Termination within Oligo(dT) Tracts in Template DNA by DNA Polymerase γ Occurs with Formation of a DNA Triplex Structure and Is Relieved by Mitochondrial Single-stranded DNA-binding Protein*

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Victor S. Mikhailov‡ and Daniel F. Bogenhagen§

From the Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794

Xenopus laevis DNA polymerase γ (pol γ) exhibits low activity on a poly(dT)-oligo(dA) primer-template. We prepared a single-stranded phagemid template containing a dT41 sequence to test the ability of pol γ to extend a primer through a defined oligo(dT) tract. pol γ terminates in the center of this dT41 sequence. This replication arrest is abrogated by addition of single-stranded DNA-binding protein or by substitution of 7-deazadenosine 5′-triphosphate (dATP) for dATP. These features are consistent with the formation of a TA:T DNA triplex involving the primer stem. Replication arrest occurs under conditions that permit highly processive DNA synthesis by pol γ. A similar replication arrest occurs for T7 DNA polymerase, which is also a highly processive DNA polymerase. These results suggest the possibility that DNA triplex formation can occur prior to dissociation of DNA polymerase. Primers with 3′-oligo(dA) termini annealed to a template with a longer oligo(dT) tract are not efficiently extended by pol γ unless single-stranded DNA-binding protein is added. Thus, one of the functions of single-stranded DNA-binding protein in mtDNA maintenance may be to enable pol γ to successfully replicate through dT-rich sequences.

Replication of vertebrate mtDNA is accomplished by DNA polymerase γ (pol γ) through a strand displacement mechanism. Although the overall features of this replication scheme have been known for some time (1, 2), the replication machinery remains poorly understood. The central component of the replication apparatus, pol γ, displays a processive polymerase activity and a tightly associated proofreading 3′→5′ exonuclease (3–8). The sequences of pol γ genes reveal both polymerase and exonuclease domains within a single catalytic subunit of ~140 kDa (9–11). Both domains show a primary sequence relationship with Escherichia coli DNA polymerase I as the prototype of the family A DNA polymerases.

The only well studied accessory protein known to be involved in mtDNA replication is the single-stranded DNA-binding protein (mtSSB). mtSSBs have been characterized in a number of organisms as tetrameric proteins with homology to the amino-terminal portion of E. coli SSB (12–14). The functional properties of mtSSBs also resemble those of E. coli SSB. These similarities include a binding site size of ~30–40 nucleotides that increases with increasing ionic strength and a preference for binding pyrimidine-rich sequences (15–18). mtSSBs are likely to be essential for mtDNA maintenance in virtually all eukaryotes, although this has been established with genetic methods only in the facultative anaerobe Saccharomyces cerevisiae (19). The mitochondrial and E. coli SSB proteins have similar effects on DNA polymerase activity in vitro. mtSSB stimulates DNA synthesis catalyzed by mitochondrial pol γ (see below) and also by E. coli DNA pol I (15), while E. coli SSB can stimulate Drosophila pol γ (20).

Previous reports of the effect of mtSSB on the activity of pol γ have revealed inconsistencies. For example, Mignotte et al. (21) reported that Xenopus mtSSB could stimulate the activity of the homologous partially purified pol γ up to 1.5-fold on poly(dA)oligo(dT) and ~3-fold on poly(rA)oligo(dT), while it inhibited pol γ activity on singly primed single-stranded M13 DNA. This is in apparent contradiction to the report by Hoke et al. (15) that rat liver mtSSB did not affect the activity of homologous pol γ on poly(dA)oligo(dT), although it increased the activity on poly(dT)oligo(dA) by ~10-fold. It is difficult to compare these studies since these two groups did not use the same templates and the pol γ used by Mignotte et al. (21) was not highly purified.

