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Eucaryotic DNA Primase Does Not Prefer To Synthesize Primers at Pyrimidine Rich DNA Sequences When Nucleoside Triphosphates Are Present at Concentrations Found in Whole Cells[†]

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ABSTRACT: The critical role of NTP concentration in determining where calf thymus DNA primase synthesizes a primer on a DNA template was examined. Varying the concentration of NTPs dramatically altered the template sequences at which primase synthesized primers. At the low NTP concentrations typically used for *in vitro* experiments (100 μM), primase greatly preferred to synthesize primers at pyrimidine rich DNA sequences. However, when the concentrations of NTPs were increased to levels typically found in whole cells, primers were now synthesized in all regions of the template. Importantly, synthesis of primers in all regions of the DNA template, not just the pyrimidine rich sequences, is the pattern of primer synthesis observed during DNA replication in whole cells. With low concentrations of NTPs (i.e., $V_{\max}/K_M$ conditions), primers are only synthesized at the most preferred synthesis sites, namely, those that are pyrimidine rich. In contrast, under conditions of high NTP concentrations, primer synthesis will occur at the first potential synthesis site to which primase binds. Now, the primase·DNA complex will be immediately converted to a primase·DNA·NTP·NTP complex that is poised for primer synthesis.

DNA polymerase α-primase (pol α-primase)[1] is essential for eucaryotic DNA replication and is capable of initiating both leading strand synthesis as well as Okazaki fragment synthesis on the lagging strand. This four-subunit complex contains two enzymatic activities: DNA primase and pol α (Hu et al., 1984; Kaguni et al., 1983; Tseng & Ahlem, 1983). On single-stranded DNA, the DNA primase activity catalyzes the synthesis of RNA primers of approximately 7−10 nucleotides in length. The primers are transferred intramolecularly to the pol α active site and then elongated via dNTP polymerization (Sheaff et al., 1994). Pol α-primase is unique in that it is the only nuclear enzyme complex capable of binding single-stranded DNA and initiating the synthesis of DNA *de novo*.

Unlike prokaryotic DNA primases (e.g., from *Escherichia coli*) that generally synthesize primers at specific sequences on the template (Kitani et al., 1985; Yoda et al., 1988), eucaryotic primase initiates primer synthesis at many different sequences (Bullock et al., 1994; Hay et al., 1984; Suzuki et al., 1993; Tseng & Prussak, 1989; Yamaguchi et al., 1985). Importantly, there exists a dramatic dissimilarity between the template sequences utilized when primers are synthesized in whole cells compared to *in vitro* (Hay et al., 1984; Sheaff & Kuchta, 1993; Suzuki et al., 1993; Yamaguchi et al., 1985). Both purified enzyme and whole cell assays reveal that primase preferentially initiates synthesis opposite a template thymidylate or deoxycytidylate by incorporating ATP or GTP as the 5′-terminal nucleotide of the primer (Hay et al., 1984; Sheaff & Kuchta, 1993; Suzuki et al., 1993; Yamaguchi et al., 1985). However, whereas purified enzyme studies have shown that primase greatly prefers that the remainder of the template be pyrimidine rich, whole cell studies showed that primers are synthesized in all regions of the template, not just those that are pyrimidine rich (Bullock et al., 1994; Harrington & Perrino, 1995; Yamaguchi et al., 1985).

There exist multiple factors that could affect primase start site selection, including (1) NTP and dNTP concentrations, (2) DNA sequence, (3) DNA structure, (4) accessory proteins, and (5) buffer conditions. For example, increasing the concentration of a single NTP increases the frequency with which it is found as the second nucleotide of the primer (Sheaff & Kuchta, 1993; Suzuki et al., 1993). This result is predicted by the mechanism of primer synthesis where the first NTP bound by the E·DNA complex becomes the second nucleotide of the primer (Sheaff & Kuchta, 1993). Most previous work has focused on the role of DNA sequence and structure in determining where primase initiates synthesis. *In vitro* studies demonstrate that primase prefers to initiate primer synthesis in pyrimidine rich regions of the DNA template (Suzuki et al., 1993; Yamaguchi et al., 1985) and at junctions between single-stranded and double-stranded regions of the DNA (Harrington & Perrino, 1995; Tseng & Ahlem, 1984). Unfortunately, none of these studies have demonstrated why primase initiation is dramatically different in whole cells compared to *in vitro*.

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[1] Abbreviations: BSA, bovine serum albumin; dsDNA, double-stranded DNA; EDTA, ethylenediaminetetraacetic acid, sodium salt; nt, nucleotide; pol α-primase, DNA polymerase α-primase; RPA, replication protein A; ssDNA, single-stranded DNA; Tris, tris(hydroxymethyl)aminomethane (HCl salt).

