	5′-TCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCC
$d(ACT)_{20}$	5′-ACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACT
DNA_G	TCC ATA TCA CAT-3′
	AGG TAT AGT GTA GAT CTT ATC ATC T

EXPERIMENTAL PROCEDURES

Materials

Unless noted, all materials and methods were as described previously (Kuchta et al., 1990). Polymerase α·primase was purified from calf thymus with the use of immunoaffinity chromatography (Kuchta et al., 1990). The synthetic oligonucleotides $d(TC)_{30}$, $d(TCC)_{20}$, $d(ACT)_{20}$, and DNA_G were all from Oligos ETC. Inc. Synthetic oligonucleotides of defined sequence are shown in Table I. Poly(dT), poly(dC), poly(dTdC), oligo$(dT)_{25-30}$, and calf thymus DNA were from Sigma or U.S. Biochemical Corp. The average strand length of poly(dT) and poly(dTdC) is 2000–5000 nucleotides. In all cases, the concentration of substrate DNAs was determined by spectrophotometry and is expressed in terms of total nucleotide. Activated calf thymus DNA was prepared as described previously (Baril et al., 1977). DNAse I came from U.S. Biochemical Corp. and was stored according to Maniatis et al. (1982). Cytidine-containing templates used as primase substrates were first heated to 80 °C and immediately transferred to ice. We found that one such treatment gave good primase activity in subsequent assays.

Methods

Trapping Assay on Poly(dT) and Poly(dTdC). All trapping experiments were performed at 25 °C. The initial mixture (2–5 μL) contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1–5 μM template (total nucleotide), and 2–10 nM primase. This mixture was incubated 2–20 min and then diluted into the trap solution (5–10 μL) containing 50 mM Tris, 5 mM $MgCl_2$, activated calf thymus DNA (1 mg mL^{-1}), and the required [α-^{32}P]NTPs (specific activity ca. 5×10^4 to 1×10^5 cpm $pmol^{-1}$). Reactions were quenched at various times by the addition of 2.5 volumes of gel loading buffer (90% formamide). Products were analyzed by gel electrophoresis (18% acrylamide, 8 M urea) followed by phosphorimagery (Molecular Dynamics) as described previously (Sheaff et al., 1991). The background due to trap failure as well as any impurities in the radiolabeled nucleotide were accounted for simultaneously, as follows. Enzyme was added to the trap solution containing calf thymus DNA, template, and [α-^{32}P]NTP and quenched as usual. Any product generated was then subtracted from the identical time points in the burst experiment. The amount of product synthesized was <1% of the product synthesized in the absence of the trap, indicating that the calf thymus DNA prevented primase from binding the template.

The product of primase activity, an RNA primer, serves as a substrate for the polymerase component of the enzyme complex. We considered the possibility that pol α·primase could bind this RNA primer·template in solution and then transfer it to the primase site, thereby allowing subsequent rounds of primer synthesis. To rule out this mechanism, we repeated the above control with RNA primers bound to the poly(dT). No product was observed, indicating that the calf thymus DNA trap also prevented any product formation through this pathway.

The relative molar amount of the various products was determined by correcting for the number of NMPs incorporated in each length primer. An aliquot of the reaction was analyzed by phosphorimagery in order to determine the specific activity of the reaction (phosphorimager units/pmol of NTP).

The amount of the various length products synthesized in the reaction was at least 2-fold above background and in general 5–10-fold higher. In some cases the radiolabeled nucleotides were too impure for quantitation of the primers 2–4 nucleotides long. This varied significantly between lots.

Data Simulation. Pre-steady-state rates were determined by computer simulation using the program Simul (Barshop et al., 1983) that was modified to allow input of data as x, y pairs (Anderson et al., 1988).

Measurement of Primase·DNA Dissociation Rates. The k_{off} for both poly(dT) and poly(dTdC) was determined using the trapping assay. The initial mixture contained 5 μM template and ca. 6 nM primase. The mixture was incubated 5 min at 25 °C and then diluted into a solution containing 1 mg mL^{-1} calf thymus DNA. Aliquots were withdrawn at 1 min intervals and diluted into an equal volume of 25 μM [α-^{32}P]NTP. The reaction was allowed to proceed to completion (20 min), after which the samples were quenched in 2.5 volumes of gel loading buffer. Data were plotted as log (primers synthesized) versus time (interval between dilution into calf thymus DNA and addition of [α-^{32}P]ATP).

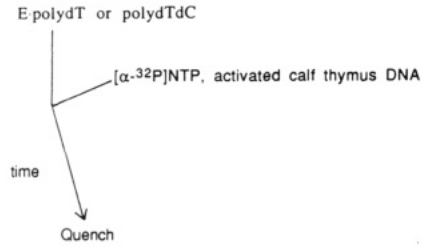
Primase Movement along Poly(dTdC). The trapping assay was performed as above, but with either 10 μM [α-^{32}P]GTP or 10 μM [α-^{32}P]GTP and 10 μM [α-^{32}P]ATP in the trap solution. In both reactions the specific activities were the same so that the amount of products synthesized was directly comparable.

Primase Activity on Poly(dT) and Poly(dTdC) in the Absence of Trap DNA. Reactions (5–15 μL) contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.1–50 μM template, 0.5–5 nM pol α·primase, 0.1 mg mL^{-1} bovine serum albumin, and [α-^{32}P]NTPs as required. Reactions were initiated by the addition of enzyme. Assays were performed at 15, 25, or 37 °C and quenched with either 1.5 volumes of 50 mM EDTA [for DE81 filter binding assays (Sheaff et al., 1991)] or 2.5 volumes of gel loading buffer (for polyacrylamide gel electrophoresis). Background values were determined with identical reactions in the absence of enzyme.

Primase Activity on Short Synthetic Templates. Reactions (5–15 μM) contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10–100 μM template DNA, 2–10 nM pol α·primase, and [α-^{32}P]NTPs, as required. Reactions were initiated by the addition of enzyme and incubated at 37 °C. Unless stated otherwise, reactions were carried out in the absence of trap DNA. Aliquots were withdrawn at various times and quenched with 2.5 volumes of gel loading buffer. Background values were determined as above.

Identity of the 5′-Terminal Nucleotide of Primers. Reactions were performed at 37 °C in the absence of trap DNA as described above for the short synthetic templates. On $d(TC)_{30}$ and $d(TCC)_{20}$, the amount of product containing a particular 5′-terminal NTP was determined in assays containing either [γ-^{32}P]ATP or [γ-^{32}P]GTP at 25 μM. The concentration of unlabeled GTP and ATP, respectively, was

Scheme I: Primase Trapping Assay



varied from 50 to 5000 μM. Products were analyzed by gel electrophoresis and phosphorimagery, and the amount of [^{32}P] incorporated into each length product measured. The total amount of product synthesized was determined the same way except [α-^{32}P]NTPs were utilized. After 30 min the reactions were quenched by the addition of 2.5 volumes of gel loading buffer. All product lengths (2–10 nucleotides) were quantitated in order to calculate the fraction of total products containing a given nucleotide at the 5′-terminal position. The background was determined as above. The $MgCl_2$ concentration was always maintained at least 2-fold above the NTP concentration. Experiments were performed in an analogous manner when $d(ACT)_{20}$ was used. However, in those experiments where only two different NTPs were included in the reaction, the only product synthesized (and hence quantitated) was the dinucleotide.