We investigated the effect of Xenopus mtSSB on the activity of highly purified Xenopus pol γ on a variety of templates. In our experiments, Xenopus mtSSB markedly stimulated the activity of homologous pol γ on poly(dT)oligo(dA) and singly primed single-stranded M13 DNA. In the course of these experiments, we discovered that a relatively short oligo(dT) tract can present an effective barrier to elongation by pol γ. Experiments are presented that suggest that this block is caused by the dynamic formation during replication of a triple-stranded structure reminiscent of H-DNA. Previous studies with other DNA polymerases have shown that sequences with dinucleotide repeats capable of forming H-DNA can block DNA synthesis (22, 23). The H-DNA sequences that have been studied most thoroughly include C-G·C+ base triads, which require protonation of C residues (C+). The observation reported here, that oligo(dT) tracts can block replication in a similar manner, has not been reported previously to our knowledge. mtSSB abrogates this block to elongation.
EXPERIMENTAL PROCEDURES

Nucleotides and Nucleic Acids—[γ-32P]ATP, [α-32P]dATP, and [α-32P]dCTP were from ICN Radiochemicals. Deoxyribonucleoside 5’- triphosphates were from Pharmacia Biotech, Inc. Oligonucleotides dA16, dT16, and dT16 were generously donated by Dr. N. Bulychev (IBC, Novosibirsk, Russia). Poly(dT) and poly(dA) were purchased from Sigma.

Concentrations of the oligonucleotides and polynucleotides were determined spectrophotometrically using extinction coefficients of 8,100 M⁻¹ cm⁻¹ for poly(dT) and 10,000 M⁻¹ cm⁻¹ for poly(dA) (24). Single-stranded M13 DNA was prepared by a standard method (25). All other chemicals were from commercial sources and were of analytical grade.

Single-stranded pSK DNA templates with oligopyrimidine stretches were separated on an 8% polyacrylamide/8 M urea sequencing gel. After dTMP on poly(dA) acid-insoluble radioactivity was measured by Cerenkov counting. 1 unit changes of cold 5% trichloroacetic acid, 1% sodium pyrophosphate. The pieces of Whatman No. 3MM paper, which were washed in several mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM MgCl₂, 2 mM dithiothreitol, 200 mM glycerol-containing buffers; thus, the final concentration of glycerol ranged 0.1 M NaCl. The mixtures were incubated at 65°C for 10 min and allowed to cool slowly to room temperature. For elongation assays with prelabeled primers, the oligonucleotides were labeled at 5’-ends using [γ-32P]ATP and T4 polynucleotide kinase before annealing to the templates.

DNA Polymerase γ Assay—The standard pol γ assay contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM MgCl₂, 2 mM dithiothreitol, 200 μg/ml bovine serum albumin, 5 μg/ml primer-template, and 50 μM concentrations of each dNTP except for the radiolabeled one, which was 5–25 μM (25–50 μM) in a 30-μl reaction. Reactions utilizing poly(dA) or poly(dT) as template contained only dTTP or dA, respectively, as dNTP substrate. pol γ and mtSSB were added to the reaction in glycerol-containing buffers; thus, the final concentration of glycerol ranged from 10 to 15%. Reactions were carried out at 30°C for 30 min and were terminated by placing in an ice bath and adding 3 μl of 0.25 M EDTA in saturated sodium pyrophosphate. The samples were transferred onto pieces of Whatman No. 3MM paper, which were washed in several changes of cold 5% trichloroacetic acid, 1% sodium pyrophosphate. The acid-insoluble radioactivity was measured by Cerenkov counting. 1 unit of DNA polymerase activity was defined as incorporation of 1 nmol of dTTP in poly(dA)·dT16 in 60 min at 30°C.