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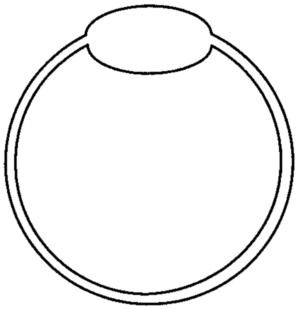
Table 1: Synthetic Templates

d(TC)$_{30}$	5'-TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT-3'
d(TCC)$_{20}$	5'-TCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCC-3'
d(ACT)$_{20}$	5'-ACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACT-3'
C3R	5'-CCCTACGATGCCCTACGATGCCCTACGATGCCCTACGATG-3'
DNA$_G$	5'-TCCATATCACAT-3' 3'-AGGTATAGTGTAGATCTTATCATCT-5'
'Bubble' Template	

We have used synthetic templates of defined sequence as well as more natural DNA sequences to examine the role of NTP concentration in determining where primase initiates primer synthesis. Increasing the concentration of NTPs from the typically low levels used for *in vitro* experiments to those found *in vivo* eliminated the preference of the enzyme for pyrimidine rich sequences. The likely mechanism of this effect and its implications are discussed.

EXPERIMENTAL PROCEDURES

Materials.

Unless noted, all materials and methods were as described previously (Sheaff & Kuchta, 1993). Pol α-primase was purified from calf thymus with the use of immunoaffinity chromatography (Kuchta et al., 1990). Synthetic oligonucleotides of defined sequence were from Oligos Etc. Inc. (Table 1). The 458 nt 'bubble' template was as previously described (Harrington & Perrino, 1995). Poly(dT) was from Sigma. The concentration of single-stranded DNAs was determined spectrally and is expressed in terms of total nucleotide. The concentration of the 458 nt 'bubble' template is expressed in terms of the number of 458 nt ssDNA sequences, and the concentration of DNA$_G$ is given in terms of primer 3′-termini. All other reagents were of the highest purity available.

Methods

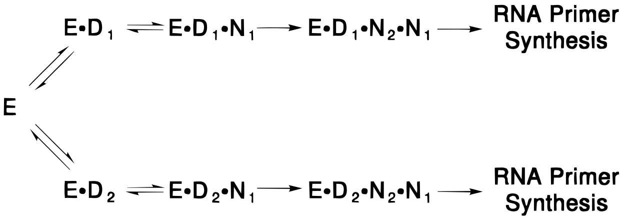
Primase Assays. Reaction mixtures (10 μL) contained 50 mM Tris-HCl, pH 7.5, MgCl$_2$ concentrations 5 mM in excess of the total NTP concentration, 60 μM single-stranded template DNA, 10 nM pol α-primase, 0.05 mg mL^{-1} BSA, and 0.1−2 mM [α-^{32}P]NTPs. To examine the effects of dNTPs (10−200 μM) on primase activity under conditions where pol α could not polymerize the dNTPs, 5 μM DNA$_G$ and 50 μM aphidicolin were included in the assays. DNA$_G$ and aphidicolin bind to the pol α active site and form a ternary complex that inhibits pol α activity but not primase activity (Sheaff et al., 1991). Reactions were initiated by the addition of enzyme and incubated at 37 °C for 1 h. After the assays were quenched by adding 2.5 volumes of gel loading buffer (90% formamide), products were separated by denaturing polyacrylamide gel electrophoresis (18% polyacrylamide, 8 M urea) and analyzed via phosphorimagery (Molecular Dynamics). Background values were determined from identical reactions that lacked enzyme.

Primase-Coupled Pol α Assays on the Short Synthetic Templates of Defined Sequence. Reaction mixtures (10 μL) typically contained 50 mM Tris-HCl, pH 7.5, MgCl$_2$ concentrations 5 mM in excess of the total NTP plus dNTP concentration, 60 μM single-stranded template DNA, 10 nM pol α-primase, 0.05 mg mL^{-1} BSA, 0.1−2 mM NTPs, and 5−10 μM [α-^{32}P]dNTPs. Reactions were initiated by the addition of enzyme and incubated at 37 °C for 1 h. After the reactions were quenched with gel loading buffer, products were separated and analyzed as described above.

Competition Reactions on Synthetic Templates. Competition assays were performed under primase-coupled pol α assay conditions with two different single-stranded DNA templates in the reaction. Template concentrations varied from 5 to 150 μM and were adjusted so that in the presence of 100 μM NTPs, approximately 50% of the primers were synthesized on each template. After the reactions were quenched with gel loading buffer, products were separated by denaturing gel electrophoresis and analyzed by phosphorimagery. The products due to primase-coupled pol α activity on each template could be distinguished because they were of different lengths.