Primase Concentration. The primase concentration was estimated by determining the amount of active polymerase in a trapping assay. Pol α·primase was incubated with 5 μM DNA_G labeled with [^{32}P] on the 5′ end of the primer strand. The pol α·DNA complex was then diluted into a trap solution containing 1 mg mL^{-1} activated calf thymus DNA and dCTP, thus allowing elongation of DNA_G by a single nucleotide. Control experiments showed that the calf thymus DNA prevented binding of any free DNA_G to pol α. Varying the dCTP concentration from 10 to 50 μM did not change the magnitude of the burst, indicating that dCTP was saturating. The burst size was measured as the DNA_G concentration varied from 0.5 to 5 μM at 20 μM dCTP. Then, the burst was extrapolated to infinite DNA_G to determine the amount of active pol α. Assuming that polymerization is faster than DNA dissociation, the burst size at saturating DNA and dNTP concentrations gives the E·DNA concentration (Kuchta et al., 1987). Since pol α is a mildly processive enzyme, polymerization must be faster than DNA dissociation (Hohn & Grosse, 1986; Sheaff et al., 1991).

RESULTS AND DISCUSSION

Initiation

Primase Binds DNA Before NTPs. Work on DNA polymerases has shown that enzyme typically binds the DNA primer·template before nucleotides (Fisher & Korn, 1981; Kuchta et al., 1987). We investigated whether primase[2] binds template first using the trapping assay depicted in Scheme I. An E·poly(dT) complex is diluted into a solution containing 25 μM [α-^{32}P]ATP and 1 mg mL^{-1} activated calf thymus DNA. Upon dilution the enzyme can either dissociate from poly(dT) and bind the calf thymus DNA or bind ATP and synthesize primers. Diluting a primase·poly(dT) complex into the trap solution resulted in a burst of primer synthesis that

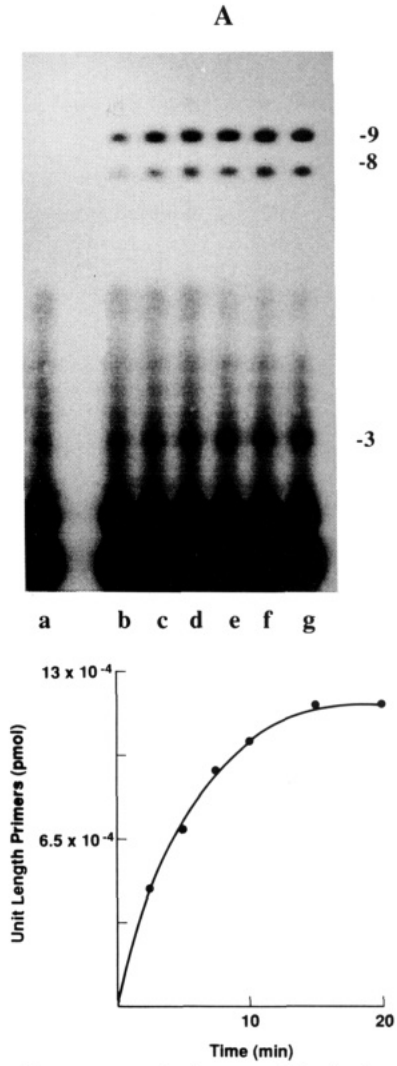


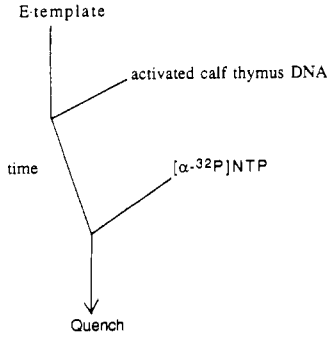
FIGURE 1: Time course of primase activity in the trapping assay. Trapping assays were performed as described under Experimental Procedures. Panel A shows the burst of primase activity after dilution of primase·poly(dT) into calf thymus DNA plus 25 μM [α-^{32}P]ATP. Lane a shows the background after 20 min, which was measured as described in under Experimental Procedures. Lanes b–g are products after 2.5, 5, 7.5, 10, 15, and 20 min, respectively. The product lengths are as noted. Panel B shows the quantitation of the unit-length primers, with the burst size = 11 × 10^{-4} pmol. For comparison, in the absence of trap DNA the amount of unit-length product after 10 min = 18 × 10^{-4} pmol and after 20 min = 34.2 × 10^{-4} pmol.

was complete in 15 min (Figure 1). In the absence of calf thymus DNA, primer synthesis was linear over 60 min (see Figure 7B).

The above data indicate that primase can bind DNA prior to nucleotides. We investigated whether free primase could bind NTPs using two different methods. First, various concentrations of GTP were included when the initial primase·poly(dT) complex was formed. If the E·GTP complex forms, it requires a template containing cytidines for product formation. Any E·GTP complex formed should therefore decrease the amount of poly(dT) bound; hence the size of the burst should decrease as well. Including up to 2 mM GTP had no effect on the rate of primer synthesis or the size of the burst (data not shown). Second, we preincubated primase with 10 μM [α-^{32}P]ATP (1 × 10^5 cpm pmol^{-1}) and initiated the reaction by the addition of 5 μM poly(dT) plus 5 mM unlabeled ATP. We observed no [^{32}P]-labeled products, consistent with either DNA binding before NTPs or rapid NTP dissociation before polymerization (data not shown).

[2] We shall use "primase" or "enzyme" to denote the primase component of the polymerase α·primase complex.

Scheme II: Trapping Assay for k_{off} Determination



Primase Binds DNA Tightly. We attempted to determine the K_D for poly(dT) using the trapping assay. The E·poly(dT) complex was preformed at various poly(dT) concentrations and the magnitude of the primase burst measured. However, the burst size remained constant over a range of 100–1000 nM poly(dT), indicating $K_D \ll 100$ nM.[3] A low K_D predicts that the dissociation rate (k_{off}) for poly(dT) from primase should be very slow. We determined k_{off} for E·poly(dT) using the methodology depicted in Scheme II. The E·poly(dT) complex was diluted into a solution containing calf thymus DNA. At various times thereafter, aliquots were withdrawn and added to 25 μM [α-^{32}P]ATP so that any remaining E·poly(dT) could form primers. The amount of primer formed reflects the amount of E·poly(dT) remaining; hence k_{off} can be calculated. With poly(dT), $k_{off} = 0.006$ s^{-1} (data not shown). Assuming a lower limit for DNA binding to be 1×10^6 M^{-1} s^{-1}, $K_D \leq 6$ nM. [By comparison, the rate of DNA binding to Klenow fragment = 1.2×10^7 M^{-1} s^{-1} (Kuchta et al., 1987).]

Using the trapping assay, we investigated the template length required for tight binding by forming the initial primase·poly(dT) complex in the presence of various inhibitor DNAs. This mixture was incubated for 15 min to ensure that equilibrium was achieved. If the inhibitors and poly(dT) bind equally well to primase, then at equal concentrations of poly(dT) and inhibitor DNA the burst size should decrease by half. Poly(dC) and poly(dTdC) are both similar in length to poly(dT) and gave 47% and 52% inhibition at equimolar concentrations, respectively. Furthermore, we determined $k_{off} = 0.005$ s^{-1} for primase·poly(dTdC), similar to k_{off} for poly(dT). However, two 60-nucleotide-long templates [d(TCC)$_{20}$ and d(ACT)$_{20}$] had no effect on the size of the primase burst, even with a 50-fold excess (data not shown). [As will be described later, both are good substrates for primase.] Thus, primase appears to bind longer templates much more tightly than short ones.

We examined the Mg^{2+} requirement for DNA binding by omitting the Mg^{2+} and adding 1 mM EDTA during formation of the initial primase·poly(dT) complex. Neither the rate of primer synthesis nor the magnitude of the burst changed upon dilution into the trap solution (5 mM $MgCl_2$), indicating that Mg^{2+} is not required for binding of template.

