Initiation of RNA-primed DNA synthesis *in vitro* by DNA polymerase α -primase

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ABSTRACT

The initiation of new DNA strands at origins of replication in animal cells requires *de novo* synthesis of RNA primers by primase and subsequent elongation from RNA primers by DNA polymerase α . To study the specificity of primer site selection by the DNA polymerase α -primase complex (pol α -primase), a natural DNA template containing a site for replication initiation was constructed. Two single-stranded DNA (ssDNA) molecules were hybridized to each other generating a duplex DNA molecule with an open helix replication 'bubble' to serve as an initiation zone. Pol α -primase recognizes the open helix region and initiates RNA-primed DNA synthesis at four specific sites that are rich in pyrimidine nucleotides. The priming site positioned nearest the ssDNA–dsDNA junction in the replication 'bubble' template is the preferred site for initiation. Using a 40 base oligonucleotide template containing the sequence of the preferred priming site, primase synthesizes RNA primers of 9 and 10 nt in length with the sequence 5'-(G)GAAGAAAGC-3'. These studies demonstrate that pol α -primase selects specific nucleotide sequences for RNA primer formation and suggest that the open helix structure of the replication 'bubble' directs pol α -primase to initiate RNA primer synthesis near the ssDNA–dsDNA junction.

INTRODUCTION

In animal cells, the initiation of DNA replication occurs at multiple origins throughout the genome. Two origins have been identified and are located near the dihydrofolate reductase and the β -globin genes (1–4). Initiation at these sites occurs over large regions of the DNA within the initiation zone (4–6). The *de novo* synthesis of RNA primers by primase provides the 3'-hydroxyl group for elongation by DNA polymerase (7–9). The tight physical association between primase and DNA pol α implicate this enzyme in RNA-primed DNA synthesis at origins (10–13). In addition, it is likely that pol α -primase functions during Okazaki fragment synthesis on the lagging DNA strand.

In vitro, pyrimidine homopolymers are the preferred templates for primase. Using natural ssDNA, RNA primers are synthesized at, or near, the 3'-end of pyrimidine-rich sequences (14–16). A purine nucleotide is the 5' residue of the RNA primer (8,14,17). The length of RNA primers is between 2 and 10 nt using poly(dT) and around 10 nt using natural DNA templates (16,18). The presence of a 3'-CC(C/A)-5' sequence positioned downstream from the primer site affects site selection and frequency of primer synthesis (19). The pol α -primase binds more tightly to longer polynucleotides than to shorter oligonucleotides (20). Thus, DNA sequence and structure might be important in origin recognition.

Primase synthesizes RNA primers that are elongated by DNA pol α . Synthesis of RNA primers occurs as a processive event (18) and the switch to DNA polymerization occurs without enzyme dissociation (21,22). The rate of RNA primer synthesis is slow relative to the rate of DNA polymerization (20) and pol α requires a primer of at least 7 nt to support DNA synthesis (18,23). The mechanism of primer synthesis is ordered with ssDNA template binding first, followed by two NTPs, such that the first NTP bound becomes the second NTP of the primer (20). Both primase subunits contain NTP binding sites and are required for optimal primer synthesis (21,24,25).

To investigate the DNA sequence and structure requirements for RNA-primed DNA synthesis by pol α -primase, we constructed a DNA template containing a replication 'bubble'. Using this DNA we demonstrate that pol α -primase initiates synthesis within the open helix region in a sequence-specific manner and that pol α -primase recognizes the secondary structure of the ssDNA–dsDNA boundary.

MATERIALS AND METHODS

Materials

Radiolabeled nucleotides were from Amersham. Unlabeled dNTPs and bovine serum albumin were from Sigma. The NTPs were from Pharmacia. Enzymes were from Promega or United States Biochemical. The four 21mer oligonucleotides (Oligos 1–4) were synthesized in the Cancer Center of Wake Forest University. The 40mer was synthesized and purified by Operon Technologies. The 10mer oligoribonucleotide was from Oligo's Etc. Phagemid DNA pBluescript II KS(–) and KS(+) were from

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Stratagene. Sequencing reactions were with T7 17mer primer hybridized to ssKS(+) DNA (26). The DNA markers were from Boehringer Mannheim and BRL. Fresh calf thymus was from Veal Co. (Madison, NC) and the DNA pol α -primase was purified as described (27). One unit of primase or DNA polymerase activity is the amount of enzyme required to catalyze the formation of 1 pmol acid-insoluble product/min at 37°C using poly(dT) for primase (7) and activated calf thymus DNA for pol α (27).

