Calf Thymus DNA Polymerase α–Primase: "Communication" and Primer·Template Movement between the Two Active Sites†

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ABSTRACT: The DNA polymerase α–primase complex replicates single-stranded DNA by first synthesizing a short RNA primer (primase) which is then further elongated by the incorporation of dNTPs (DNA polymerase α). While primase and pol α function independently prior to synthesis of an RNA primer, the two activities become coordinated after primer synthesis. After primase generates a primer·template, it moves from the primase active site to the pol α active site for further elongation without dissociating into solution. Intramolecular transfer occurs immediately after primer synthesis and is employed on both long templates such as poly(dT) and short synthetic templates (≤60 nucleotides). Primer·template transfer and elongation by pol α are rapid compared to primer synthesis. After pol α elongates the primer, primase reinitiates primer synthesis, and the cycle is repeated. However, if dNTPs are absent such that primer elongation cannot occur, further primase activity is inhibited after a single round of primer synthesis. This "negative regulation" of primase activity is mediated by the newly generated primer·template provided the following conditions are met: (1) Primase synthesizes the primer; (2) the primer is 7–10 nucleotides long and remains bound to the template; (3) the template is of sufficient length; (4) the primer·template dissociates slowly from the enzyme complex; and (5) the primer·template interacts with the pol α active site. Polymerization of multiple dNTPs by pol α rapidly reactivates primase; hence, negative regulation of primase activity likely ensures a new primer is not synthesized until the previous one has been elongated by pol α.

During eukaryotic DNA replication, the lagging strand is synthesized as short segments (Okazaki fragments) which require frequent initiation (Kornberg & Baker, 1992). Since DNA polymerases cannot initiate synthesis *de novo*, a 3′-OH is initially provided for them by primase, which generates short RNA primers. In eukaryotes, the primase and DNA polymerase α (pol α)[1] activities are tightly associated in a highly conserved four-subunit complex (Tseng & Ahlem, 1983; Kaguni et al., 1983; Hu et al., 1984). It is generally thought that the association of the two catalytic activities facilitates the complex task of replicating the lagging strand, perhaps by coordinating primer synthesis and pol α elongation (Fry & Loeb, 1986). Physical association might also permit "channeling" of the primer·template from the primase active site to the pol α active site for efficient DNA synthesis. However, direct mechanistic evidence supporting these hypotheses is lacking.

The primase and polymerase catalytic activities have been physically separated and are associated with different polypeptides within the complex: primase[2] with the 48-kDa subunit and pol α with the 180-kDa subunit (Holmes et al., 1986; Grosse & Nasheuer, 1988; Santocanale et al., 1993). Thus, the 48- and 180-kDa subunits each contain a functioning active site as well as DNA binding domains that are at least partially independent. The functions of the 58- and 70-kDa subunits are unknown.

On single-stranded DNA *in vitro*, primase synthesizes short oligoribonucleotides that provide the 3′-OH required by pol α. Although primase generates primers 2–10 nucleotides long, primers 7–10 nucleotides long (unit-length primers) are greatly preferred as substrates for purified pol α (Chang et al., 1984; Kuchta et al., 1990; Podust et al., 1991).[3] Furthermore, primers attached to Okazaki fragments *in vivo* are primarily 8–10 nucleotides long (Hay et al., 1984; Kitani et al., 1984; Paff & Fernandes, 1990). However, under some steady-state reaction conditions, pol α appears to elongate primers as short as two nucleotides (Yamaguchi et al., 1985a,b; Vishwanatha et al., 1986; Tseng & Prussak, 1989). This could be due to (i) the 100-fold greater rate of nucleotide polymerization for pol α, such that pol α may eventually use even very short primers if given sufficient time (Chang et al., 1984), (ii) the presence of accessory proteins, (iii) the nucleotide composition of the primer·template, or (iv) the high ratios of dNTPs to NTP used compared to the ratio *in vivo*.

The primase mechanism appears specifically geared toward providing pol α with a unit-length primer. Previously we found that in the absence of dNTPs, generation of a unit-length primer·template inhibits further primase activity (Sheaff & Kuchta, 1993). This "negative regulation" of primase activity requires that the unit-length primer remain stably bound to the template, and occurs both on synthetic templates of defined sequence and on more natural templates such as single-stranded M13 DNA. Conversely, if primase initiates primer synthesis but fails to synthesize a unit-length primer, the enzyme reinitiates rather than dissociating from the template. These results suggest that the physical association of primase and pol α allows mechanistic coordination of the two activities, which may be crucial for replication of the lagging strand.

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[1] Abbreviations: pol α, DNA polymerase α; Tris, tris(hydroxymethyl)-aminomethane, HCl salt; EDTA, ethylenediaminetetraacetic acid, sodium salt; ddNTP, 2′,3′-dideoxynucleoside triphosphate.

[2] We shall use "primase" and "pol α" to denote the primase and polymerase components of the pol α–primase complex, respectively.

[3] In the absence of dNTPs, primase also generates primer dimers, products that are multiples of unit-length primers (20–30 nucleotides long). They are not observed *in vivo*.

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Table 1: Synthetic DNAs

DNA	sequence
Pol α primer:templates	
DNA_G	TCCATATCACAT-3' AGGTATAGTGTACATCTTATCATCATCT
RP860	GGAGGGAG-3' TCGTCCTCCCTCCCATCCTCCTCCATCTTTCTTTACTTCTTCTTATCTCTCTCTGCTGCT
Primase templates	
$d(TC)_{30}$	5'-TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$d(TCC)_{20}$	5'-TCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCC
R60	5'-TCGTCGTCTCTCTCTATTCTTCTTCATTTCTTTCTACCTCCTCCTACCCTCCCTCCTGCT

We therefore used defined-sequence templates and pre-steady-state kinetics to obtain a detailed mechanistic description of what happens in a single reaction cycle of primer synthesis, primer·template movement to pol α, and dNTP polymerization. We have examined how a primase generated primer·template negatively regulates primase activity, as well as the requirements for primase reactivation. Whereas the activities of primase and pol α are independent prior to primer synthesis, after generation of the primer·template they are coordinated.