Elongation Assay— Primer utilization and elongation of nascent chains were monitored by electrophoresis of the reaction products on polyacrylamide-urea gels. Reactions were carried out in 10-μl samples containing 5–32P-labeled primer annealed to unlabeled template and other ingredients as indicated above except that the radioactive dNTP was omitted. After incubation at 30°C, the reactions were terminated by placing the samples in an ice bath followed by the addition of 7 μl of loading buffer (90% formamide, 25 mM EDTA, 0.01% each bromphenol blue and xylene cyanol). Reaction products were boiled for 10 min and separated on an 8% polyacrylamide/8 M urea sequencing gel. After electrophoresis, gels were dried onto Whatman DE81 paper under vacuum and exposed at –80°C to Kodak X-Omat AR film. Radioactivity was analyzed using a Phosphorimage (Molecular Dynamics).

Protein Assay—Protein was quantified using bovine serum albumin as a standard in the Bradford dye-binding assay (26).

RESULTS

The effect of xl-mtSSB on the rate of DNA synthesis by pol γ was investigated using a variety of DNA primer-templates (Fig. 1). MtSSB markedly increased polymerization on poly(dT)·oligo(dA) and singly primed single-stranded phage M13 DNA at 50 mM KCl. On both templates, the stimulatory effect was highly dependent on the monovalent salt concentration in the reaction mixture, since little stimulation was observed at 100 mM KCl (data not shown). Even in the presence of mtSSB, the maximal rate of DNA synthesis is considerably lower on poly(dT)·dA16 than on M13 DNA. In contrast, mtSSB did not significantly affect the pol γ activity on poly(dA)·dT16 (Fig. 1C). This is a very favorable template for pol γ even in the absence of mtSSB, as evidenced by the absolute rates of DNA synthesis reflected by the different scales used for the three plots in Fig. 1. Our results with highly purified pol γ are in agreement with those reported by Hoke et al. (15) using rat liver pol γ. We suggest that the lack of stimulation by mtSSB of X. laevis pol γ activity on M13 DNA reported by Mignotte et al. (21) is due to the use of a crude pol γ fraction that may have been contaminated with mtSSB.

The stimulation by mtSSB of DNA synthesis on singly primed M13 DNA may be explained by the ability of this accessory protein to prevent the formation of secondary structures in the template strand that impede the progress of pol V. mtSSB increases the sensitivity of replication but does not increase primer utilization (18). The poor template activity of poly(dT) presented a mystery, since this template lacks stable secondary structure. We constructed a single-stranded DNA template containing an oligo(dT) sequence, pSK-T41, to determine whether this pyrimidine tract would present a barrier to replication by pol γ. In the experiment shown in Fig. 2, the elongation of a 5’-end-labeled primer by pol γ on pSK-T41 was monitored by analysis of the extension products on a DNA sequencing gel. A prominent pause or termination site was observed in the center of the oligo(dT) tract representing nascent DNA strands containing –18 3’-terminal dAMP residues (Fig. 2, lanes 4–6; band P). This replication arrest resembles the block to replication in a

![Graph](image-url)
Replication Arrest with DNA Triplex Formation

**Fig. 2.** Pol γ pauses during polymerization inside an oligo(dT) motif in template DNA. Three standard polymerase reactions (each with a final volume of 21 μl) were assembled containing 5 μg/ml pSK-T41 DNA primed by 32P-labeled 17-mer M13 (-20) primer. Reaction 1 (lanes 4–6) contained no additions; reaction 2 (lanes 7–9) contained 20 μg/ml mtSSB; reaction 3 (lanes 10–12) contained 0.1 mM 7-deaza-dATP instead of dATP. After preincubation for 3 min at 30°C, 1 μl of pol γ (0.03 unit) was added to each reaction, and incubation at 30°C was continued. Portions from the reactions were removed at indicated times, mixed with the loading buffer, and analyzed by electrophoresis in an 8% polyacrylamide, 8M urea gel. Migration of the standard, 5'-32P-labeled MspI digest of pUC18 DNA and sequencing ladders, C and A are shown, respectively, in lanes 1–3. Lanes 4–9, mobility of strands arrested at a duplex hairpin (band D) or in the oligo(dT) tract (band P) as discussed in the text.