Primase-Coupled Pol α Assays on 458 nt 'Bubble' Template. Assays (15 μL) to determine primase initiation sites on a natural sequence were performed identically to

Scheme 1



the primase-coupled pol α assays on synthetic templates, except each reaction contained 0.028 pmol of the 'bubble' template (Harrington & Perrino, 1995). After incubation for 1 h at 37 °C, the reaction was quenched with an equal volume of 50 mM EDTA. The quenched reaction mixture was concentrated to approximately 5 μL *in vacuo* and then diluted with 10 μL of gel loading buffer. Products were separated by denaturing polyacrylamide gel electrophoresis (8% polyacrylamide, 8 M urea) and analyzed as described above.

RESULTS

Initially we examined the role of NTP concentration in determining where primase initiates primer synthesis. Previous studies on purified pol α-primase have used NTP concentrations much lower than those found *in vivo*. Typically, these assays contained 0.1−2 mM ATP and 100−200 μM CTP, GTP, and UTP (Bullock et al., 1994; Suzuki et al., 1993; Yamaguchi et al., 1985; Yoda & Okazaki, 1983). In contrast, *in vivo* ATP and GTP levels usually range from 1−4 mM, and CTP and UTP are typically 0.5−1 mM (Hauschka, 1973). Previous studies have already demonstrated that varying the concentration of a single NTP will increase the frequency with which that nucleotide becomes the second nucleotide of the primer (Sheaff & Kuchta, 1993; Suzuki et al., 1993). Together, these data raised the possibility that by simultaneously increasing the concentration of all of the NTPs to the levels found in whole cells, one would dramatically alter where primase initiates primer synthesis.

Scheme 1 depicts how varying the NTP concentration could greatly alter where primase initiates synthesis under conditions where primase could bind to either of two different binding sites in order to initiate primase synthesis. At low NTP concentrations (i.e., V_{max}/K_M conditions), the rate of E•DNA dissociation will be rapid compared to the rate of E•DNA•NTP•NTP quaternary complex formation. Under these conditions, primase will have the opportunity to bind to (and dissociate from) both template sites prior to primer synthesis such that primer synthesis will occur at the preferred site. In contrast, high NTP concentrations will serve to trap primase at whichever initiation site it initially binds; the high NTP concentration immediately converts the E•DNA binary complex into a E•DNA•NTP•NTP quaternary complex that is poised to initiate primer synthesis. This model predicts that increasing the NTP concentration from the low values typically used for purified enzyme studies to levels similar to those found in whole cells will greatly alter where primase initiates primer synthesis. In order to test this model, we first examined how NTPs affect the rate of primer synthesis. Then, we examined how varying the NTP concentration affects primase's choice of initiation sites on both synthetic templates as well as a more natural DNA template.

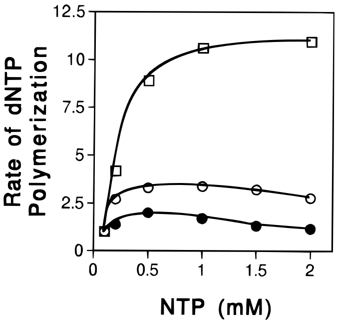


FIGURE 1: Effect of NTP concentration on the relative rate of primer synthesis during primase-coupled pol α activity on d(TC)$_{30}$. Primase-coupled pol α assays were performed as described under Experimental Procedures. Initial conditions consisted of 60 μM d(TC)$_{30}$ and 100 μM ATP and GTP. ATP (○), GTP (●), or both ATP and GTP (□) were varied from 100 to 2000 μM. The amount of product formed with 100 μM ATP and GTP was normalized to 1. This value of 1 corresponds to a rate of 1.6 pmol of dNTP polymerized h^{-1}.

Effects of NTP Concentration on the Rate of Primase-Coupled Pol α Activity. The effect of varying the concentration of one or more NTPs on the rate of primase-coupled pol α activity was examined using d(TC)$_{30}$, a template that requires both ATP and GTP for primer synthesis (Figure 1). Since synthesis of the RNA primer is the rate-limiting step during coupled activity (Sheaff & Kuchta, 1993), the effect of NTPs reflects changes in primase activity, not pol α activity. Elevating the ATP concentration while keeping the GTP concentration at 100 μM stimulated primase-coupled pol α activity approximately 3.5-fold at maximum stimulation, but further increasing the ATP concentration then gave partial inhibition. Increasing the GTP concentration while maintaining the ATP concentration at 100 μM maximally stimulated primase-coupled pol α activity approximately 2-fold (Figure 1) and then gave moderate inhibition at higher GTP concentrations. In contrast, simultaneously elevating the levels of ATP and GTP resulted in a much greater increase in primase-coupled pol α activity, approximately 11-fold, and demonstrated saturation behavior. Adding high concentrations (2 mM) of either UTP or CTP, neither of which are required for primer synthesis, had either a slight inhibitory effect (10−15%) or no effect on the rate of primase−coupled pol α activity (data not shown). Increasing the NTP concentrations on other templates [(ACT)$_{20}$ and d(TCC)$_{20}$] gave qualitatively similar results (data not shown). If the concentration of a single NTP was increased and that NTP was required for initiation, i.e., dinucleotide synthesis, we observed a 2−3-fold increase in primase-coupled pol α activity followed by inhibition. If both NTPs required for initiation were increased simultaneously, a 5−15-fold increase in primase-coupled pol α activity was observed and the data demonstrated saturation behavior. Finally, elevating the concentration of a single NTP not required for initiation affected the rate of primer synthesis by <15%.