Primase Can Slide Along ssDNA. We used poly(dTdC) to determine if primase can slide along the template to find a start site. Poly(dTdC) is a long (2500–5000 nucleotides), random polymer of thymidines and cytidines generated by terminal transferase. Primase synthesized products on poly(dTdC) in the trapping experiments when either 25 μM [α-^{32}P]ATP or 25 μM [α-^{32}P]GTP alone was included in the assay, showing that the primase·DNA complex binds at both thymidine and cytidine tracts of the template. Notably, only abortive (2–6 nucleotides long) products were synthesized with just ATP present, while with GTP alone, the majority of products were unit-length primers (7–10 nucleotides long; data not shown). We therefore compared the amount of unit-length product synthesized with GTP alone to the amount synthesized when both GTP and ATP were included in the reaction. If primase cannot slide along the DNA, then with GTP alone only those enzyme molecules bound at cytidine tracts can generate unit-length primers. With GTP and ATP present, however, more product should be generated since all bound enzyme molecules can now synthesize unit-length primers.[4] If primase can slide along the DNA to find a cytidine tract, the total amount of unit-length primers generated should be the same in both cases. When ATP and GTP were present, 43% of the primers contained an ATP at the 5′ terminus (see below). The amount of unit-length primers and their rate of formation were similar with ATP plus GTP (2.7×10^{-4} pmol of product, 3.7×10^{-3} s^{-1}) or with GTP alone (2.3×10^{-4} pmol of product, 4.1×10^{-3} s^{-1}), consistent with enzyme sliding along the DNA to find a start site.

Table II: Identity of the 5′-Terminal NTP of Primers[a]

	% of primers with indicated NTP at 5′ terminus		
template	ATP	GTP	UTP
poly(dTdC)	43	57	
d(TCC)$_{20}$	54	46	
d(TC)$_{30}$	>99	<1	
d(ACT)$_{20}$	>99	<1	<1

[a] The NTPs required for primer synthesis were present at equimolar concentrations (25 μM). The percentage of primers containing ATP, GTP, or UTP at the 5′ terminus was determined as described under Experimental Procedures.

Start Site Selection. We utilized defined templates of mixed-base composition to determine if primase prefered certain nucleotides when initiating primer synthesis. Primase initiates RNA synthesis de novo, which requires the formation of an E·DNA·NTP·NTP quaternary complex. Importantly, the enzyme could therefore prefer a particular NTP at either of the two NTP binding sites. [γ-^{32}P]NTPs were used to identify the 5′-terminal nucleotide of a primer, since only this nucleotide retains its triphosphate group in the product. Table II shows the percentage of total products containing ATP, GTP, or UTP as the 5′-terminal NTP on different templates at equimolar NTP concentrations. On d(TCC)$_{20}$ and the mixed-pyrimidine template poly(dTdC), primase utilized both ATP and GTP as the 5′-terminal NTP. However, primase behaved quite differently on the templates d(TC)$_{30}$ and d(ACT)$_{20}$, where >99% of the products contained ATP as the 5′-terminal nucleotide.

One unifying explanation for these disparate results is that primase does not significantly discriminate between ATP and GTP as the 5′-terminal NTP, but instead has a preference for guanosine as the second nucleotide of the primer. On d(TC)$_{30}$ and d(ACT)$_{20}$, ATP is required as the 5′-terminal nucleotide if guanosine is to become the second nucleotide of the primer. Thus >99% of the products synthesized on these templates

[3] At poly(dT) concentrations below 100 nM, it was not possible to maintain a constant DNA:primase ratio and still observe product in the trapping experiments. As will be described later, at high enzyme:poly(dT) ratios the size distribution of products changes. Thus, at poly(dT) concentrations less than 100 nM the amount of product synthesized cannot be accurately quantified due to the small amount of enzyme that must be used.

[4] Importantly, when both ATP and GTP were present, the amount of abortive primer decreased, and adenosine was now incorporated in unit-length primers. Thus, primase molecules bound at thymidine tracts are capable of synthesizing unit-length primers.

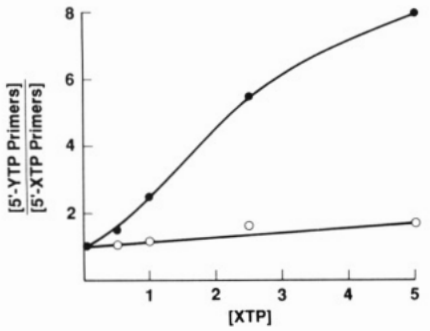


FIGURE 2: Effect of increasing NTP concentration on the identity of the 5′-terminal NTP. Reactions contained 50 μM d(TCC)$_{20}$, and the amount of primers containing a 5′-terminal ATP or GTP was determined. [γ-^{32}P]YTP was used to determine the amount of primer containing a 5′-terminal YTP. The total amount of primer was determined using [α-^{32}P]YTP. The concentration of one NTP (millimolar XTP) was increased while the concentration of the other NTP (YTP) was held constant at 25 μM. The ratio of primers containing a 5′-terminal YTP versus a 5′-terminal XTP was determined as described under Experimental Procedures. (○) XTP was GTP and YTP was ATP. (●) XTP was ATP and YTP was GTP.

contain ATP as the 5′-terminal NTP. In contrast, on d(TCC)$_{20}$ primase can utilize either ATP or GTP as the 5′-terminal NTP and still ensure that GTP becomes the second nucleotide of the primer. Consistent with the model, ATP and GTP were found with equal frequency as the 5′-terminal NTP of the products synthesized on this template. Although the identity of the second nucleotide of the primer synthesized on poly(dTdC) is unknown, the fact that ATP and GTP are both utilized as the 5′-terminal nucleotide is consistent with this hypothesis.

Order of Nucleotide Binding. We next examined the apparent order of NTP binding to the E·DNA complex. Data discussed earlier showed that primase does not remain fixed upon binding DNA but instead can slide along the template to find a start site for primer initiation. Therefore, varying the ratio of required nucleotides using the mixed-base templates should influence the identity of the 5′-terminal nucleotide. Surprisingly, with d(TC)$_{30}$, increasing the *ATP* concentration increased the frequency of *GTP* as the 5′-terminal nucleotide of the primers relative to ATP as the 5′-terminal nucleotide.[5] At 25 μM ATP and GTP, <1% of the total products contained GTP as the 5′-terminal NTP. When the concentration of ATP was increased to 6 mM, however, 6% of the products contained GTP as the 5′-terminal NTP. This result suggested that the first NTP to bind the E·DNA complex becomes the second nucleotide of the primer.

To test this model in greater detail, we utilized d(TCC)$_{20}$. The template sequence is designed so that if the second nucleotide of a primer is adenosine, the 5′-terminal nucleotide must be GTP. If the second nucleotide of a primer is guanosine, however, the 5′-terminal nucleotide can be either ATP or GTP. A high ATP:GTP ratio should result in ATP binding the E·DNA complex first; hence a greater fraction of the primers should contain a 5′-terminal GTP. This indeed is what occurs (Figure 2, closed circles). Conversely, a high GTP:ATP ratio should result in GTP binding the E·DNA complex first. Since the initial E·DNA·GTP complex can form at either template cytosine, the 5′-terminal NTP of the products can be either ATP or GTP. Thus as the GTP:ATP ratio increases, the ratio of primers containing a 5′-terminal ATP versus a 5′-terminal GTP should remain constant (Figure 2, open circles). In measuring the total amount of product at high GTP concentration, we monitored [α-^{32}P]ATP polymerization. However, this does not allow us to measure GG dinucleotide synthesis. If the amount of GG dinucleotide changes dramatically with increasing GTP concentration, the ratio of primers containing a 5′-terminal ATP versus a 5′-terminal GTP may also change and yet not be detected.