The ssDNA templates and RNA primer

Nucleotides 2–5 of pBluescript II KS(–) were changed to 5'-CTTG-3', generating a unique *S*tyI site in FP5(–) (28). The ssKS(+) and ssFP5(–) DNAs were prepared as described (29). Linear ssFP5(–) DNA was prepared by hybridizing Oligo 1 to nucleotides 709–729 and cleaving the partial duplex with *Hind*III. To generate the 830 nt DNA template, Oligo 1 and Oligo 2 (position 2840–2860) were hybridized to ssFP5(–) and cleaved with *Hind*III and *S*spI. The 470 nt DNA template was generated using Oligo 3 (position 2981–10) and Oligo 4 (position 462–482) and *S*tyI and *B*glI. The sequence of the 40mer is identical to nucleotides 77–116 of ssFP5(–). The synthetic RNA 10mer (5'-GGAAGAAAGC-3') was labeled with 32 P at the 5'-end and purified on a 20% urea-polyacrylamide gel.

RNA-primed DNA synthesis

Reactions (30 μ l) containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM ATP, 200 μ M CTP, GTP and UTP, 100 μ M dATP, dGTP and dCTP, 25 μ M [α - 32 P]TTP, pol α -primase (9.3 U pol α , 29 U primase) and the indicated amount of template were incubated at 37°C for the indicated times. Reactions were processed for scintillation spectroscopy by collecting acid-insoluble products on glass fiber discs. For agarose gels, reactions were precipitated with ethanol and resuspended in sample buffer containing 5% glycerol. For urea-polyacrylamide gels, reactions were stopped with 10 mM EDTA, eluted through Sephadex G-100 columns, dried and resuspended in 95% formamide. Dried gels were exposed to Kodak XAR-5 film and quantitated using Pharmacia ImageMaster.

Primase reactions

Reactions (10 μ l) containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM dithiothreitol, 0.1 mg/ml BSA, 100 μ M NTPs with [α - 32 P]ATP, 100 pmol 40mer DNA template and pol α -primase (0.7 U pol α , 6.3 U primase) were incubated at 37°C for the indicated times and stopped with 5 mM EDTA. Samples were dried, resuspended in 95% formamide and subjected to electrophoresis through urea-polyacrylamide gels.

RESULTS

Generation of the replication 'bubble' DNA template

A DNA replication 'bubble' template was designed with an open helix region (Fig. 1). The ssFP5(–) DNA is linearized using *Hind*III to cleave at the duplex region generated by hybridizing Oligo 1. The linearized ssFP5(–) DNA is incubated with the covalently closed ssKS(+) DNA to allow hybridization of complementary regions. This results in a 2503 nt region of dsDNA from nucleotide 460 to 1. The sequences at 2–459 in the ssKS(+)

Figure 1. Generation of the replication 'bubble' template. The Oligo 1 was hybridized to ssFP5(–) DNA and linearized with *Hind*III. The direction of the *f*1ori in each phagemid DNA is indicated by $\rightarrow$. In separate reactions, 1 μ g ssKS(+) was incubated at 37°C for 16 h with 0, 0.2, 0.4, 0.6, 0.8 or 1.0 μ g ssFP5(–) and the products were resolved on a 1% agarose gel (lanes 1–6). Lane 7 contains 1 μ g linear ssFP5(–) DNA only. Lane 8 contains the dsDNA plasmid KS(+). The positions of migration of the 'bubble' DNA, ssDNA and Form I, II and III DNA are indicated. In reactions containing (+) and (–) DNA (lanes 2–6), a small amount of DNA of unknown structure is detected in a band that migrates more slowly than the 'bubble' DNA.