EXPERIMENTAL PROCEDURES

Materials

Unless noted, all materials and methods were as described previously (Sheaff & Kuchta, 1993). Polymerase α–primase was purified from calf thymus with the use of immunoaffinity chromatography (Kuchta et al., 1990). Synthetic oligonucleotides of defined sequence (Table 1) were from Oligos ETC. Inc. $(dC)_{28}$ was a gift from Drs. Bill Marshall and Marv Caruthers. ddNTPs, poly(dT), poly(dTdC), $(dT)_{25-30}$, single-stranded M13, M13 universal primer (17-mer), and calf thymus DNA were from Sigma or U.S. Biochemical. The average strand length of poly(dT) and poly(dTdC) is 2000–5000 nucleotides. The concentration of single-stranded DNAs was determined spectrally and is expressed in terms of total nucleotide, while concentrations of primer templates are expressed as 3'-termini. Activated calf thymus DNA was prepared according to Baril et al. (1977). DNase I, Klenow fragment, and T7 RNA polymerase were from U.S. Biochemical, RNase T2 was from Sigma, and RNase H was from either U.S. Biochemical or Gibco–BRL. Each was stored according to Maniatis et al. (1982).

Methods

Primer·Template Construction. The primer and template strands were annealed in a 1:1 ratio and quantified as previously described (Kuchta et al., 1987). Primer·templates containing a 5'-^{32}P-labeled primer strand were annealed with a 10% excess of the template strand. The RNA primer for the R60 template was synthesized using T7 RNA polymerase (Milligan et al., 1987).

Gel Electrophoresis and Phosphorimagery. Electrophoresis (18% polyacrylamide, 8 M urea) and phosphorimagery (Molecular Dynamics) were performed as described previously (Sheaff et al., 1991).

Primase Activity in the Absence of Trap. Assays were performed at 37 °C as described previously (Sheaff & Kuchta, 1993).

Coupled Pol α–Primase Activity in the Absence of Trap. Reactions were indentical to those measuring primase activity, except the dNTPs required for the pol α catalyzed elongation of the primase-synthesized primers were included (2–20 μM). Assays contained either [α-^{32}P]NTPs or [α-^{32}P]dNTPs, as indicated. The switch efficiency on $d(TC)_{30}$ was determined using [α-^{32}P]NTPs so that both unit-length primers and pol α elongated products could be quantified by separating the products on a polyacrylamide gel. The amount of pol α elongated products divided by the amount of pol α elongated products plus unelongated unit-length primers gives the switch efficiency (Kuchta et al., 1990). In some cases, products were quantified by a DE81 filter binding assay (Sheaff et al., 1991). Background values were determined in the absence of enzyme, and all products were quantified unless stated otherwise.

To demonstrate that primers synthesized on $(dT)_{25-30}$ or $(dC)_{28}$ are translocated intramolecularly to the pol α active site, assays contained DNA, unlabeled NTPs, [α-^{32}P]dNTPs, and RNase T2 or H, respectively. The amount of RNase was sufficient to hydrolyze any RNA primers that were released into solution after synthesis by primase (see below for control reactions).

Primase Trapping Assay. Assays and quantitation were performed as previously described (Sheaff & Kuchta, 1993). The effect of an occupied pol α site on the primase burst was determined by including the indicated DNAs (0.5–5 μM) in the initial preincubation. To determine the effect of primase-synthesized primers, pol α–primase was incubated for 5–20 min with 25 μM template and the NTPs (25 μM) required for unit-length primer synthesis. Poly(dT) (5 μM) was then added and the mixture diluted into the trap solution.

Pol α Trapping Assay. For DNA primer·templates, 1–5 μM DNA (5'-^{32}P-labeled on the primer strand) was preincubated for 2 min with 5 mM $MgCl_2$, 50 mM Tris, pH 7.5, and 5–15 nM pol α–primase. This mixture was then diluted into a trap solution containing 5 mM $MgCl_2$, 50 mM Tris, pH 7.5, 1 mg mL^{-1} activated calf thymus DNA, and 5–20 μM of the dNTP required to elongate the primer by one nucleotide. Several time points were taken, quenched in gel loading buffer, and subjected to electrophoresis. The amount of elongated primer was quantified to give the burst size. Background due to trap failure was accounted for by diluting pol α–primase alone into the trap solution plus the 5'-^{32}P-labeled primer·template. The amount of product due to trap failure was <2% of the amount observed in the burst.

For RNA-primed templates, the trap solution contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, RNase H (0.2 unit), and 2–10 μM [α-^{32}P]dNTPs required to fully elongate the primer. When primer·template alone was added to the trap solution containing pol α–primase, the amount of product was <2% of the product synthesized in the absence of RNase H. Thus, RNase H prevented pol α from elongating any primer·template free in solution. The products of the burst were shorter than those synthesized in the absence of RNase H due to hydrolysis