The K_M for NTPs during primase-coupled pol α activity was determined on several different templates (Table 2). In each case, the K_M is significantly greater than the concentration of NTPs typically used for *in vitro* assays (Bullock et al., 1994; Suzuki et al., 1993; Yamaguchi et al., 1985; Yoda & Okazaki, 1983).

Effects of NTP Levels on Primase Start Site Selection with Synthetic Templates. The different effects of changing one or multiple NTP concentrations on the rate of primer

Table 2: K_M(NTPs) under Primase-Coupled Pol α Assay Conditions for a Variety of Templates[a]

template	K_M(NTPs) (mM)
d(TC)$_{30}$	0.8 ± 0.3
d(TCC)$_{20}$	0.7 ± 0.1
d(ACT)$_{20}$	1.7 ± 0.5
poly(dT)	1.7 ± 0.5
C3R	0.9 ± 0.3
bubble template	0.3 ± 0.03

[a] Rates were determined as described under Experimental Procedures. All of the required NTPs for a given template were titrated from 100 μM to 2 mM while maintaining the required dNTPs at 10 μM.

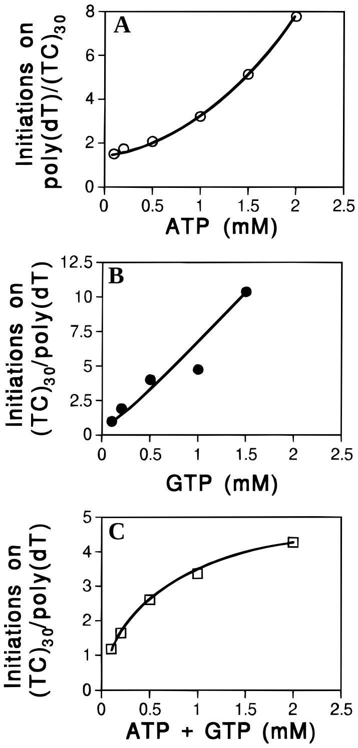


FIGURE 2: Effect of NTP concentration on utilization of d(TC)$_{30}$ versus poly(dT) during primase-coupled pol α activity. Competition assays between d(TC)$_{30}$ and poly(dT) were performed as described under Experimental Procedures. The concentrations of ATP (A), GTP (B), or both ATP and GTP (C) were varied as shown. The relative amount of product formed on each template in the presence of 100 μM ATP and GTP was normalized to 1. This value of 1 corresponds to total rates of dNTP polymerization on both templates of 1.0 (A), 1.2 (B), and 1.2 (C) pmol of dNTP polymerized h^{-1}.

synthesis suggested that changing the concentration of NTPs would greatly influence start site selection. In order to test this hypothesis, competition assays were performed where primase could synthesize a primer on either of two different templates. Since dNTPs were present in the assays such that pol α could elongate the primase-synthesized primer, the assays measured primase-coupled pol α activity, the physiological reaction sequence of pol α-primase. The data shown in Figure 2 demonstrate that elevating the concentration of a single NTP can have a dramatic effect on which template is utilized by primase. In a competition experiment between poly(dT) and d(TC)$_{30}$, increasing the ATP concentration from 100 μM to 2 mM while maintaining the GTP concentration at 100 μM resulted in a 5.2-fold increase in the number of primers synthesized on poly(dT) relative to d(TC)$_{30}$. Conversely, increasing GTP to 2 mM while holding the ATP concentration at 100 μM resulted in d(TC)$_{30}$ being utilized exclusively. Since the second nucleotide of primers synthesized on poly(dT) is adenylate whereas the second nucleotide of the primer synthesized on d(TC)$_{30}$ is guanylate, these data are consistent with previous work showing that one can influence primase initiation by increasing the NTP that will become the second nucleotide of the primer (Sheaff & Kuchta, 1993; Suzuki et al., 1993). Finally, we examined the effect of simultaneously raising the concentrations of ATP and GTP (Figure 2). Even though we are now increasing the concentration of the NTPs that will become both the second and 5′-terminal nucleotide of the primer synthesized on each template, increasing the concentrations of ATP and GTP from 100 μM to 2 mM resulted in a 3.6-fold increase in the number of initiations on d(TC)$_{30}$ relative to poly(dT).