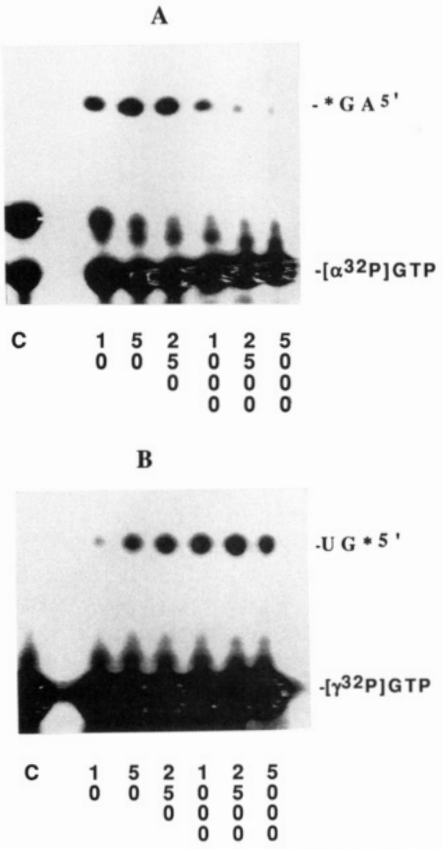


FIGURE 3: The NTP that becomes the second nucleotide of the primer binds the primase·DNA complex first. Reactions contained 50 μM d(ACT)$_{20}$, 10 μM [^{32}P]NTP, and the indicated concentrations (micromolar) of the unlabeled NTP. The products were then separated by gel electrophoresis. The reaction shown in lane C lacked enzyme. Panel A shows the effect of increasing the ATP concentration at 10 μM [α-^{32}P]GTP on the amount of *GA-$^{5'}$. The product running directly above [α-^{32}P]GTP in the control lane (c) and which disappears as a function of increasing ATP concentration was particular to this lot of [α-^{32}P]GTP. Panel B shows the effect of increasing the UTP concentration at 10 μM [γ-^{32}P]GTP on the amount of UG*-$^{5'}$.

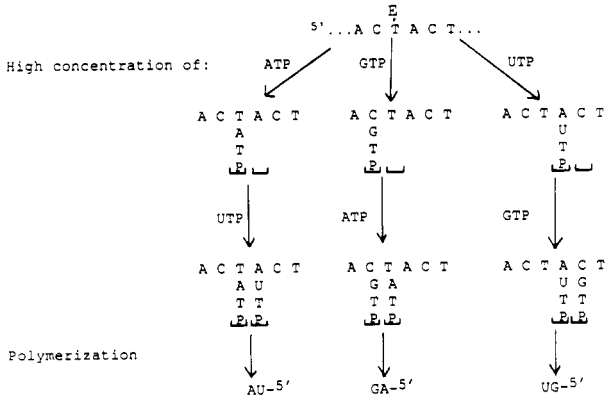
Therefore, to further distinguish between random and ordering binding of NTPs to the E·DNA complex, we assayed primase activity on d(ACT)$_{20}$. This template is designed so that when two NTPs are included in the reaction, only one type of dinucleotide can be synthesized. When ATP and GTP alone are present, the product is the GA-$^{5'}$ dinucleotide, where ATP is always the 5′-terminal NTP.[6] Increasing the concentration of GTP from 10 μM to 5 mM in the presence of 10 μM [γ-^{32}P]ATP increased the amount of GA*-$^{5'}$ dinucleotide synthesized by 57% (data not shown). Conversely, increasing the ATP concentration at 10 μM [α-^{32}P]GTP resulted in a slight initial increase in the amount of *GA-$^{5'}$ dinucleotide, followed by strong inhibition (Figure 3A).

These results are consistent with the model in which a high GTP concentration drives primase to form the E·d-

[5] Unfortunately, it was not possible to do the converse experiment because >99% of products contain ATP as the 5′-terminal nucleotide at equimolar concentrations of ATP and GTP.

[6] GA*-$^{5'}$ refers to the GA dinucleotide with ATP at the 5′ terminus. The asterisk denotes which nucleotide was [^{32}P]-labeled.

Scheme III: The First NTP To Bind Primase·DNA Becomes the Second NMP of the Primer



$(ACT)_{20}$·GTP complex, with GTP bound in position to become the second nucleotide of the primer (Scheme III). Thus, ATP is required as the 5′-terminal nucleotide. Since ATP is present in the reaction, the dinucleotide can be generated. In contrast, a high ATP concentration drives primase to form the E·d-$(ACT)_{20}$·ATP ternary complex but with ATP bound in position to become the second nucleotide of the primer. Due to the template sequence, UTP is now required as the 5′-terminal nucleotide. Since UTP is not present in the reaction, the AU-$^{5'}$ product cannot be synthesized and synthesis of GA-$^{5'}$ is inhibited. By comparison, random binding of the NTPs to the E·DNA complex predicts that the ratio of ATP:GTP should not affect the amount of dinucleotide synthesized. Alternatively, if ATP had to bind first (as the 5′-terminal NTP), then increasing the ratio of ATP:GTP should have increased the amount of product.

Earlier results indicated (Table II) that >99% of the primers synthesized on $d(ACT)_{20}$ contained ATP as the 5′-terminal NTP. We therefore attempted to force primase to incorporate GTP as the 5′-terminal nucleotide with high concentrations of UTP. With 10 μM [γ-^{32}P]GTP and high UTP concentrations, the UG*-$^{5'}$ dinucleotide was now synthesized (Figure 3B). This result is consistent with the model in which the high UTP concentration drives primase to form the E·-$(ACT)_{20}$·UTP ternary complex. UTP is bound in position to become the second nucleotide of the primer, which requires that GTP become the 5′-terminal NTP (Scheme III). Conversely, high GTP concentrations (1–5 mM) at constant [α-^{32}P]UTP (10 μM) gave <1% of the *UG-^{5} dinucleotide as was observed in Figure 3B (data not shown).

Primase was much less likely to incorporate UTP as the 5′-terminal NTP with increasing ATP concentrations. At 5 mM ATP, we observed <5% of the AU*-$^{5'}$ dinucleotide relative to the GA*-^{5} product under identical conditions, consistent with earlier results indicating that primase prefers a purine as the 5′-terminal NTP (Tseng & Ahlem, 1984; Yamaguchi et al., 1985; Grosse & Krauss, 1988; Davey & Faust, 1990). In summary, binding of the two NTPs required for initiation appears to be ordered but with the NTP that ultimately becomes the second nucleotide of the primer binding the E·DNA complex first or with much greater affinity.

Polymerization

Rate of Product Formation. We measured the rate of primer synthesis using poly(dT) in the trapping assay. The observed rate constant (k_{obs}) of unit-length primer formation at 20 μM ATP was remarkably slow, 0.0027 s^{-1}. With increasing ATP concentration, k_{obs} increased as well (Table III). Varying the time of the primase·poly(dT) preincubation from 2 to 20 min had no effect on k_{obs}, indicating the rate-determining step was after binding of the first NTP.[7] We considered the possibility that the rate-limiting step occurred after synthesis of the dinucleotide; however, the size distribution of products did not change during the time course of the burst (data not shown). If the slow step were after generation of the 2mer, there should have been a burst of dinucleotide synthesis followed by its slow conversion to longer products. Therefore, the rate-determining step in the reaction most likely occurs at or before formation of the 2mer.

Table III: Rate of Primer Synthesis on Poly(dT) as a Function of ATP Concentration

ATP (μM)	k_{obs}[a] (s^{-1})	ATP (μM)	k_{obs}[a] (s^{-1})
10	0.0027	50	0.0049
20	0.0027	70	0.0075
30	0.0040		

[a] Data were fit to a one-step process (A (k_{obs}) → B) as described under Experimental Procedures. Since the size distribution of products does not change over time, identical k_{obs} values are obtained for each length product. Here the rates are measured on the basis of the amount of 9mer synthesized.

As will be described in greater detail later, the burst of unit-length primers likely reflects a single round of unit-length primer synthesis per template binding event. An alternative possibility, however, is that only a small fraction of the total enzyme is active but can synthesize multiple unit-length primers on poly(dT). [In this scenario product formation is fast and k_{obs} likely reflects dissociation from the template.] We used short synthetic templates of defined sequence to rule out this possibility as well as to locate the rate-limiting step of the reaction. They are ideal substrates due to their length (60 nucleotides), which likely permits the synthesis of only one or a few products per template, and their defined sequences, which allow steps after DNA binding to be examined.