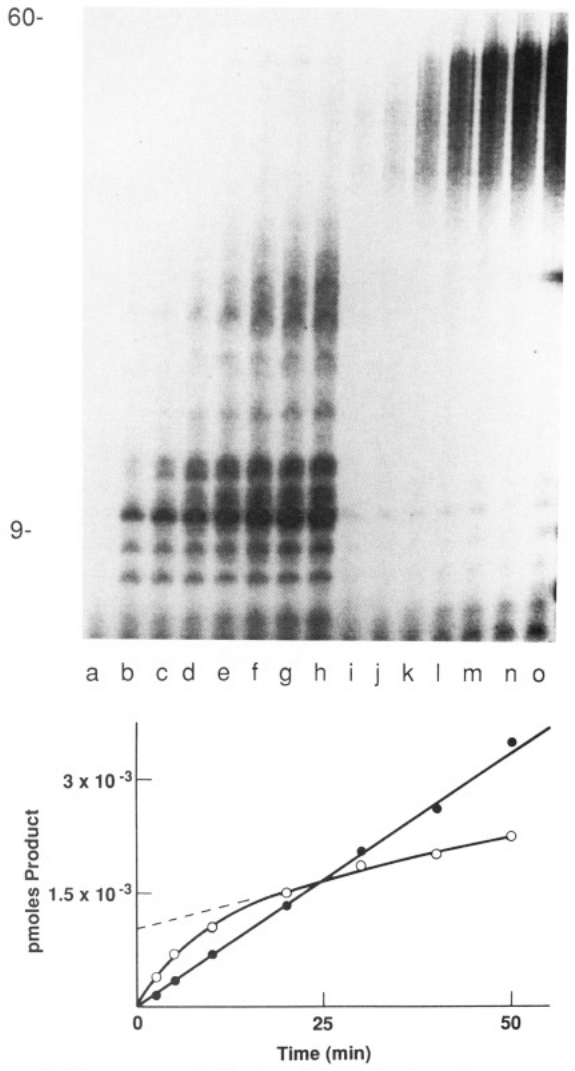


FIGURE 1: Time course of primase activity and pol α–primase-coupled activity on d(TC)$_{30}$. Reactions (37 °C) contained 4 nM pol α–primase, 50 μM d(TC)$_{30}$, 20 μM [α-^{32}P]ATP, and 20 μM [α-^{32}P]-GTP. The gel (top panel) lanes b–h) shows the products after 2.5, 5, 10, 20, 30, 40, and 50 min. The position of a nine-nucleotide-long primer is noted. The longer products above these primers are primer dimers. Lanes i–o show the pol α elongated products synthesized at identical times when 5 μM dATP and 5 μM dGTP were included in the reaction. Lane a is a control where enzyme was omitted. In the graph (bottom panel), the amount of unit-length primers and primer dimers was quantified [(○) primase activity (lanes b–h)]. The dashed line indicates the burst size (9×10^{-4} pmol). Data were simulated using the model E·S (k_1) → E·P (k_2) → E·S + P, where $k_1 = 2 \times 10^{-3}$ s^{-1} and $k_2 = 4 \times 10^{-4}$ s^{-1}, as described under Experimental Procedures. The solid line is E·P + P. The amount of pol α elongated primers [(●) lanes i–o] was also quantified. The slightly slower rate in the coupled assay likely reflects inhibition of primase by the dNTPs (Kuchta et al., 1992).

of the RNA primer. This does not affect quantitation since [^{32}P]dNTPs were incorporated.

Coupled Trapping Assay. Reactions were performed at 25 °C. An initial mixture (3 μL) containing 5 mM $MgCl_2$, 50 mM Tris, pH 7.5, and 5–15 nM pol α–primase was preincubated 2–10 min with 5 μM poly(dT). An aliquot (2 μL) was then added to a trap solution (10 μL) containing 5 mM $MgCl_2$, 50 mM Tris, pH 7.5, 30 μM unlabeled ATP, 2 μM [α-^{32}P]dATP (final concentration, specific activity ca. 5×10^4–10^5 cpm pmol^{-1}), 0.5 mM poly(dC), and 1.5 unit of RNase T2. Aliquots (1–3 μL) were withdrawn at the time points indicated and quenched by the addition of 2.5 volumes of gel loading buffer. An identical reaction was performed in the absence of poly(dC) and RNase T2 to obtain the steady-state rate. The backgrounds due to poly(dC) trap failure and any impurities in the radiolabeled nucleotide were accounted for in identical reactions, except the poly(dT) was added to the trap solution rather than the preincubation mix. 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 trap, indicating poly(dC) prevented primase from rebinding poly(dT). The effectiveness of RNase T2 at hydrolyzing any primers released into solution was tested by preincubating pol α–primase with poly(dT) and 30 μM [α-^{32}P]ATP (specific activity ca. 5×10^4–10^5 cpm pmol^{-1}) in order to synthesize ^{32}P-labeled unit-length primers. The amount of primers synthesized was 2-fold greater than the total amount synthesized in the actual trapping assay. This mixture was heated to 80 °C for 5 min to inactivate pol α–primase and then added to a trap solution containing 2 μM unlabeled dATP, poly(dC), RNase T2, and 1.5 nM pol α–primase. No pol α elongated primers were observed, indicating RNase T2 prevented pol α from binding primers free in solution. Gel electrophoresis of the products showed the ^{32}P-labeled RNA primers were hydrolyzed by RNase T2, while in its absence the labeled primers were elongated.

To measure the elongation of primase-synthesized primers *after* primers were synthesized, pol α–primase was first preincubated with 25 μM template (total nucleotide) and 20 μM NTPs, as required for unit-length primer synthesis. This mixture was then diluted into a trap solution containing 2–10 μM [α-^{32}P]dNTPs and 0.2 unit of RNase H. No further primase activity occurred after dilution due to the low concentration of NTPs (5 μM) and template (4 μM). Products were analyzed by gel electrophoresis and phosphorimagery.

k_{off} Determination. (A) Protection Method. ^{32}P-Labeled primer·templates were generated by incubating enzyme with 25 μM template and 20 μM [α-^{32}P]NTPs. The mixture was then diluted into a solution containing 0.2 unit of RNase H, where no further primase activity occurred due to the low concentration of NTPs and template. Any primer dissociating from pol α–primase was hydrolyzed by the RNase H. At various times, aliquots were removed, and the amount of ^{32}P-labeled primer protected from digestion was determined by gel electrophoresis and phosphorimagery. k_{off} was calculated from the amount of primer remaining at various times. Control reactions indicated that RNase hydrolyzed the primers more rapidly than pol α–primase could bind them (see Coupled Trapping Assay).