In competition experiments between d(ACT)$_{20}$ and poly(dT), the results were qualitatively similar to the competition experiments between d(TC)$_{30}$ and poly(dT) (Table 3). Increasing the concentration of only ATP resulted in a 17.5-fold increase in the number of initiations on poly(dT), a template where adenylate will become the second nucleotide of the newly synthesized primer, relative to d(ACT)$_{20}$, a template where guanylate will become the second nucleotide of the newly synthesized primer (Sheaff & Kuchta, 1993). Increasing the concentration of only GTP resulted in a 5-fold increase in the number of primers generated from d(ACT)$_{20}$ relative to poly(dT). Increasing the ATP and GTP concentrations simultaneously while keeping the concentration of UTP at 100 μM resulted in a 2.5-fold increase of primer synthesis on d(ACT)$_{20}$ relative to poly(dT). Likewise, simultaneously increasing the concentrations of ATP, GTP, and UTP resulted in a 5-fold increase in the number of primers synthesized on d(ACT)$_{20}$ relative to poly(dT). In order to observe the effect of elevating the concentration of an NTP required for primer synthesis but not involved in dinucleotide formation, the concentration of UTP was increased to 2 mM while holding the concentrations of ATP and GTP constant at 100 μM. This resulted in a 1.2-fold increase of primers synthesized on d(ACT)$_{20}$ relative to poly(dT) (Table 3).

Competition experiments were performed on several additional templates to demonstrate that increasing the concentration of one or both of the NTPs required for initiation could greatly alter where primase synthesized primers (Table 3). With each of these pyrimidine rich templates, initiation involves the polymerization of two purine NTPs (Sheaff & Kuchta, 1993). In each of the competition experiments, altering the concentration of NTPs altered which template was preferred for initiation, although the magnitude of the effect varied in the different competition experiments.

Effects of NTPs on Primase Start Site Selection with Natural Template Sequences. To confirm that these results were not an artifact of the short synthetic templates and that NTPs could dramatically alter initiation site selection on more natural templates, we utilized a circular plasmid DNA that contained a bubble region of 458 nt (Harrington & Perrino, 1995). Two single-stranded copies of a 458 nt long natural sequence were incorporated into a dsDNA plasmid. The two identical stretches cannot hybridize to each other; thus a 'bubble' is formed. Initiation sites can be determined from the length of primase-coupled pol α products, which consist of an RNA primer elongated by pol α to the end of the

Table 3: Results of Competition Experiments with Varying Concentrations of NTPs[a]

	ATP	GTP	CTP	UTP
d(TC)$_{30}$:poly(dT)	5.2 [poly(dT)]	>20 [d(TC)$_{30}$]		
d(TCC)$_{20}$:poly(dT)	>20 [poly(dT)]	>20 [d(TCC)$_{20}$]		
d(ACT)$_{20}$:poly(dT)	17.5 [poly(dT)]	5.0 [d(ACT)$_{20}$]	1.3 [d(ACT)$_{20}$]	1.2 [d(ACT)$_{20}$]
d(TCC)$_{20}$:C3R	1.4 [d(TCC)$_{20}$]	1.6 [C3R]	1.4 [C3R]	1.5 [d(TCC)$_{20}$
d(ACT)$_{20}$:C3R	1.8 [C3R]	6.4 [C3R]	1.4 [C3R]	1.9 [d(ACT)$_{20}$]

	ATP/GTP	ATP/GTP/UTP	all NTPs
d(TC)$_{30}$:poly(dT)	3.6 [d(TC)$_{30}$]		
d(TCC)$_{20}$:poly(dT)	>20 [d(TCC)$_{20}$]		
d(ACT)$_{20}$:poly(dT)	2.5 [d(ACT)$_{20}$]	5.0 [d(ACT)$_{20}$]	
d(TCC)$_{20}$:C3R	1.2 [C3R]		1.6 [C3R]
d(ACT)$_{20}$:C3R	1.5 [d(ACT)$_{20}$]		1.6 [d(ACT)$_{20}$]

[a] Competition assays where performed as described under Experimental Procedures. The frequency with which each template was used as a substrate was measured with 100 μM of all required NTPs, and this value was normalized to 1.0. The concentration of the indicated NTP(s) was increased to 2 mM, and the frequency with which each template was used as a substrate was again measured. The values shown are the frequency at 2 mM relative to the frequency at 100 μM. The preferred template with 2 mM NTP is shown in brackets.