and ssFP5(–) DNAs are identical, resulting in a 458 nt region of non-complementary DNA. The efficiency of replication 'bubble' formation was tested in reactions containing 1 μ g ssKS(+) DNA and increasing amounts of ssFP5(–) DNA (Fig. 1). Upon addition of increased amounts of ssFP5(–) DNA, an increased amount of DNA migrates to a position in the agarose gel that would be expected for the replication 'bubble' construct (Fig. 1, lanes 2–6). The phosphodiester bond between nucleotides 719 and 720 is reformed using T4 DNA ligase, generating covalently closed DNA in both strands. Greater than 95% of the molecules are ligated, as determined by the appearance of a 139 nt *B*ssHII–*S*acI DNA fragment (nucleotides 658–796) that contains the ligation site in the replication 'bubble' DNA template (data not shown).

Verification of the replication 'bubble' structure

Formation of the replication 'bubble' requires that complementary nucleotides of the two ssDNAs hybridize and that non-complementary nucleotides remain unhybridized. The replication 'bubble' DNA was digested with restriction enzymes and a ssDNA-specific endonuclease to verify its structure (Fig. 2). The enzymes *Hinc*II and *S*spI cleave at positions 736 and 2850 respectively, resulting in two DNA fragments of 2114 and 847 nt (Fig. 2A). Cleavage at the *S*spI site at position 442 is not expected,

Figure 2. Verification of the replication 'bubble' DNA structure. (A) The 'bubble' DNA and the plasmid pFP5(-) DNA were digested with the indicated enzymes. (B) The positions of migration of uncut 'bubble' DNA (lane 1) and the Form I and II plasmid pFP5(-) DNA (lane 2) are indicated. The products generated using *HincII* and *SspI* with the 'bubble' DNA (lane 3) and the plasmid pFP5(-) DNA (lane 4) and using *HincII* and *HaeII* with the 'bubble' DNA (lane 5) and the plasmid pFP5(-) DNA (lane 6) are shown. Lane M contains size standards and fragment sizes are indicated. (C) A reaction was prepared containing the 'bubble' DNA and mung bean nuclease. Aliquots were removed prior to nuclease addition (lane 1) and after 15, 30, 45, 60 and 180 s of incubation at 37°C (lanes 2-6). Lane M contains the 1 Kb DNA ladder.

since this site is positioned within the ssDNA region of the 'bubble' DNA. The 2114 and 847 nt fragments are detected upon incubation of the 'bubble' DNA with *HincII* and *SspI* (Fig. 2B, lane 3). As expected, digestion of the plasmid pFP5(-) DNA with *HincII* and *SspI* results in fragments of 2114, 553 and 294 nt (Fig. 2B, lane 4). Digestion of the 'bubble' DNA with *HincII* and *HaeII* results in fragments of 2296, 370 and 294 nt (Fig. 2B, lane 5) and digestion of the plasmid pFP5(-) DNA with these enzymes results in fragments of 1639, 649, 370 and 294 nt (Fig. 2B, lane 6). The 'bubble' and plasmid DNAs were also digested with *RsaI*, *BglI*, *NaeI* and *DraIII* enzymes (data not shown). For all 13 sites tested, the 'bubble' DNA template was cleaved at sites predicted to be dsDNA and not cleaved at sites predicted to be ssDNA. To directly test for ssDNA, the 'bubble' DNA was incubated with mung bean

Figure 3. RNA-primed DNA synthesis by pol α -primase using replication 'bubble' DNA. (A and B) Reactions containing 50 fmol 'bubble' DNA (■) or plasmid pFP5(-) DNA (●) were incubated at 37°C for the indicated times. Incorporation of nucleotide was determined by scintillation spectroscopy (A) and by agarose gel electrophoresis (B). Lane M contains 5' ³²P-labeled linear ssFP5(-) DNA. The 'bubble' DNA and linear ssFP5(-) are indicated. (C) A reaction was stopped after 10 min at 37°C, digested with *RsaI* and *BglI* and subjected to agarose gel electrophoresis (lane 4) with DNA size standards (lane 1), 'bubble' DNA digested with *RsaI* and *BglI* (lane 2) and ³²P-labeled DNA size standards (lane 3). Lanes 1 and 2 were stained with ethidium bromide and lanes 3 and 4 were dried and autoradiographed. The position and sizes of the DNA fragments and the 'bubble' fragment generated in the digest (lanes 2 and 4) are indicated.