(B) Elongation Method. In a second method, the NTPs present during primer synthesis were unlabeled. The mixture containing newly synthesized primers was diluted into 0.2 unit of RNase H, and at various times thereafter [α-^{32}P]-dNTPs were added. Any primers still bound to pol α–primase were elongated via [α-^{32}P]dNTP polymerization. After an additional 5 min, the reactions were quenched by the addition of gel loading buffer and the amount of polymerization products quantified by gel electrophoresis and phosphorimagery. Since the amount of polymerization products reflects the amount of remaining pol α–primase·[primer·template], k_{off} can be obtained.

Data Simulation. Rates were determined by computer simulation using the program KINSIM (Barshop et al., 1983) that was modified to allow input of data as x,y pairs (Anderson et al., 1988). Biphasic primer synthesis data were simulated using the model E·S → E·P → E·S + P.

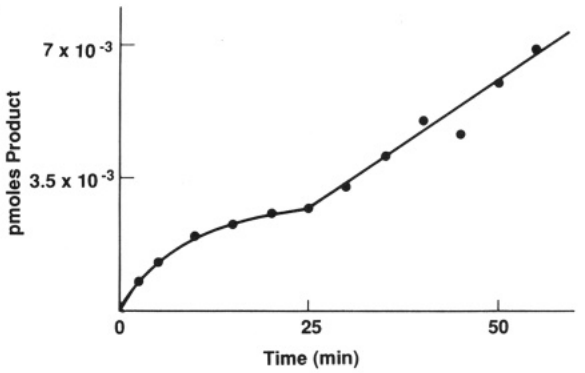


FIGURE 2: Adding dNTPs alleviates primase inhibition. The reaction contained 4 nM pol α–primase, 50 μM d(TC)$_{30}$, 20 μM [α-^{32}P]ATP, and 20 μM [α-^{32}P]GTP. After 25 min at 37 °C, 10 μM dATP and 10 μM dGTP (final concentration) were added. The amount of ^{32}P-labeled unit-length primers and pol α elongated primers was determined at the indicated times.

RESULTS

Pol α Elongation of the Primase-Generated Primer·Template Reactivates Primase. Negative regulation of primase can be described kinetically as a change to a slower rate-limiting step after an initial "burst" of unit-length primer synthesis. In the absence of dNTPs, the time course of unit-length primer synthesis on d(TC)$_{30}$ is biphasic (Sheaff & Kuchta, 1993). To determine if the failure to elongate the primer was responsible for primase regulation, we included the required dNTPs and measured coupled pol α–primase activity on d(TC)$_{30}$. Unit-length primers were efficiently elongated by pol α, and the time course of product formation became linear (>99% elongated, Figure 1), indicating the enzyme complex was undergoing multiple turnover with a single rate-limiting step.

To demonstrate that dNTP polymerization actually reactivated primase, we first incubated pol α–primase with d(TC)$_{30}$, [α-^{32}P]ATP, and GTP for 25 min to generate the typical biphasic kinetics of unit-length primer synthesis (Figure 2). Upon addition of dATP and dGTP, the previously synthesized primers were rapidly elongated by pol α. We then observed a linear rate of coupled pol α·primase activity, which corresonds to the "rapid" phase of the biphasic curve observed when only NTPs are included. The mere presence of dNTPs was not sufficient to reactivate primase, as the biphasic time course did not change when the noncognate dNTPs were included (data not shown). Similar results were obtained on longer templates such as poly(dTdC) and single-stranded M13 (data not shown); hence, it is unlikely that these results are due to pol α simply polymerizing to the end of the short templates. Thus, elongation of the previously generated primer·template rapidly reactivates primase.

We included ddNTPs in the primase reaction on d(TC)$_{30}$ to determine if simply "activating" pol α was sufficient to alleviate inhibition. The RNA primers can only be elongated by one ddNTP, since the absence of a 3′-OH results in chain termination. Importantly, pol α readily polymerizes ddNTPs onto RNA primers, while primase cannot incorporate ddNTPs (Kuchta et al., 1992). When 5 μM ddATP plus ddGTP was included in a primase reaction on d(TC)$_{30}$, the rate of product formation remained biphasic (Figure 3). The rapid phase (k_{obs} = 0.002 s^{-1}) and the slow phase (0.0004 s^{-1}) were identical to those observed in the absence of ddNTPs, indicating that polymerization of a single ddNMP does not reactivate primase. To allow elongation of primers by one or two nucleotides, one dNTP (dGTP or dATP) and one ddNTP (ddATP or ddGTP, respectively) were included. In each case, data were similar to when both ddNTPs were present in the reaction (data not shown).