**Fig. 3.** Structure of a T-A-T DNA triplex. A, as polymerization proceeds through an oligo(dT) tract, interaction of the template strand head of the replication fork with the nascent DNA duplex permits formation of a DNA triplex. B, Hoogsteen base pairing of the third strand. C, structure of a T-A-T base triad adapted from Ref. 23.

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oligo(dT) tract as observed for pol γ. Each enzyme generates a different characteristic set of partially replicated products. The pattern of arrested strands produced by pol γ most closely resembled the pattern produced by T7 DNA pol. For T4 DNA pol, only a minor pause was observed in the oligo(dT) tract. It is interesting to contrast the behavior of the large (Klenow) fragment of pol I, which exhibits low processivity, with those of the other three polymerases, all of which are replicative enzymes capable of highly processive DNA synthesis. At the earliest time point, the Klenow polymerase arrested replication after incorporation of only 11–14 dA residues (Fig. 5, lane 12). These strands were slowly elongated to the midpoint of the oligo(dT) tract. With continued incubation, a fraction of the nascent chains arrested in the oligo(dT) tract were extended (Fig. 5, lane 14). It appears that a more distributive mode of replication favors arrest within the oligo(dT) tract. Nevertheless, the main conclusion provided by the data in Fig. 5 is that DNA polymerases from a variety of sources share a common propensity to arrest DNA replication within oligo(dT) stretches in the DNA template.

Several experiments were performed to characterize further the phenomenon of DNA synthesis arrest in an oligo(dT) tract. First, polymerase reactions were performed at a range of salt concentrations. The processivity of pol γ is increased at low ionic strength, so that chains of several hundred nucleotides can be synthesized without dissociation of the enzyme (20). The results shown in Fig. 6 indicate that a significant fraction of nascent chains paused or terminated within the oligo(dT) tract in pSK-T14, in reactions performed at salt concentrations ranging from 20 to 120 mM KCl. At early time points, ~55–60% of nascent chains arrested in the oligo(dT) tract at every salt concentration tested (Fig. 6B). These results are consistent with the possibility that the arrest of DNA synthesis results from formation of a triplex structure while the DNA polymerase is still engaged on the primer-template.

We performed the experiment shown in Fig. 7 to determine whether the synthesis of aborted nascent chains represents premature termination of replication or pausing of the polymerase. Polymerase reactions were set up to follow elongation of an end-labeled primer annealed to pSK-T14, as in Fig. 2. After 1, 3, or 10 min of incubation with pol γ, a 20-fold excess of unlabeled singly primed M13 template was added as a trap for free polymerase, and the reactions were incubated for a further 10 or 20 min. The effectiveness of this competitor “trap” procedure is confirmed by the lack of continued utilization of 17-mer primers during the “chase.” We reasoned that if the DNA polymerase had paused in the center of the oligo(dT) tract and was still capable of resuming processive DNA synthesis, it would be able to continue elongation during the chase. If this occurred, band P containing arrested strands would become less intense during the chase. To quantify the extension of arrested strands, we measured the ratio of the intensity of band P to that of unused 17-mer primers during the chase following each of the three pulse intervals, as shown in Fig. 7B. The three lines graphed in Fig. 7B indicate that nascent chains arrested in the center of the oligo(dT) tract (band P) during the initial pulse were not utilized during the chase interval in the presence of an excess of competitor primer-templates. We conclude that most replication products arrested in the center of the oligo(dT) tract represent true termination events.