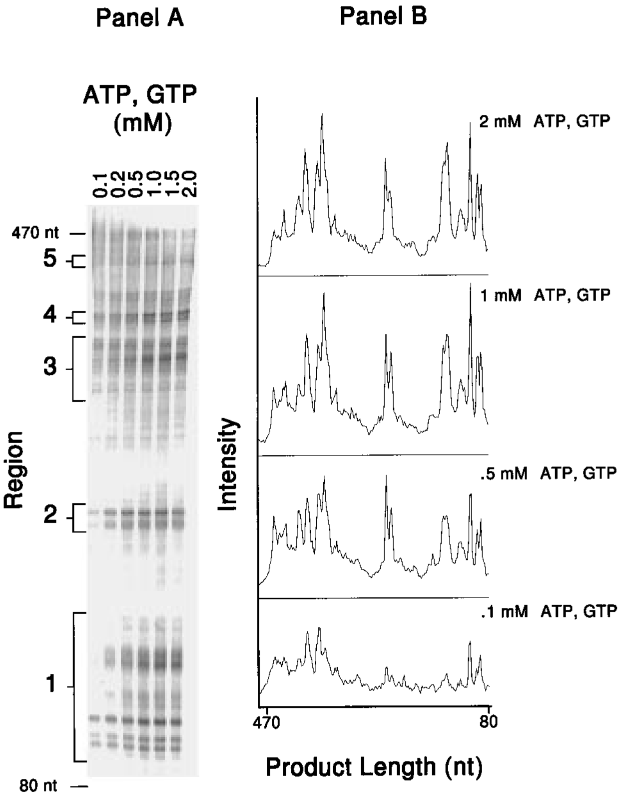


FIGURE 3: Effect of simultaneously changing NTPs on primase start site selection on a natural template sequence. Primase-coupled pol α assays were performed and analyzed as described under Experimental Procedures. Panel A: Initial conditions consisted of 0.028 pmol of ‘bubble’ template and 100 μM ATP, CTP, GTP, and UTP. ATP and GTP were simultaneously increased from 100 to 2000 μM. Panel B: Data from panel A were quantified using phosphorimager analysis.

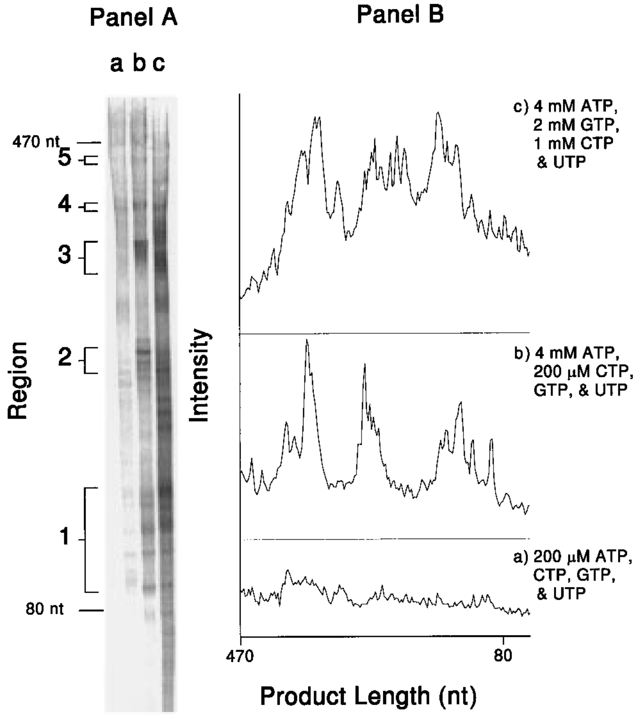


FIGURE 4: Effect of *in vitro* versus *in vivo* NTP concentrations on primase start site selection on a natural template sequence. Assays were performed and analyzed as described under Experimental Procedures. Panel A: NTP concentrations for each reaction were (lane a) 200 μM ATP, CTP, GTP, and UTP, (lane b) 4 mM ATP and 200 μM CTP, GTP, and UTP, (lane c) 4 mM ATP, 2 mM GTP, and 1 mM CTP and UTP. Panel B: Data in panel A were quantified using phosphorimager analysis.

single-stranded region. The longer the product, the closer initiation was to the 3′-end of one strand of the bubble.[2]

Figures 3 and 4 show the distribution of initiation sites utilized by pol α-primase in assays containing either 100 μM of each NTP or 200 μM of each NTP. Primer synthesis occurs primarily in five different regions of the template. Regions 1−4 correspond to primer synthesis in pyrimidine rich regions of the template. Region 5 corresponds to products of approximately 458 nt in length and represents initiation events that occurred adjacent to the junction between single- and double-stranded DNA. These data are consistent with previous *in vitro* studies showing that primase prefers to initiate primer synthesis in pyrimidine rich regions of a template or in regions where ssDNA is adjacent to dsDNA (Bullock et al., 1994; Harrington & Perrino, 1995; Suzuki et al., 1993; Tseng & Prussak, 1989; Yamaguchi et al., 1985; Yoda & Okazaki, 1983).

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[2] Control reactions were performed under primase-coupled pol α assay conditions with the ‘bubble’ template as described under Experimental Procedures, except 1 U of Klenow fragment (−Exo) was also added. The product distribution in the absence and presence of Klenow fragment was identical, indicating that pol α had elongated the primase-synthesized primers to the end of the single-stranded template.