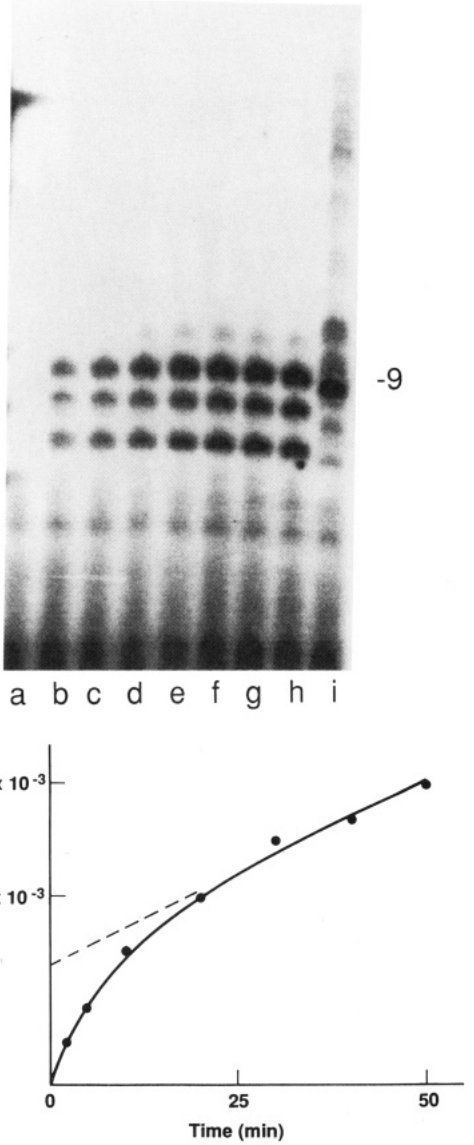


FIGURE 3: Primer synthesis on d(TC)$_{30}$ in the presence of ddNTPs. The reaction (37 °C) contained 4 nM pol α–primase, 50 μM d(TC)$_{30}$, 20 μM [α-^{32}P]ATP, 20 μM [α-^{32}P]GTP, 5 μM ddATP, and 5 μM ddGTP. The top panel shows the background prior to adding enzyme (lane a) and after 2.5, 5, 10, 20, 30, 40, and 50 min (lanes b–h). For comparison, lane i shows the primers synthesized after 30 min in the absence of ddNTPs. A nine-nucleotide-long primer is noted. The bottom panel is the quantitation of the unit-length primers and primer dimers. The dashed line indicates the burst size (7 × 10^{-4} pmol). Data were simulated using the model E·S (k_1) → E·P (k_2) → E·S + P, where k_1 = 1.8 × 10^{-3} s^{-1} and k_2 = 4 × 10^{-4} s^{-1}, as described under Experimental Procedures. The solid line is E·P + P.

Interestingly, the presence of ddNTPs changed the size distribution of the products in a complex fashion. Some primers were shorter than the unit-length primers synthesized in the absence of ddNTPs, and no primer dimers were synthesized (Figure 3, compare lane f with lane i). To verify that the products contained a ddNTP at the 3′-terminus, we added dNTPs in an attempt to further elongate the primers. The ^{32}P-labeled products were not extended, indicating each contained a 3′-ddNMP (data not shown). In contrast, primers synthesized in the absence of ddNTPs were efficiently elongated by pol α upon addition of dNTPs. Thus, elongation of the primer by one or two nucleotides cannot alleviate primase inhibition.

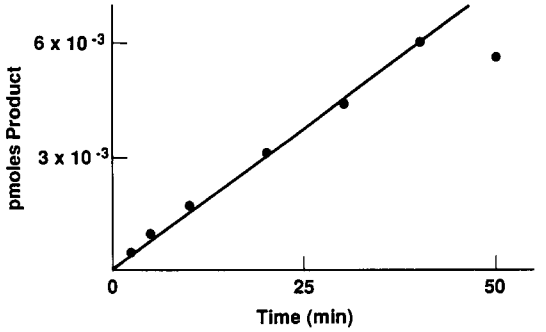


FIGURE 4: Effect of DNA_G and aphidicolin on the kinetics of primer synthesis. The reaction (37 °C) contained 4 nM pol α–primase, 50 μM $d(TC)_{30}$, 20 μM [α-^{32}P]ATP, 20 μM [α-^{32}P]GTP, 10 μM DNA_G, and 50 μM aphidicolin. The amount of unit-length primers plus primer dimers was quantified.

The Newly Generated Primer·Template Must Interact with Pol α for Primase Inhibition. We next examined the kinetics of primase activity when the newly generated primer·template was prevented from binding in the pol α active site. Aphidicolin and an exogenously added primer·template (DNA_G, Table 1) were utilized to block the pol α active site. Aphidicolin potently inhibits pol α by binding the pol α·[primer·template] complex and preventing both dNTP binding and DNA dissociation, but has little effect on the rate of primer synthesis on poly(dT) (Sheaff et al., 1991).[4] The time course of primer synthesis on $d(TC)_{30}$ was linear when the primase-generated primer·template could not relocate to the pol α active site due to the presence of DNA_G and aphidicolin, and the ratio of unit-length primers to primer dimers remained constant (Figure 4). This lack of primase inhibition suggests the primer·template must interact with the pol α active site to negatively regulate further primase activity.

In contrast, aphidicolin alone had no effect on the burst size or the time course of unit-length primer synthesis on $d(TC)_{30}$, although primer dimer formation was inhibited 53%. Aphidicolin likely "locks" the newly generated primer·template in the pol α active site, thereby favoring the interaction required for negative regulation of primase activity. These results provide evidence that inhibition of primase activity by the newly generated primer·template is not simply a consequence of the primase reaction mechanism, but rather a coordination of primase and polymerase activities.

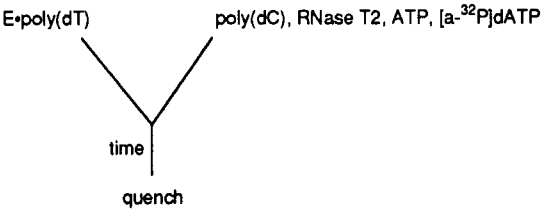
Relationship between Primer·Template Dissociation and Primase Regulation. We utilized a pre-steady-state trapping assay to measure primase inhibition by a newly generated primer·template. Pol α·primase, a template DNA, and NTPs were preincubated to allow unit-length primer synthesis on the template DNA. Poly(dT) was then added, and the mixture was diluted into a trap solution which permitted a single turnover of primase activity on poly(dT). If primase first synthesized a primer on $d(TCC)_{20}$ or R60, subsequent primase activity on poly(dT) was inhibited 86% or 82%, respectively, compared to control reactions lacking $d(TCC)_{20}$ or R60. These results are consistent with the formation of an inhibited enzyme·[primer·template] complex. Surprisingly, however, when the template DNA was $(dC)_{28}$, the burst of primer synthesis on poly(dT) was only inhibited 11%.[5]

Table 2: Dissociation Rates of Newly Generated Primer·Templates

	k_{off}[a] (s^{-1})
$(dC)_{28}$	>0.1
$d(TC)_{30}$	1.2×10^{-3}
$d(TC)_{30}$ + aphidicolin	$<1 \times 10^{-4}$
$d(TC)_{30}$ + aphidicolin + DNA_G	1.4×10^{-3}

[a] Primers were synthesized on each template, and the dissociation rate of the E·[primer·template] complex was measured as described under Experimental Procedures (Protection Method). Assays contained 10 μM aphidicolin and 5 μM DNA_G as noted.