nuclease and aliquots were removed in a time course reaction (Fig. 2C). An increase in mobility of the DNA is detected upon incubation with the ssDNA nuclease (Fig. 2C, lanes 2-6). The migration of the digested DNA indicates that the nuclease cleaves the ssDNA region, generating a fragment of 2500 nt. In contrast, the ssDNA nuclease does not affect the mobility of the plasmid pFP5(-) DNA (data not shown). These results indicate that the complementary regions between the (+) and (-) DNA strands hybridize to form stable dsDNA, while the non-complementary regions form an open helical ssDNA region.

RNA-primed DNA synthesis

Pol α -primase recognizes the ssDNA of the replication 'bubble' template and initiates RNA-primed DNA synthesis in this region (Fig. 3). Upon incubation of pol α -primase with the 'bubble' DNA, the amount of nucleotide incorporated into the DNA template increases with time and reaches a maximum of ~45 pmol (Fig. 3A). In reactions containing plasmid DNA, no synthesis is detected (Fig. 3A). These data indicate that pol α -primase synthesizes DNA fragments in the ssDNA region of the replication 'bubble'

Figure 4. DNA fragments synthesized by pol α -primase using the replication 'bubble' DNA. RNA-primed DNA synthesis reactions were incubated at 37°C for the indicated times and products were processed for electrophoresis on an 8% urea-polyacrylamide gel. The sizes of the DNA fragments were determined by comparison with the products of a ddNTP sequencing reaction (lanes A, C, G and T).

template. To demonstrate that polymerization is in the replication 'bubble' template and not in contaminant ssDNA, the products of RNA-primed DNA synthesis reactions were examined by agarose gel electrophoresis and autoradiography (Fig. 3B). Quantitation indicates that ~90% of the radiolabel in each lane migrates with the replication 'bubble' DNA. In addition, the amount of radiolabel in the band increases during the time course reaction (Fig. 3B). Approximately 5% of the radiolabel is detected in a slower migrating band that is generated during replication 'bubble' preparation (see Fig. 1). To further verify that the nascent DNA was synthesized within the open helix region, the radiolabeled DNA was localized to a DNA fragment that contains the replication 'bubble'. The products of an RNA-primed DNA synthesis reaction were digested with *RsaI* and *BglII* and subjected to agarose gel electrophoresis (Fig. 3C). The 871 nt fragment produced by the digest contains the replication 'bubble' region of the DNA template. The 'bubble' structure in this fragment causes it to migrate more slowly than a double-stranded 871 base fragment

Figure 5. Mapping of the pol α -primase initiation sites. In separate reactions, the purified 98, 141 and 254 nt fragments were hybridized to the 830 nt ssFP5(-) template. The DNA fragments were elongated from 98 to 210 (lane 1), 141 to 287 (lane 2) and 254 to 412 (lane 3) nt using Klenow (exo-) and dNTPs. A schematic of the 830 nt template and the deduced positions of the hybridized DNA fragments (1, 2 and 3) are shown. The nucleotide position 2-459 are the region of ssDNA in the 'bubble' DNA construct used to generate the three RNA-primed DNA fragments. The sizes of the DNA fragments were determined by comparison with the products of a ddNTP sequencing reaction (lanes A, C, G and T).

(Fig. 3C, lane 2). Autoradiography of the products shows a single radiolabeled band that migrates to the same position as the 'bubble' DNA fragment containing nascent DNA (Fig. 3C, lane 4). These results confirm that pol α -primase synthesizes DNA within the open helix region of the replication 'bubble' DNA.