Table 4: Result of Competition Experiments with Varying Concentrations of dNTPs[a]

	dATP	dGTP	dTTP
d(TC)$_{30}$:poly(dT)	1.4 [d(TC)$_{30}$]	1.1 [poly(dT)]	
d(TCC)$_{20}$:poly(dT)	1.6 [d(TC)$_{30}$]	1.0	
d(ACT)$_{20}$:poly(dT)	1.1 [d(ACT)$_{20}$]	1.0	1.0

[a] Competition assays where performed as described under Experimental Procedures. The frequency with which each template was used as a substrate was measured with 100 μM of all required NTPs and 10 μM dNTPs, and this value was normalized to 1.0. The concentration of the indicated dNTP was increased to 135 μM, and the frequency with which each template was used as a substrate was measured. The values shown are the frequency at 135 μM relative to the frequency at 10 μM. The preferred template with 135 μM dNTP is shown in brackets.

Increasing the concentration of ATP from 200 μM to 4 mM while maintaining the concentrations of CTP, GTP, and UTP at 200 μM resulted in only a 30% increase in activity on the 'bubble' template, and the pyrimidine rich regions were still favored for initiation (Figure 4). Similarly, increasing the concentrations of ATP and GTP to 2 mM while maintaining the CTP and UTP concentrations at 100 μM increased the rate of DNA synthesis by 2-fold and subtly altered where primase synthesized primers (Figure 3). Regions 1−3, three template regions which are pyrimidine rich, were now utilized with much greater frequency relative to regions 4 and 5.

The template competition experiments described earlier showed that pyrimidine triphosphates could significantly alter where primers were synthesized, strongly suggesting that increasing the concentrations of UTP and CTP would affect where primers were synthesized on the 'bubble' template. Therefore, primase-coupled pol α activity was measured when the concentrations of the NTPs were adjusted to values similar to those found in whole cells [4 mM ATP, 2 mM GTP, and 1 mM CTP and UTP (Hauschka, 1973)]. Raising the NTP concentration to these values increased the rate of coupled activity by 3.8-fold (Figure 4). More importantly, primer synthesis now occurred along the entire single-stranded template, not just the pyrimidine rich regions. It should be noted that even within the preferred regions, specific sites are preferentially utilized (Figure 4), as opposed to initiation occurring equally at every template base. Most significantly however, utilization of the entire template for initiation of primer synthesis is qualitatively similar to what occurs during DNA replication in whole cells (Hay et al., 1984).

dNTPs Appear To Play a Minor Role in Primase Initiation. We previously demonstrated that dNTPs can significantly inhibit primase activity and that only dNTPs complimentary to the template are strong inhibitors (Kuchta et al., 1992). Therefore, we used template competition assays to determine if dNTPs could alter where primase initiates primer synthesis. Assays contained 100 μM NTPs, and the concentration of a dNTP was varied from 10 to 135 μM. Table 4 shows that the highest dNTP concentration altered template selection by less than 60% in each case, while lower dNTP concentrations had much less effect (data not shown). Since these results occurred when the dNTP concentration was greater than the NTP concentration, conditions that are physiologically irrelevant, these data indicate that dNTPs play a minor role in determining where primase initiates primer synthesis.

DISCUSSION

We have used synthetic templates of defined sequence as well as a more natural DNA template to examine the role of NTPs and dNTPs in determining where primase initiates primer synthesis. At low NTP concentrations, primase greatly prefers to initiate primer synthesis at pyrimidine rich regions of a template. However, at higher NTP concentrations similar to those found in whole cells, primase readily initiates primer synthesis in all regions of the template. Importantly, initiation in all regions of the template is qualitatively similar to what is observed during DNA replication in whole cells. In contrast to the large changes in initiation site selection caused by varying the NTP concentration, varying the dNTP concentration had little effect on start site selection.

A mechanism by which the concentration of NTPs will dramatically affect where primase initiates primer synthesis is provided in Scheme 1, a simplified situation where primase can synthesize a primer at either of two sites. At low NTP concentrations (i.e., $V_{\max}/K_M$ conditions, Table 2), primer synthesis will occur at the most preferred site since primase will have the opportunity to bind to and dissociate from both potential primer synthesis sites prior to primer synthesis. In contrast, high NTP concentrations will serve to 'trap' primase at the initiation site to which it initially binds by immediately converting the E•DNA binary complex into an E•DNA•NTP•NTP quaternary complex that is poised to initiate primer synthesis. As predicted by this model, primase initiates primer synthesis over all regions of the template at high NTP concentrations. Furthermore, while the concentrations of NTPs typically used for *in vitro* primase assays are much below the K_M (Table 2), the concentrations of NTPs found in whole cells are similar to or greater than the K_M.