Scheme 1: Coupled Pol α–Primase Trapping Assay



To determine why the primer·template generated on $(dC)_{28}$ failed to inhibit further primase activity, we examined the dissociation rates (k_{off}) of primase-generated primer·templates from the enzyme complex. We first measured k_{off} for the primer·template generated on R60 and $d(TCC)_{20}$ [0.0020 and 0.0018 s^{-1}, respectively (Elongation Method)]. We next attempted to measure k_{off} for the primer·template generated on $(dC)_{28}$ using this methodology, but observed no product formation. This indicates rapid dissociation of the primer·template from the enzyme complex ($k_{off} > 0.1\ s^{-1}$), and suggests that slow dissociation of the primer·template is required for regulation of primase activity.

As shown earlier, preventing the primase-generated primer·template from binding pol α obviates negative regulation of primase and gives the "rapid" rate of primer synthesis. Curiously, the rates of primer·template dissociation were similar in the presence and absence of DNA_G and aphidicolin (Table 2). However, aphidicolin alone greatly reduced the rate of primer·template dissociation, even though it had no effect on the time course of primer synthesis on $d(TC)_{30}$ (Table 2). These data indicate that slow dissociation of the primer·template is required, but not by itself sufficient, for negative regulation of primase activity.

Primase-Synthesized Primers Are Transferred to Pol α without Dissociating from the Enzyme Complex. Previous steady-state experiments suggested that the newly generated primer·template moves directly from primase to pol α without dissociating into solution (Kuchta et al., 1990; Eki & Hurwitz, 1991). To directly test this hypothesis, we examined primase-dependent pol α activity using the pre-steady-state trapping assay depicted in Scheme 1. The incorporation of [α-^{32}P]-dATP by pol α was measured after diluting an E·poly(dT) complex into ATP, [α-^{32}P]dATP, poly(dC), and RNase T2. Importantly, primase must synthesize a unit-length primer before [α-^{32}P]dATP can be incorporated by pol α. The primase trap [poly(dC)] ensures single-turnover conditions by preventing free primase from rebinding poly(dT) once the primase·poly(dT) complex dissociates. RNase T2 functions

[4] Aphidicolin binds 10-fold tighter to the E·DNA complex when the next template base to be replicated is dG than when the next base to be replicated is dA, dC, or dT (Sheaff et al., 1991). Thus, aphidicolin will select for binding to E·DNA_G complexes. Control experiments showed that the amount of DNA_G and aphidicolin used was sufficient to completely inhibit pol α.

[5] Unit-length primer synthesis on $(dC)_{28}$ was linear with time, consistent with the newly generated primer·template not regulating further primer synthesis. However, the rate of primer synthesis (0.0003 s^{-1}) is much slower than the rate on other templates; hence, it is possible that this slow initial rate could obscure regulation.

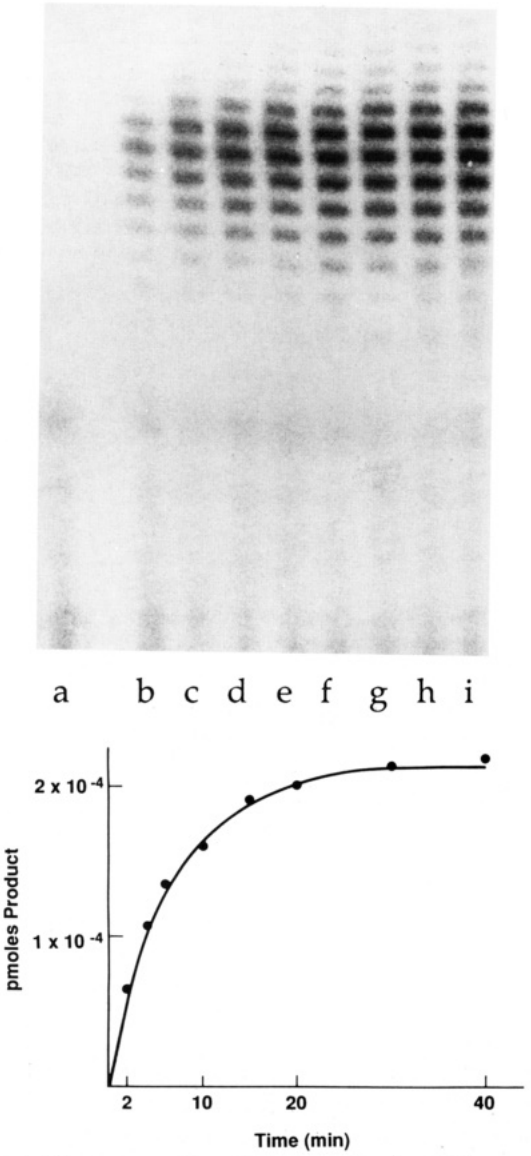


FIGURE 5: Time course of coupled pol α–primase activity on poly(dT). Trapping assays and controls were performed as described under Experimental Procedures. The top panel shows the products synthesized in the coupled pol α–primase assay. In lane a, pol α-primase was diluted into RNase T2, poly(dC), 30 μM ATP, 3 μM [α-^{32}P]dATP, and poly(dT) and then quenched after 40 min. Lanes b–i show the burst of pol α–primase-coupled activity after diluting primase·poly(dT) into the same trap solution [no poly(dT)] ater 2, 4, 6, 10, 15, 20, 30, and 40 min. The bottom panel shows the quantitation of the pol α elongated products (burst size = 2.1×10^{-4} pmol). The curve in the bottom panel was determined by fitting the data to the model E·S (k) $\rightarrow$ E + P (k = 0.002 s^{-1}).

as a primer·template trap by hydrolyzing any primer which dissociates into solution, thereby preventing [α-^{32}P]dATP incorporation onto these primers. However, if the primer·template moves intramolecularly from primase into the pol α active site, [α-^{32}P]dATP can be polymerized. The appearance of pol α elongated primers (Figure 5, lanes b–i) indicates that the primer·template moves to the pol α site by an intramolecular mechanism.[6] Similar results were obtained on poly(dTdC), $(dT)_{25-30}$, and $(dC)_{28}$, indicating that the template length does not affect the mechanism of transfer (data not shown).