We first compared primer synthesis on the mixed-base templates in the absence of trap DNA to that on poly(dT). With 1 μM poly(dT) and 25 μM [α-^{32}P]ATP, product formation was linear for 80 min (see Figure 7B). In contrast, the rate of unit-length primer synthesis on the synthetic DNAs was clearly biphasic, with an initial burst of synthesis followed by a much slower rate of product formation (Figure 4). The data in Figure 4B were accurately fit using rate constants of 1.9×10^{-3} s^{-1} for the rapid phase and 2.2×10^{-4} s^{-1} for the slow phase.

We performed a number of controls to determine if the burst represented a single turnover by primase. Doubling the enzyme concentration doubled the size of the burst (data not shown). However, the burst size remained constant as either the DNA concentration varied (50–1000 μM) or the NTP concentration varied (10–50 μM). The enzyme concentration is small compared to the DNA concentration; hence these data exclude the possibility that primers accumulate free in solution and then compete with either template or NTP for binding to primase. Finally, we observed a 1:1.5 ratio of primase molecules to unit-length primers in the initial burst.

We attempted to employ the short synthetic templates in the trapping assay (i.e., form the E·DNA complex and dilute

[7] If the rate-determining step were after binding the DNA and prior to binding of the first NTP, then increasing the enzyme·DNA preincubation time should have resulted in an increase in the rate of product formation. An alternative possibility we cannot exclude is that the rate-determining step does occur after DNA binding and prior to binding of the first NTP but with an unfavorable K_{eq} for this step.

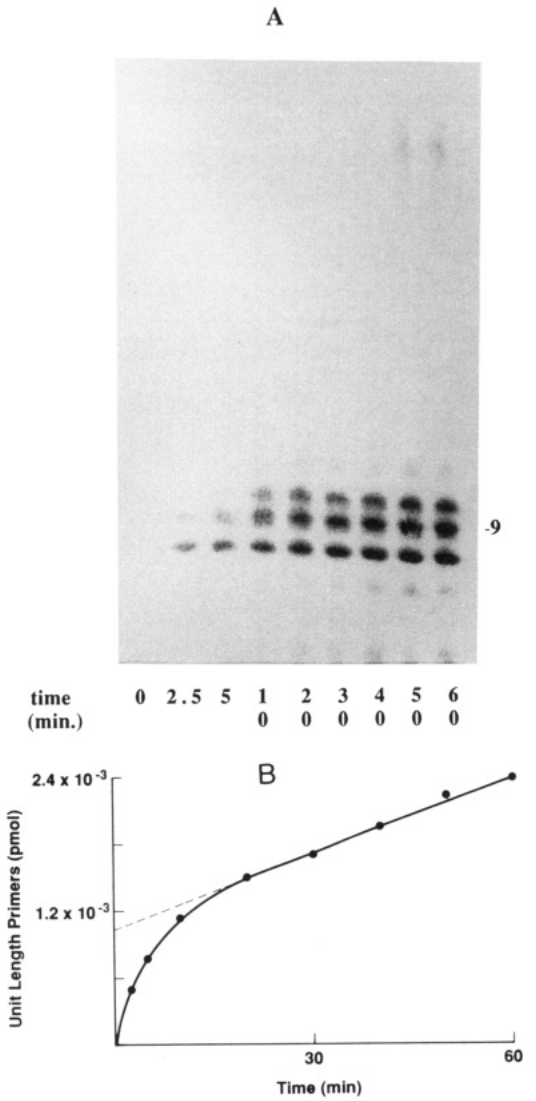


FIGURE 4: Time course of primase activity on $d(TCC)_{20}$. Reactions (37 °C) contained 50 μM $d(TCC)_{20}$, 25 μM [α-^{32}P]GTP, and 25 μM [α-^{32}P]ATP. Panel A shows the products generated during the reaction. Panel B is the quantitation of the unit-length primers. Times are as indicated. The dashed line indicates the size of the burst. Data were simulated using the model E·S (k_1) → E·P (k_2) → E·S + P, where $k_1 = 1.9 \times 10^{-3}$ s^{-1} and $k_2 = 2.2 \times 10^{-4}$ s^{-1}, as described under Experimental Procedures. The solid line is E·P + P.

it into [α-^{32}P]NTPs and 1 mg mL^{-1} activated calf thymus DNA) but observed <1% of the product synthesized in the absence of trap DNA. Experiments described earlier showed that the K_D's for short templates are much higher than for longer DNAs such as poly(dT) or poly(dTdC). Given the slow rate of primer formation, upon dilution into the trap solution primase is much more likely to dissociate from the short template and bind trap DNA rather than synthesize primers. To ensure that the failure to generate product was not due to the template sequence, we attempted the trapping experiment using oligo$(dT)_{25-30}$. No products were observed, consistent with short templates binding much less tightly to primase than longer DNAs.

Rate-Limiting Step. The studies with poly(dT) indicated that the rate-determining step is probably after binding of the first NTP and before or during generation of the dinucleotide. To define its position more precisely, we utilized $d(TC)_{30}$. The template sequence allows formation of a stable E·d-$(TC)_{30}$·GTP ternary complex. To first show that primase actually formed this complex, we examined the ability of GTP and $d(TC)_{30}$ to synergistically inhibit the primase burst on poly(dT) in the trapping assay. While 500 μM GTP or 2 μM $d(TC)_{30}$ alone gave no inhibition of the burst on poly(dT), together they inhibited the burst size 50%, consistent with formation of the E·$d(TC)_{30}$·GTP ternary complex.

We therefore preincubated primase with $d(TC)_{30}$ in the presence of 1 mM ATP or 1 mM GTP to preform the E·DNA·NTP complex. This complex was then diluted (no trap DNA) into a solution containing either 25 μM [α-^{32}P]-GTP or [α-^{32}P]ATP, respectively. In both cases the rate of primer synthesis was identical to the rate when primase was added directly to DNA and NTPs (data not shown). We repeated the experiment using $d(TCC)_{20}$ and ATP and again observed similar rates whether or not we preformed the E·d-$(TCC)_{20}$·ATP complex (data not shown). If the rate-limiting step were after binding of the first NTP but before binding of the second NTP, preforming the ternary complex should have given a much faster rate of synthesis relative to the control. The data therefore indicate that the rate-determining step of the reaction is most likely after formation of the E·DNA·NTP·NTP complex.[8]

Termination

Primase "Knows" When a Unit-Length Primer Has Been Synthesized. Using poly(dT) in the trapping assay, we measured the effect of increasing ATP concentrations on the product distribution. Figure 5A shows a sigmoidal increase in the molar amount of unit-length primers as the ATP concentration increased. However, the effect was quite different when we measured the total moles of product (abortive plus unit length) synthesized. The total amount of product increased until it reached a maximum at 25 μM ATP, after which it decreased with further increases in the ATP concentration (Figure 5B). The decrease in total product at higher ATP concentration was due solely to a decrease in the amount of abortive product being generated. Importantly, this decrease was not compensated for by an analogous increase in the amount of unit-length primers. These data indicate that primase likely synthesizes multiple abortive products per DNA binding event; i.e., primase reinitiates primer synthesis rather than dissociate from the DNA after generating an abortive primer. Once a unit-length primer is generated, however, the likelihood of further reinitiations diminishes. Thus, the decrease in total products at higher ATP concentrations arises from a decrease in the number of attempts required to synthesize a unit-length primer.

We also compared the molar amounts of the various length products synthesized in the trapping assay to the estimated primase concentration. On poly(dT) with 50 μM ATP, the ratio of unit length primers to primase was 2:1. However, the ratio of abortive primers to primase was 35:1, further evidence that primase synthesizes multiple abortive products per DNA binding event but only one or a few unit-length primers.