Mapping the primase initiation sites

Pol α -primase initiates at specific sites within the open helix region of the replication 'bubble'. The size of the RNA-primed DNA fragments generated within the replication 'bubble' vary from ~80 to 350 nt (Fig. 4). Four distinct clusters of bands are detected, corresponding to DNA fragments of 80-110, 130-150, 240-260 and 340-360 nt. The clusters of radiolabeled bands result from RNA priming at unique initiation sites. The locations of the initiation sites were determined for three of the DNA fragments synthesized by pol α -primase using the replication 'bubble' template (Fig. 5). In separate reactions the purified DNA fragments were hybridized to an 830 nt template containing the 458 nt sequence of the open helix region of the replication 'bubble' DNA and elongated to the end of the template. The 98mer DNA fragment is elongated to a 210mer (Fig. 5, lane 1), the 141mer to

Figure 6. The replication 'bubble' structure affects primer site selection. RNA-primed DNA synthesis reactions containing 50 fmol 470 nt ssDNA (lanes 1-4) or 'bubble' DNA (lanes 5-10) were incubated at 37°C for the indicated times. Products were processed for electrophoresis on an 8% urea-polyacrylamide gel and the amount of DNA present in each of the clusters of bands was quantitated. The sizes of the DNA fragments were determined by comparison with the products of a ddNTP sequencing reaction (lanes A, C, G and T).

a 287mer (Fig. 5, lane 2) and the 254mer to a 412mer (Fig. 5, lane 3). From these data the initiation sites are located to approximately nucleotides 99, 176 and 301 within the ssDNA region of the replication 'bubble' template. These three sites contain 63-90% pyrimidine nucleotides (Table 1), demonstrating that pol α -primase initiates RNA-primed DNA synthesis at pyrimidine-rich nucleotide sequences in the replication 'bubble' region.

Table 1. Position of pol α -primase initiation sites

DNA fragment ^a	DNA template sequence at the initiation sites
80-110	cgcccgctccttcgctttctccctct
130-150	ctcccttaggtggtccgatttagtgcttta
240-260	cacgttctttaatagtggactctgttcca

^aSee Figure 5 for details.

DNA template structure affects primer site selection

To test the effect of the replication 'bubble' structure on RNA-primed DNA synthesis, we measured nucleotide incorporation in time course reactions using the 'bubble' DNA template and a 470 nt ssDNA template of identical sequence (Fig. 6). The products obtained using the ssDNA template are similar to those obtained using the replication 'bubble' DNA, indicating that pol α -primase recognizes the same four sites in both templates. Quantitation indicates that the rates of accumulation of the four

Figure 7. Site-specific priming using a 40 base oligonucleotide template. Primase reactions were incubated at 37°C for the indicated times. NE indicates no enzyme. The 9mer and 10mer products in the 20% urea-polyacrylamide are indicated. The DNA sequence of the 40mer DNA template is shown.

different sizes of DNA fragments using the 470 nt ssDNA are approximately equal (Fig. 6, lanes 1-4). In contrast, using the replication 'bubble' DNA, the 80-110 and the 130-150 nt fragments accumulate 20- and 10-fold more rapidly respectively than the larger DNA fragments (Fig. 6, lanes 5-10). It has been demonstrated that the rate of RNA primer synthesis by primase is 'slow' relative to DNA synthesis by pol α (20). Therefore, the more rapid accumulation of products from initiation sites proximal to the ssDNA-dsDNA junction in the replication 'bubble' DNA likely reflects a greater rate of RNA primer synthesis relative to the rate at the two more distal sites. These results suggest that pol α -primase recognizes the junction between ssDNA and dsDNA in the replication 'bubble' template.

Site-specific priming using a 40 base oligonucleotide

The precise size and position of RNA primers synthesized by primase were determined using a 40 base oligonucleotide template (Fig. 7). The sequence of the oligonucleotide corresponds to the initiation site of DNA fragment 1 using the replication 'bubble' DNA template (see Fig. 5). The ability of the 40mer DNA to support primer synthesis is demonstrated by monitoring the incorporation of [α -³²P]ATP into RNA oligomers (Fig. 7). The major products generated in a time course reaction correspond to oligomers 9 and 10 nt in length (Fig. 7). Whether primer synthesis initiates at the same nucleotide using the 40mer and the replication 'bubble' templates was not determined. However, the RNA oligomers generated using the 40mer template and the position of DNA fragment 1 generated using the replication 'bubble' template indicate that primase recognizes the same pyrimidine-rich sequence.