[6] No products were observed when ATP was excluded from the trap solution (data not shown), indicating that primer synthesis is required for pol α activity.

The size of the coupled pol α–primase burst corresponds to 0.8 elongated primer per enzyme molecule, similar to the size of a primase burst (Sheaff & Kuchta, 1993). The rate of product formation was 0.002 s^{-1}, compared to a rate of 0.003 s^{-1} for unit-length primer synthesis alone (30 μM ATP, data not shown). Thus, the rate of primer·template movement to pol α and subsequent dNTP polymerization is likely fast relative to primer synthesis. To explicitly show that primer elongation by pol α is rapid, ^{32}P-labeled primers were first synthesized on poly(dT) or $d(TCC)_{20}$ and then diluted into the trap solution containing dNTPs. All of the primers were elongated at the earliest time point (15 s), indicating rapid dNTP polymerization.

The above data suggest that the newly generated primer·template relocates from primase to the pol α active site immediately after primer synthesis. We explicitly examined its location by attempting to block the pol α active site (using DNA_G plus aphidicolin) either before or after primer synthesis. Primase was first preincubated with $d(TC)_{30}$, ATP, and GTP to generate a primer·template. Upon dilution into [α-^{32}P]-dATP, dGTP, 10 μM aphidicolin, and 5 μM DNA_G, the amount of pol α elongated products only decreased 10 ± 3% compared to when DNA_G was not present. The low concentration of NTPs after dilution prevents further primer synthesis, while aphidicolin alone decreases the rate of primer elongation but not the amount of elongated primers. In contrast, if DNA_G and aphidicolin were present when the primers were synthesized as well as when dNTPs were added, the amount of pol α elongated primers decreased by 78 ± 6%. Thus, blocking the pol α active site *prior* to primer synthesis strongly inhibits primer elongation, whereas attempting to block the pol α active site *after* primer synthesis has little effect on primer elongation. This suggests the newly generated primer·template enters the pol α active site immediately after synthesis.

Primase and Pol α Active Sites Are Independent Prior to Primer Synthesis. Earlier studies (steady state) using poly(dT) as template indicated that exogenously added DNA primer·templates have little effect on primase activity (Sheaff et al., 1991). We examined this result in greater detail using the pre-steady-state trapping assay. Neither a short (DNA_G) nor a long (singly-primed M13 DNA) primer·template bound to pol α affected pre-steady-state primase activity on poly(dT) (data not shown). Similar results were obtained when dNTPs (no dATP) were included so that the exogenously added primer·template could be elongated.

Since pol α interacts very differently with RNA and DNA primers (Kuchta et al., 1992), it was possible that a primer·template similar to that generated by primase was required to inhibit primase activity. We generated RP860 (Table 1) by synthesizing an RNA primer complementary to a position near the 3′ end of R60. A 5-fold molar excess of RP860 (50 nM) over enzyme had no effect on the pre-steady-state primase burst on poly(dT). The addition of 25 μM aphidicolin to enhance RP860 binding to the pol α active site also had no effect. Control experiments showed pol α elongated RP860 in both the steady and pre-steady states, and importantly, the length of these products was similar to those synthesized in a pol α–primase-coupled assay on R60. Since the primase start site determines the length of pol α elongated products, this suggests that the primer on RP860 is positioned on the template similarly to where primase initiates synthesis on R60. Thus, an exogenously added primer·template similar to that generated by primase fails to inhibit primase activity.

We also found that pol α activity on DNA_G in a pre-steady-state trapping assay was unaffected by poly(dT) bound in the primase active site. Enzyme [±poly(dT)] was incubated with DNA_G (5′-^{32}P-labeled on the primer strand) and then diluted into a trap solution containing dCTP and activated calf thymus DNA. The amount on DNA_G elongated via dCTP polymerization reflects the amount of E·DNA_G formed in the preincubation. Poly(dT) had no effect on the amount of DNA_G elongated (data not shown), and including ATP in the trap solution to allow primer synthesis on poly(dT) likewise had no effect. Thus, *prior* to unit-length primer synthesis, primase and pol α can bind their DNA substrates simultaneously and perform catalysis. Once primase has generated a primer·template, however, the two activities become coordinated to prevent further primer synthesis.

DISCUSSION

Coordinating the various enzymatic activities thought to be involved at the replication fork is likely a daunting task. Initiation of the leading strand and each Okazaki fragment requires synthesis of a new RNA primer and then elongation by a DNA polymerase. The physical association of primase and polymerase within an enzyme complex makes pol α–primase ideally suited for these "initiation" events. Here we present evidence that the two activities are functionally coordinated such that the newly generated primer·template is transferred intramolecularly from primase to pol α, whereupon it negatively regulates further primase activity until elongated by pol α.

The coordination between primase and pol α requires that the following conditions be satisfied: (i) Primase synthesizes the primer; (ii) the primer is "long enough" (i.e., a unit-length primer) and remains bound to the template (Sheaff & Kuchta, 1993; (iii) the template is of sufficient length; and (iv) the primer·template can interact with the pol α active site. Negative regulation of primase after the initial round of primer synthesis can be described as either complete inhibition of primase followed by reactivation or conversion of primase from a "high-activity" form to a "low-activity" form—these two possibilities cannot be distinguished from the data.