High ratios of primase:poly(dT) hindered the synthesis of unit-length primers (data not shown). In a trapping experiment with 0.7 μM poly(dT) in the initial mixture and 50 μM [α-^{32}P]ATP in the trap solution, the amount of unit-length primers decreased 2-fold compared to an identical experiment with 5 μM poly(dT). However, the total amount of product increased 3-fold due to the enhanced synthesis of abortive primers. Again, conditions that selectively inhibit unit primer

[8] An alternative possibility we cannot exclude is that the rate-determining step does occur between binding of the first and second NTPs but with an unfavorable K_{eq} for this step.

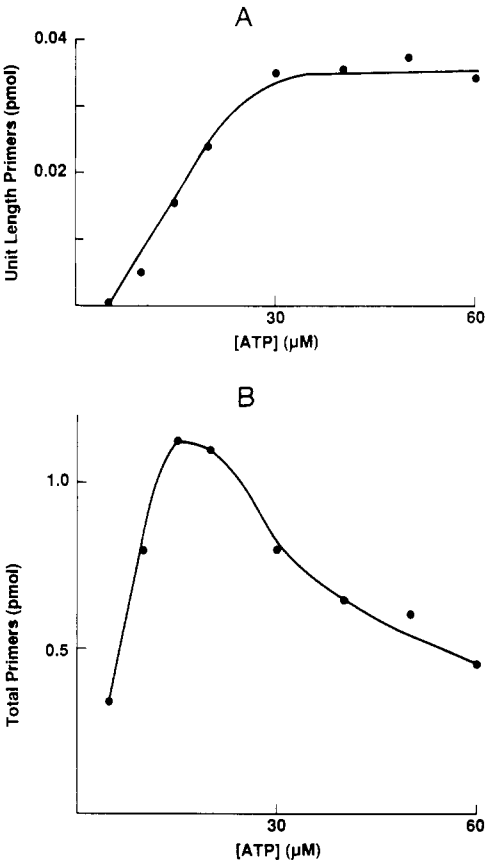


FIGURE 5: Amount of primer synthesized on poly(dT) as a function of ATP concentration. The trapping assay and quantitation of the various length primers was performed as described under Experimental Procedures. Panel A shows the moles of unit-length primers vs ATP concentration; panel B shows the total moles of primer (abortive plus unit length) vs ATP concentration.

synthesis on poly(dT) result in increased synthesis of abortive products.

As a further test of these ideas, we examined the time course of primer synthesis on poly(dTdC) in the trapping experiment. As mentioned earlier, this template supports unit-length primer synthesis with GTP alone, suggesting it contains cytidine tracts at least 7–10 nucleotides long. However, with ATP alone only abortive products are generated. The time course with GTP should therefore be qualitatively similar to that on poly(dT) since unit-length primers are generated in both cases. In contrast, primase generates abortive products with only ATP present; hence the time course should reflect the continual reinitiations which occur in the absence of unit-length primer synthesis. Figure 6A shows that when unit-length primers are synthesized, the reaction is over in less than 15 min (k_{obs} = 0.003 s^{-1}), while the synthesis of abortive products (only ATP present) continues over 50 min (Figure 6B). Consistent with reinitiation after abortive primer synthesis, the abortive products are in large excess over primase.

If the enzyme does not reinitiate after synthesizing a unit-length primer, then the unit-length primer must be viewed differently from the abortive products. An intriguing possibility is that completion of a unit-length primer acts as a termination signal indicating that no further reinitiations are required; i.e., primase has accomplished its goal of providing a substrate for pol α.

The ability of primase to synthesize multiple products per template binding event raises the possibility that primase is in either an active or inactive mode. After an initial slow step to "activate" the enzyme, all subsequent reinitiations are fast.

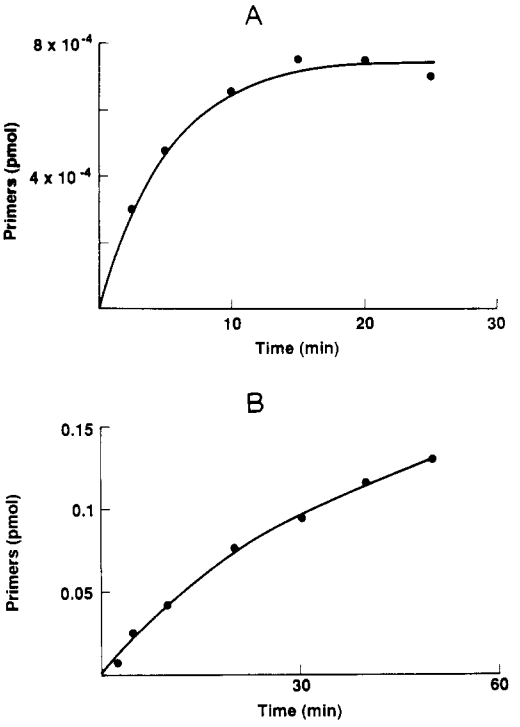


FIGURE 6: Primase burst on poly(dTdC). The trapping assay using 5 μM poly(dTdC) was performed as described under Experimental Procedures, except either 25 μM [α-^{32}P]ATP or [α-^{32}P]GTP was included in the trap solution. Panel A shows the time course of unit-length primer synthesis with only GTP in the reaction. Panel B shows the time course of abortive primer synthesis when only ATP is present.

We therefore attempted to "preactivate" primase before measuring the rate of product formation. Enzyme was incubated with poly(dT) and 5 μM ATP and then diluted into the standard trap solution containing calf thymus DNA and [α-^{32}P]ATP at 25 μM. In the presence of 5 μM ATP, primase is active but mainly synthesizes the dinucleotide. Upon dilution into a higher concentration of ATP, processivity increases and unit-length primers are synthesized. Thus the rate of unit-length primer synthesis should be much faster if the slow step involves enzyme activation. We observed no change in the rate of unit-length primer formation (data not shown).

We also attempted to "activate" primase using d(TCC)$_{20}$ as template. The enzyme was preincubated with 2 μM GTP, which allowed synthesis of mainly the GG dinucleotide. [α-^{32}P]ATP and [α-^{32}P]GTP (20 μM) were then added to the reaction so that unit-length primers could be synthesized. Again, there was no change in the rate of primer formation compared to enzyme not preincubated with GTP, consistent with primase going through the rate-limiting step during each initiation event (data not shown).

A Stable Unit-Length Primer·Template Is Required To Mediate Primase Activity. Using poly(dT) as template in the absence of trap DNA (37 °C), we observed a linear increase in the amount of unit-length primers for 80 min, indicating that primase generates multiple unit-length primers per enzyme molecule. In the absence of trap DNA on the short, cytidine-containing templates, however, primase undergoes an initial burst of unit-length primer synthesis followed by a slower rate of product formation (compare Figures 4B and 7B). The burst corresponds to approximately one unit-length primer per enzyme molecule. We considered two characteristics of the DNA which might be responsible for the different kinetics: template length and template composition. We first examined primase activity on oligo(dT)$_{25-30}$, since

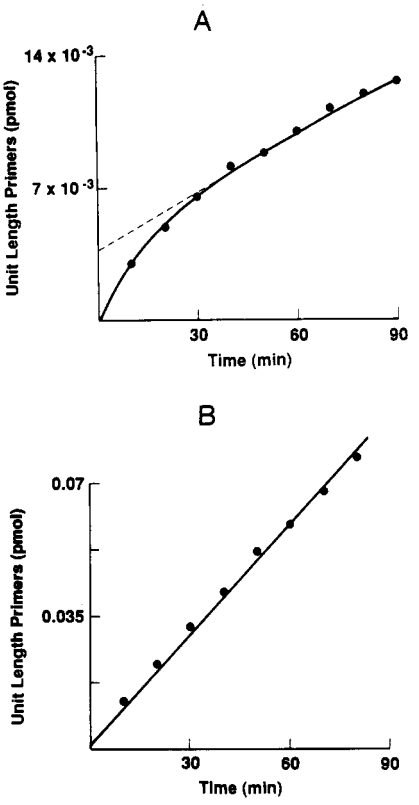


FIGURE 7: Temperature effects on the kinetics of primase activity using poly(dT) as template. Reactions contained 1 μM poly(dT) and 25 μM [α-^{32}P]ATP and were performed as described under Experimental Procedures (no trap DNA). Panels A and B show the time course of unit-length primer synthesis at 15 and 37 °C, respectively. The dashed line in panel A indicates the size of the burst.