Figure 8. The 3' nucleotides of the 9mer and 10mer products. The 9mer and 10mer generated in primase reactions (A) and the chemically synthesized 10mer (B) were extended with Klenow (exo⁻) and 100 μ M ddGTP (lane 2), dGTP and ddATP (lane 3), dGTP, dATP and ddCTP (lane 4), dGTP, dATP, dCTP and ddTTP (lane 5) and dNTPs (lane 6). The DNA sequence on the right of each panel indicates nascent DNA. The positions of ddNTP incorporation are indicated. The deduced positions of the 9mer and 10mer RNA on the 40 base template are shown.

The 3'-terminal nucleotide of the 9mer and 10mer products correspond to a cytidine residue incorporated opposite a guanosine at position 22 in the 40mer DNA template. The identity of the 3' nucleotides was determined by extending the 9mer and 10mer products in reactions containing Klenow (exo⁻) and ddNTPs (Fig. 8). The migration of the terminated products identifies the position of the 3'-terminus of the RNA primer. The products of the primase reaction are predominantly 9 and 10 nt (Fig. 8A, lane 1). Elongation in the presence of ddGTP results in an 11 nt product (Fig. 8A, lane 2). With dGTP and ddATP, a 12 nt product is detected (Fig. 8A, lane 3). In the presence of dGTP, dATP and ddCTP two bands are detected, corresponding to termination opposite guanosine in the DNA template (Fig. 8A, lane 4), and in the presence of dGTP, dATP, dCTP and ddTTP a band is detected opposite adenosine in the DNA template (Fig. 8A, lane 5). In addition, in the ddCTP- and ddTTP-containing reactions, two bands are detected migrating to adjacent positions in the gel (Fig. 8A, lanes 4 and 5). These results indicate that the 9mer and 10mer result from initiation at one of the two adjacent cytidine residues located at positions 30 and 31 in the 40mer template and terminate at the guanosine residue located at position 22. To confirm this result, an RNA 10mer corresponding to the deduced nucleotide sequence of the primase-generated 10mer was synthesized, hybridized to the 40mer template and extended using the same ddNTP mixtures (Fig. 8B). The similar products obtained using the chemically synthesized 10mer and the primase-generated primers verifies the identity of the 3'-terminal nucleotides.

The positions of the 9mer and 10mer products indicate that a guanosine nucleotide is the first nucleotide incorporated. To

Figure 9. The 5' nucleotides of the 9mer and 10mer products. The 9mer and 10mer primase products were purified, 5' 32 P-labeled and subjected to alkaline hydrolysis in 0.1 M NaOH for 15 min at 100°C. The 32 P-labeled 10mer before (lane 3) and after hydrolysis (lane 4) and the 32 P-labeled 9mer before (lane 5) and after hydrolysis (lane 6) were subjected to electrophoresis on a 23% urea-polyacrylamide gel. Lanes 1 and 2 contain 32 P-A-p and 32 P-G-p markers respectively. The deduced sequences of the 9mer and 10mer primers on the 40 base template are shown.

identify the 5'-terminal nucleotide, the 9mer and 10mer products were purified and subjected to alkaline hydrolysis (Fig. 9). A single band of radioactivity is detected upon hydrolysis of the 9mer and 10mer primers and the radiolabeled bands migrate to the same position as a guanosine nucleotide. These results demonstrate that the 5'-terminal nucleotides of both the 9mer and 10mer primase products are guanosines. Thus, primase initiates RNA primer synthesis at a cytidine residue, synthesizes a 9 or 10 nt RNA primer and terminates at a guanosine residue.