Polymerization of multiple dNMPs by pol α is required to rapidly overcome negative regulation and reactivate primase, although reactivation will slowly occur in the absence of dNTPs. In contrast, pol α catalyzed polymerization of either a single ddNMP or a dNMP followed by a ddNMP did not overcome inhibition, indicating that simply activating pol α is insufficient to reactivate primase. An alternative possibility is that primers containing ddNMPs at the 3′-terminus bind very tightly to pol α and prevent reactivation. However, this seems unlikely since primers containing a 3′-terminal ddNMP bind 2-fold less well than primers containing a 3′-terminal dNMP (Kuchta et al., 1992). Furthermore, preventing primer·template dissociation by the addition of aphidicolin does not affect the slow rate of primer synthesis.

Previous studies with *Drosophila* pol α–primase also demonstrate that the pol α subunit is required for regulation of primase activity after unit-length primer synthesis (Cotterill et al., 1987). The kinetics of unit-length primer synthesis by the *Drosophila* pol α–primase complex were biphasic on single-stranded M13, similar to the calf thymus enzyme. However, with only the primase subunit present, the time course of unit-length primer synthesis was linear.

We used a single-turnover trapping assay to show directly that after unit-length primer synthesis the primer·template relocates into the pol α active site via an intramolecular pathway. Two additional observations suggest that the primer·template moves between the primase and pol α active sites. (1) Aphidicolin inhibited synthesis of primer dimers but not unit-length primers. Primer dimers are multiples of unit-length primers synthesized by primase in the absence of dNTPs. Once a primer reaches ca. 7 nucleotides long, it becomes a substrate for pol α and may move between the primase and polymerase active site. After the primer·template enters the pol α site, aphidicolin binds the pol α·[primer·template] complex and prevents the primer from returning to primase for conversion into a primer dimer. (2) When ddNTPs were included in a primase reaction on $d(TC)_{30}$, some products were actually shorter than the unit-length primers synthesized in their absence (see Figure 3). The incorporation of ddNTPs may "trap" primers as soon as they transfer to pol α and prevent further elongation. However, in the absence of ddNTPs, primase can add additional NTPs to the primer as it moves between the two active sites.

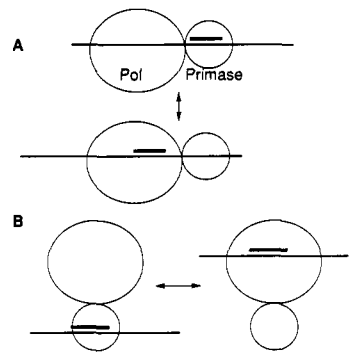


FIGURE 6: Two models for the orientation of the primase and polymerase active sites with respect to DNA.

An alternative mechanism consistent with these data is that primase polymerizes the first 10–20 dNTPs onto the RNA primer, as has been suggested for primase from KB cells (Hu et al., 1984). Calf thymus primase will polymerize one dNTP onto a primer, but there is no further polymerization of dNTPs (Kuchta et al., 1992). Additionally, conditions that block the pol α active site but do not prevent primase activity (e.g., DNA_G + aphidicolin) can completely inhibit polymerization of dNTPs onto primase-synthesized primers. Finally, with *Drosophila* pol α–primase, high levels of aphidicolin completely inhibited polymerization of dNTPs onto primase-synthesized primers (Cotterill et al., 1987). Thus, at least with the *Drosophila* and calf thymus enzymes, only pol α appears capable of polymerizing multiple dNTPs onto primase-synthesized primers.

There are two very different possible configurations of primase and pol α in the enzyme complex with respect to the DNA. In Figure 6A, the primase and pol α sites are lined up along the DNA axis. Each may interact with the template, and primers synthesized by primase are transferred by sliding longitudinally into the pol α active site. This scenario seems unlikely, however, as exogenously added primer·templates bound to pol α had no effect on primase activity, and poly(dT) bound in the primase site did not affect pol α activity. Figure 6B presents an alternative configuration in which the DNA binding sites are distinct. An exogenously added DNA substrate in either site, regardless of length, would not affect activity in the other site. After primer synthesis, the completed primer·template slides laterally into the pol α active site.

How does the newly generated primer·template regulate primase activity? Intriguingly, exogenously added primer·templates did not inhibit primase activity, even if they were

structurally similar to a primase-synthesized primer. Hence, one possibility is that the primase-generated primer·template interacts with both the pol α and primase active sites, thereby preventing a new DNA substrate from binding. This could account for the lack of regulation by unit-length primers synthesized on $(dC)_{28}$—the template is too short to make both interactions. Alternatively, primase may undergo a conformational change after synthesizing a unit-length primer which greatly decreases its activity. Translocation of the primer to the pol α site stabilizes this conformation, thereby ensuring a new primer is not synthesized until the previous one is elongated.

Could the high degree of communication between pol α and primase serve other functions *in vivo*? By physically assocating primase and polymerase, the short RNA primer can be stabilized and protected through continual association with the pol α–primase complex, and no time is wasted due to primer·template dissociation and rebinding by pol α. Pol α–primase initiates replication of both the leading and lagging strands, which presumably requires generating primers during formation of the replication bubble. Maintaining contact with the first primer generated may be important for fixing the start site of replication, as well as regulating the timing of initiation. Pol α–primase could bind to an initiation site and synthesize a primer to generate the stable enzyme··[primer·template] complex, but then wait for the appropriate signal before commencing DNA synthesis.

Replication forks do not move at a steady rate, but rather pause at various sites along the DNA (Tapper & DePamphilis, 1980; Hill et al., 1988; Gahn & Schildkraut, 1989; Brewer et al., 1992). Maintaining the position of the pol α–primase complex and preventing synthesis of new primers during these pauses might help ensure the integrity of the replication complex. Finally, stalled pol α–primase complexes could accumulate at initiation sites prior to firing, or perhaps when DNA damage causes replication forks to pause. The presence of these complexes could indicate to the cell that DNA replication/S phase has not been completed, and thereby prevent entry into mitosis (Dasso & Newport, 1990).

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