Table IV: Primase Processivity as a Function of the Primer·Template Composition, Expressed as the Percentage of the Total NTPs Incorporated Contained in Unit-Length Primers[a]

template	NTP	10 μM	50 μM
poly(dT)	ATP	1	17
d(TC)$_{30}$	ATP,GTP	15	33
poly(dTdC)	GTP	65	72

[a] Reactions (37 °C) contained 50 μM template and the indicated [α-^{32}P]NTP(s) at 10 or 50 μM.

this template is considerably shorter than poly(dT) yet has the same sequence. In the absence of trap DNA the time course of unit-length primer synthesis was linear for 40 min, consistent with multiple turnover of the enzyme. The products were ca. 1 nucleotide shorter than those observed on poly(dT) (data not shown). Nevertheless, simply reducing the template length does not prevent multiple turnover by primase, implicating instead the template composition.

Increasing the G·C character of a primer·template increases the primer·template stability. Table IV shows that as the guanosine content of the primer increases, the percent of dinucleotide being elongated to a unit-length primer also increases (i.e., the processivity increases). This suggests that the additional hydrogen bond in G·C base pairs provides extra stability to the dinucleotide·template complex, thus increasing the probability that the 2mer will be elongated rather than dissociate.

We employed d(ACT)$_{20}$ as the template to determine if incorporation of guanosine into an abortive primer was sufficient to give biphasic kinetics. With 25 μM ATP and 25 μM GTP in the reaction, the only product synthesized was the GA-$^{5'}$ dinucleotide. The rate of dinucleotide formation was linear over the 40-min time course, consistent with multiple turnover of the enzyme (data not shown). When 25 μM UTP was also included so that unit-length primers could be synthesized, we now observed a burst of primase activity corresponding to a single turnover of the enzyme, followed by a slower rate [The time course of unit-length primer synthesis could be described using the model E·S (k_1) $\rightarrow$ E·P (k_2) $\rightarrow$ E·S + P, where $k_1 = 3.5 \times 10^{-3}$ s^{-1} and $k_2 = 3.5 \times 10^{-4}$ s^{-1} (not shown), and was similar to that observed on d(TCC)$_{20}$ in the presence of GTP and ATP (Figure 4B)]. With only ATP and GTP present, the amount of GA-$^{5'}$ dinucleotide synthesized in 30 min was 100-fold greater than the amount of unit-length primer synthesized when ATP, GTP, and UTP were present. Therefore, it is not incorporation of guanosine per se but rather synthesis of a unit-length primer containing guanosine which is responsible for the biphasic kinetics.

Why then does primase undergo multiple turnover on poly(dT) in the absence of trap DNA when unit-length primers are synthesized? The time course at 37 °C was linear for 40 min over a wide range of poly(dT) concentrations. Surprisingly, at 0.1 μM poly(dT) the amount of ATP incorporated into unit-length primers was 6.5-fold greater than the DNA concentration. This suggested that the adenine primers were mainly free in solution or only weakly bound to the poly(dT) (Kuchta et al. 1990). We therefore reduced the temperature of the reaction in order to stabilize the primer·template. Panels A and B of Figure 7 compare the time course of unit-length primer synthesis at 15 and 37 °C, respectively. Reducing the temperature alters the kinetics of unit-length primer synthesis such that they are now biphasic, with an initial burst of activity corresponding to a 2.5:1 ratio of unit-length primers to primase. We repeated the reaction at 15 °C for 60 min to generate the biphasic time course and then raised the temperature to 37 °C. Upon increasing the temperature (and thereby destabilizing the primer·template), a rapid linear rate of primer synthesis ensued (data not shown). These results show that the instability of adenine primers on poly(dT) is responsible for the multiple turnover of primase (and hence the linear time course). When the temperature is reduced so that the unit-length primer·template is stabilized, subsequent primase activity is inhibited.

CONCLUSIONS

We have examined the steps leading to synthesis of a unit-length primer competent for elongation by pol α. Substrate binding appears ordered with DNA adding before either nucleotide. Primase binds the DNA very tightly, with $K_D \ll 100$ nM (total nucleotide). In contrast, $K_D = 500$ nM (3′ termini) for pol α binding to a primed template (Sheaff et al., 1991). The main effect of reducing the template length from >2000 to 60 nucleotides appears to be a substantial decrease in binding affinity for the DNA, suggesting that primase interacts with a large region of the template. Remarkably, the 60mers utilized in these studies correspond to >200 Å in length (Voet & Voet, 1990), yet this was insufficient for tight DNA binding by primase. This raises the interesting possibility that primase may interact with a large region of ssDNA in vivo at the replication fork.

Initiation. After binding the DNA, primase can slide along the template to find an initiation site. Although we cannot determine from our studies how far primase can slide, this characteristic may be important for Okazaki fragment synthesis. After initiating and/or completing synthesis of an Okazaki fragment, pol α·primase may need to move back to

the replication fork in order to initiate another Okazaki fragment. The ability to slide along DNA could be important for this recycling. Additionally, sliding along the DNA could also explain why the K_D's for short templates appear to be much higher than for longer templates such as poly(dT) or poly(dTdC). Primase may be simply sliding off the ends of the short DNAs.

Our data suggest primase prefers that the two nucleotides of the E·DNA·NTP·NTP quaternary complex be purines. On $d(ACT)_{20}$, primase could utilize uridine as the second nucleotide. However, the enzyme was much less likely to use UTP as the 5′-terminal NTP, suggesting the preference for purines is strongest at this position. Surprisingly, the eventual second nucleotide of the primer appears to bind the E·DNA complex first, thus fixing the identity of the 5′-terminal NTP. Primase prefers that the second nucleotide of the primer be GTP, while the 5′-terminal nucleotide can be either ATP or GTP.

A compelling reason for incorporating guanosine during initiation is the inherent difficulty of stabilizing the short products on the template strand. The formation of G·C base pairs during initiation increases the stability of the dinucleotide due to the additional hydrogen bond. This in turn increases the probability of generating a unit-length primer relative to dissociation of the abortive product. We have not investigated whether this preference extends to cytidine at the second position of the primer.

Two pieces of evidence suggest that binding of NTPs stabilizes the E·DNA complex. (i) $d(TC)_{30}$ alone did not inhibit formation of a primase·poly(dT) complex. However, when $d(TC)_{30}$ and GTP were included, formation of the primase·poly(dT) complex was strongly inhibited. (ii) Dissociation of the E·poly(dTdC) complex was essentially complete in 10 min. However, when ATP was included so that only abortive primers could be synthesized, synthesis continued for 50 min (see Figure 6B), suggesting ATP binding and/or primase activity stabilized the E·DNA complex.

Rate-Determining Step. For most polymerases, nucleotide incorporation is fast. Rates of polymerization vary from 50 s^{-1} for Klenow fragment to 1000 s^{-1} for the *Escherichia coli* pol III holoenzyme (Kuchta et al., 1987; Kornberg, 1992). In comparison, k_{obs} for unit-length primer synthesis on poly(dT) at 20 μM ATP = 0.0027 s^{-1}. The rate-determining step for primase likely occurs during the initiation of primer synthesis, after quaternary complex formation and before synthesis of the dinucleotide. It is possible that the first polymerization generating the dinucleotide is slow, after which all subsequent polymerizations are fast. It seems unlikely, however, that phosphodiester bond formation would be this slow, based on rates of catalysis for other polymerases. Additionally, this requires that the first polymerization event be different from all following ones. We prefer the hypothesis that primase undergoes a rate-limiting conformational change upon binding the two NTPs, after which all polymerization events are fast. Importantly, it appears that primase must go through the rate-limiting conformational change each time it synthesizes a dinucleotide.