DISCUSSION

The requirement for pyrimidine nucleotides at initiation sites is apparent from results obtained using natural DNA (14,16,19) and homopolymer templates (7-9). This preference is further substantiated by the ability of primase to utilize these sequences whether present in a large ssDNA or in short oligonucleotides (16, this work). However, not all pyrimidine-rich regions support RNA primer formation, suggesting that a high pyrimidine nucleotide content is not sufficient to constitute an RNA priming site. Experiments using oligonucleotides that contain priming sites indicate that primase binds to a specific position within the pyrimidine-rich region and initiates RNA primer synthesis (16, this work). A detailed analysis of the reaction mechanism indicates that primase binds ssDNA, slides along the DNA to an initiator site, binds two NTPs and initiates primer synthesis (20). Perhaps, specific initiator site sequences reduce primase sliding and promote RNA primer synthesis by decreasing the mobility of primase on ssDNA. Thus, the presence of a specific initiator site would be expected to increase the binding affinity of primase for a pyrimidine-rich oligonucleotide template.

The sequences of primer initiation sites indicate that purine nucleotides are required as the first two NTPs polymerized into

RNA primers (14–16). At the specific site identified in this study, the most prominent RNA primer synthesized is 10 nt in length, with guanosines as the first and second nucleotides. Using DNA containing the SV40 origin it was shown that high concentrations of ATP promote initiation of RNA primer synthesis opposite template thymidine residues at sites containing 3'-CTTT and high concentrations of GTP promoted initiation opposite template cytidine residues at sites containing 3'-CCC (14). The effect of nucleotide concentrations on primer site selection might reflect the different binding affinities of primase for different initiator sequences (19). It seems likely that relative binding affinities of primase for pyrimidine-rich DNA sequences and the ATP/GTP nucleotide concentrations influence priming site selection.

Synthesis of RNA-primed DNA fragments by pol α -primase *in vivo* might require the enzyme to bind a large region of ssDNA and translocate to a primer initiation site. This idea is supported by the lower binding affinity of primase for short oligonucleotides relative to longer DNA templates (20) and the lack of a detectable primase footprint at initiator sites using oligonucleotide templates (16). The apparent lack of an exact initiator DNA sequence for primase suggests that additional structural elements in the DNA template, such as inverted repeats (30) or replication forks, might be required to direct pol α -primase to initiation sites. At origins of replication, opening of the DNA helix establishes a ssDNA-dsDNA junction. It is possible that replication proteins recognize this structure and associate at these sites. The SV40 T-antigen recognizes replication fork structure (31) and T-antigen binds directly to pol α -primase (32). Our results support the concept that pol α -primase 'enters' the open helix region of DNA at the ssDNA-dsDNA junction, translocates along the ssDNA in the 5'→3' direction and initiates RNA primer synthesis at the first available RNA priming site. Using the replication 'bubble' DNA, the first RNA-primed DNA fragments that are detected initiate from the priming site positioned nearest the ssDNA-dsDNA junction. The second DNA fragment to accumulate initiates from the priming site located the next greatest distance from the junction. This result is in contrast to that obtained using the 470mer DNA that lacks replication fork structure, where the four pyrimidine-rich regions are used with equal efficiencies. The preference for priming at the site nearest the ssDNA-dsDNA junction using the replication 'bubble' template and not the ssDNA template of identical sequence indicates that the replication fork plays a role in primer site selection.

Additional proteins in cells that bind to DNA at origins or interact directly with pol α -primase might influence RNA-primed DNA synthesis (14,15,33–35). This is supported by the observation that different primer sites are utilized by pol α -primase and a multiprotein pol α -primase complex (14,15). This difference is attributed to the presence of primer recognition proteins within the multiprotein complex (15). The ssDNA binding protein RP-A interacts directly with pol α -primase and might play a role in directing primase to primer initiation sites (36,37). However, the synthesis of RNA primers by purified pol α -primase at specific sites indicates that specificity of primer site selection is at least partially inherent in the interaction of primase with ssDNA. Additional proteins that play a role in synthesis of the first RNA-primed DNA fragment might be identified as factors that affect primer site selection or stimulate primer synthesis by pol α -primase using the replication 'bubble' DNA template.

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