Varying the concentration of NTPs can also affect where primase initiates primer synthesis by specifically altering the identity of the second nucleotide of the primer (Sheaff & Kuchta, 1993; Suzuki et al., 1993). This occurs because the first NTP to bind to the primase•DNA complex becomes the second nucleotide of the primer. For example, increasing the concentration of ATP during primer synthesis on d(TC)$_{30}$ increases the frequency with which GTP becomes the 5′-terminal nucleotide of newly synthesized primers (Sheaff & Kuchta, 1993). Thus, NTPs can affect where primase initiates primer synthesis in two ways: on a macroscopic scale, high NTP concentrations allow primase to initiate synthesis in all regions of the DNA, not just pyrimidine rich regions, and on a microscopic scale, high NTP concentrations greatly influence which nucleotide is found as the second nucleotide of the primer.

A high concentration of an NTP may also influence primase initiation by an inhibition mechanism. This NTP may compete with the cognate NTP for binding to a primase•DNA•NTP complex, thus inhibiting primer synthesis at some sites. Consistent with this idea, we found that when synthesis of a primer required polymerization of at least two different NTPs, increasing the concentration of just one NTP caused stimulation of primer synthesis followed by inhibition (*vide infra*). Additionally, since primase has a remarkably low ability to discriminate against noncognate NTPs, as low as 1/30 in some cases (Sheaff & Kuchta, 1994), it can incorporate a noncognate NTP that may result in the production of an abortive primer (i.e., an RNA primer that cannot be elongated by pol α). Direct evidence that these mechanisms influence synthesis site selection comes from the ability of NTPs noncognate to the dinucleotide start site to affect where primase initiates primer synthesis (Table 3).

Immobilization of primase on a template and “trapping” it at a primer synthesis site appear to require formation of a primase•DNA•NTP•NTP complex, not just a primase•DNA•NTP complex. If formation of a primase•DNA•NTP ternary complex was sufficient to trap primase at an initiation site, addition of high concentrations of ATP should have resulted in formation of a primase•DNA•ATP complex at all thymidylate residues on a template. Since thymidylate residues are scattered throughout the ‘bubble’ template, this would have resulted in primer synthesis occurring throughout the template in primase-coupled pol α assays. This did not occur, thus indicating that formation of a primase•DNA•NTP complex is insufficient to trap primase at an initiation site. Rather, only when all four NTPs are raised to the levels found in whole cells does one observe all regions of the template being utilized for primer synthesis. Previous studies have shown that primase can slide along the DNA and likely binds DNA before NTPs (Sheaff & Kuchta, 1993). These data suggest that primase can slide along the DNA with one NTP bound and is not immobilized until it binds a second NTP.

The data herein suggest a two-stage model for primase start site selection. Initially, primase is recruited to the replication fork. This process may be facilitated by other proteins present at the replication fork, since both RPA and the SV40 large T antigen have been shown to interact directly with pol α-primase (Dornreiter et al., 1990, 1992). Next, primase may slide along the ssDNA a short distance until it has bound two NTPs. At this point the enzyme is immobilized on the template and is poised to synthesize the RNA primer. Under conditions of high NTP concentrations, the binding of the two NTPs is facilitated to such an extent that dissociation of the enzyme from the binding site is minimized. Hence, once the enzyme is bound to the template, it utilizes that binding site for initiation.

Using concentrations of NTPs similar to those found in whole cells solves the general problem of why primer synthesis occurs in all regions of the template. However, it is unclear if this is sufficient to precisely determine where primase will synthesize primers *in vivo*. Even at high NTP concentrations, some sites are greatly preferred for initiation of primer synthesis. Whole cell studies have also shown that while initiation can occur over the entire template, some sites are still greatly preferred for initiation (Hay et al., 1984). First of all, this could reflect template sequence effects that result in different rates of E•DNA•NTP•NTP polymerization as well as different K_Ds for NTPs. In addition, inhibition effects discussed earlier could lead to different efficiencies of elongation of dinucleotides to full length products. Second, the NTP concentrations that we used to mimic those found *in vivo* are averages of several different measurements using whole cells, and these values appear to vary substantially among different cell types and growth rate (Hauschka, 1973). We have also found that buffer conditions can substantially alter where primase synthesizes primers. For example, low levels of Mn^{2+} greatly increase primase activity and significantly alter start site selection on natural template sequences (B. Kirk and R. Kuchta, unpublished data). Finally, accessory proteins such as RPA and SV40 T antigen have been shown to directly interact with primase as well as to stimulate pol α-primase activity (Collins & Kelly, 1991; Erdile et al., 1991; Kenny et al., 1989; Melendy & Stillman, 1993; Tsurimoto & Stillman, 1989). Therefore, it would not be surprising if these accessory proteins also influence start site selection. Studies to understand how these other factors affect site selection are in progress.

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