Unfortunately, the ability of primase to synthesize multiple products per DNA binding event makes interpretation of rates difficult. For instance, the increase in k_{obs} for unit-length primers with increasing ATP concentration (Table III) may simply reflect an increase in the primase processivity. Since the enzyme appears to go through the slow step of the reaction during each reinitiation event, decreasing the number of attempts required to synthesize a unit-length primer will result in a faster rate.

Is the slow rate of primer synthesis observed with purified enzyme sufficient for DNA replication in vivo? In vivo, the replication fork moves at ca. 8–80 nucleotides s^{-1} (Kornberg, 1992). Assuming that leading- and lagging-strand DNA synthesis are coordinated, Okazaki fragment synthesis must also occur at this rate. With 70 μM ATP at 25 °C, the rate of primer synthesis on poly(dT) was 0.0075 s^{-1} (Table III). To compare the rates, we must consider the following: (i) In vivo, the ATP concentration is ca. 4 mM (Hauschka, 1973), and the K_M (ATP) of purified primase is 100 μM (Kuchta et al., 1990). (ii) The rate of primer synthesis at 37 °C is ca. 4-fold faster than at 25 °C [poly(dT) and 50 μM ATP (R. Sheaff and R. Kuchta, unpublished data)]. (iii) Okazaki fragments are ca. 150 nucleotides long (Blumenthal & Clark, 1977, Kornberg, 1992); hence synthesis of one primer allows polymerization of ca. 150 nucleotides. Correcting for these factors, the data with 70 μM ATP indicate that primase can support dNTP polymerization at a rate of 12 dNTPs s^{-1}, consistent with in vivo rates. Additionally, the in vivo rate may be further modified due to the different templates, accessory proteins and/or covalent modification.

Intelligent Termination. The primase synthesized RNA primer is an intermediate in the pathway which ultimately culminates in formation of a completed Okazaki fragment. Since pol α only utilizes primers 7–10 nucleotides long, it would be advantageous if primase recognizes when a unit-length primer rather than an abortive product has been synthesized. Our data indicate that primase readily distinguishes between unit-length and abortive primers. If an abortive primer rather than a unit-length primer is synthesized, primase reinitiates synthesis rather than dissociate from the DNA. Once a unit-length primer has been generated, however, the primer·template acts as a termination signal and inhibits further reinitiations.

Importantly, the data show that synthesis of a unit-length primer per se is insufficient to inhibit multiple turnover by primase. Rather, it appears that the primer must remain bound to the template and that the unit-length primer·template must remain associated with the enzyme. We are currently investigating where the primer·template resides on the pol α·primase complex and how it mediates primase function.

The ability to distinguish between abortive and unit-length primers appears to be a characteristic of eukaryotic primases in general rather than being specific to the calf thymus primase. With single-stranded M13 as a template, Cotterill et al. (1987) also observed a burst of primer synthesis using pol α·primase purified from embryos of *Drosophila melanogaster*.

Distinguishing between Abortive and Unit-Length Primers. How is primase able to distinguish between the various length products it synthesizes? Primase must form a quaternary E·ssDNA·NTP·NTP complex during initiation, so the enzyme must have at least two nucleotide binding sites. An intriguing model is that one site binds the 5′-terminal NTP and retains this contact throughout primer synthesis. The other site binds the next required NTP and performs catalysis. As primer synthesis occurs, the two sites must move apart from each other. The primer length is thus determined by the limits of movement established by fixing one site of primase at the 5′ end of the product.

Some evidence already exists suggesting that primase movement is required for unit-length primer synthesis. At high enzyme:poly(dT) ratios, primase had difficulty synthesizing unit-length primers, while the synthesis of abortive products was enhanced. At high enzyme:poly(dT) ratios the amount of primase may be sufficient to bind most of the available template. Primase can generate the dinucleotide simply upon binding the two initial NTPs, while subsequent polymerization events require realignment of the primase active site at the 3′ end of the growing primer. When most of the template strand is bound by primase, the close proximity of the enzyme molecules may inhibit this movement.

The data discussed herein illustrate the elegant methods employed by primase to ensure synthesis of a unit-length primer. The reason primase mediates its activity once a unit-length primer has been synthesized is revealed in the tight coupling of the primase and pol α subunits in a single enzyme complex. Unit-length RNA primers are transfered from the primase active site to the pol α active site without dissociating from the enzyme complex (manuscript in preparation), thereby ensuring efficient elongation of primase-synthesized primers. Further studies of primase and pol α should reveal how the enzyme complex coordinates its respective activities during lagging-strand synthesis at the replication fork.

ACKNOWLEDGMENT

We thank Drs. Carlos Catalano and Dan Herschlag for many helpful comments during the course of these studies and during preparation of the manuscript.

REFERENCES

Anderson, K. S., Sikorski, J. A., & Johnson, K. A. (1988) *Biochemistry 27*, 7395–7406.

Baril, E., Mitchener, J., Lee, L., & Baril, B. (1977) *Nucleic Acids Res. 4*, 2641–2653.

Barshop, B. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134–145.

Blumenthal, A. B., & Clark, E. J. (1977) *Cell 12*, 183–189.

Chang, L. M., Rafter, E., Augl, C., & Bollum, F. J. (1984) *J. Biol. Chem. 259*, 14679–14687.

Cotterill, S., Chui, G., & Lehman, I. R. (1987) *J. Biol. Chem. 262*, 16105–16108.

Davey, S. K., & Faust, E. A. (1990) *J. Biol. Chem. 265*, 3611–3614.

Fisher, P. A., & Korn, D. (1981) *Biochemistry 20*, 4560–4569.

Foiani, M., Lindner, A. J., Hartmann, G. R., Lucchini, G., & Plevani, P. (1989) *J. Biol. Chem. 264*, 2189–2194.

Fry, M., & Loeb, A. (1986) *in Animal Cell Polymerases*, CRC Press Inc., Boca Raton, FL.

Grosse, F., & Krauss, G. (1988) *J. Biol. Chem. 260*, 1881–1888.

Grosse, F., & Nasheuer, H. P. (1988) *Cancer Cells 6*, 397–402.

Hauschka, P. V. (1973) *Methods Cell. Biol. 7*, 362–462.

Hohn, K., & Grosse, F. (1986) *Biochemistry 26*, 2870–2878.

Huberman, J. (1981) *Cell 23*, 647–648.

Kaguni, L. S., Rossignol, J. M., Conaway, R. C., Banks, G. R., & Lehman, I. R. (1983) *J. Biol. Chem. 258*, 9037–9039.

Kornberg, A. (1992) in *DNA Replication*, W. H. Freeman and Company, New York.

Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 8410–8417.

Kuchta, R. D., Reid, B., & Chang, L. M. (1990) *J. Biol. Chem. 265*, 16158–16165.

Lehman, I. R., & Kaguni, L. S. (1989) *J. Biol. Chem. 264*, 4265–4268.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Podust, V. N., Vladimirova, O. V., & Larvrik, O. I. (1991) *Biochem. Int. 23*, 1195–1204.

Sheaff, R., Ilsley, D., & Kuchta, R. D. (1991) *Biochemistry 30*, 8590–8597.

Tseng, B. Y., & Ahlem, C. N. (1983) *J. Biol. Chem. 258*, 9845–94849.

Tseng, B. Y., & Ahlem, C. N. (1984) *Proc. Acad. Natl. Sci. U.S.A. 81*, 2342–2346.

Voet, C., & Voet, J. G. (1990) in *Biochemistry*, J. Wiley & Sons, New York.

Yamaguchi, M., Hendrickson, E. A., & DePamphilis, M. L. (1985) *J. Biol. Chem. 260*, 6254–6263.