An oligo(dT) tract is not an absolute barrier to elongation by pol γ. As shown in Fig. 6, there is a significant probability that pol γ can pass through this sequence following a single binding event. An alternative mechanism for replication through an oligo(dT) tract is that pol γ may be able to bind and extend primers generated following termination in an oligo(dT) tract. To study this latter mechanism, we reconstituted triplex struc-
tures of the sort formed during DNA synthesis on single-stranded pSK-T41. Primers with 3'-oligo(dA) ends were prepared by elongating 5'-32P-labeled 17-mer M13 primers annealed to pSK-T41 using the large (Klenow) fragment of pol I. As shown in Fig. 5, lane 12, extension for 1 min generated nascent chains averaging 87 nucleotides in length, containing 11–14 3'-terminal dA residues. Extension for ~3 min generated nascent chains averaging 91 nucleotides in length, containing 16–18 3'-terminal dA residues (Fig. 5, lane 13). Extended oligonucleotides in these two size classes were purified by electrophoresis in an 8% polyacrylamide, 8 M urea gel. A, autoradiogram of the dried gel. B, fraction of chains with 3' ends within the oligo(dT) tract (band P) following 1 min of primer extension at each salt concentration as determined by PhosphorImager analysis of the dried gel.

Fig. 6. Effects of monovalent salt and incubation time on replication arrest by pol γ at the oligo(dT) motif. Four standard polymerase reactions (each of final volume 21 µl) containing 5 µg/ml pSK-T41 DNA primed by 32P-labeled 17-mer M13 (-20) were assembled at varied KCl concentrations. After preincubation for 3 min at 30 °C, 1 µl of pol γ (0.03 unit) was added to each reaction, and incubation at 30 °C was continued. Portions from the reactions were removed at the indicated times, mixed with the loading buffer, and analyzed by electrophoresis in an 8% polyacrylamide, 8 M urea gel. A, autoradiogram of the dried gel. B, fraction of chains with 3' ends within the oligo(dT) tract (band P) following 1 min of primer extension as determined by PhosphorImager analysis of the dried gel.

Fig. 7. The arrest of DNA synthesis in the oligo(dT) tract represents termination of replication. Standard polymerase reactions (80 µl) were assembled containing 1 µg/ml pSK-T41 DNA primed by 32P-labeled 17-mer M13 (-20) primer. After preincubation for 3 min at 30 °C, the reaction was started by the addition of 0.8 µl of pol γ (0.024 unit), and incubation at 30 °C was continued. Portions (18.9 µl) from the reaction were removed after 1-min (lanes 3–5), 3-min (lanes 6–8), and 10-min (lanes 9–11) pulse and mixed with 2.1 µl of the competitor “trap” DNA (0.2 mg/ml nonradioactive singly primed M13mp7 DNA). Six-µl aliquots from the mixtures were taken immediately after mixing with the trap and after incubation at 30 °C for 10-min and 20-min chase periods. Aliquots were taken also from the initial reaction without the competitor “trap” DNA to monitor its time course after incubation for 20 min (lane 12) and 30 min (lane 13). All samples were mixed with the loading buffer and analyzed by electrophoresis in an 8% polyacrylamide, 8 M urea gel. A, autoradiogram of the dried gel. Sequencing ladders C and A are shown, respectively, in lanes 1 and 2. B, ratio of primers arrested in the oligo(dT) tract (band P) to unused 17-mer primers as a function of the chase time following initial extension for 1 min (●), 3 min (■), or 10 min (▲).
polymerases by Lapidot et al. (22) and Baran et al. (23) on templates containing CT dinucleotide repeats. Both of these template sequences appear to permit formation of DNA triplex structures based on the criterion that replication arrest does not occur in the presence of 7-deaza-dATP (or 7-deaza-dGTP for d(CT)n). Substitution of 7-deaza-dATP for dATP dramatically stimulates DNA synthesis by pol γ on oligo(dA)-poly(dT) primer-template (data not shown). We showed that replication arrest in oligo(dT) sequences is observed for other DNA polymerases as well as for pol γ. One novel feature of the T-A-T triplex studied in our experiments is that Hoogsteen base pairing in this base triad does not require low pH. We have not systematically studied the sequence features that might permit DNA triplex arrest. We have found that oligo(dT) tracts as short as 22 bases can induce a significant amount of replication arrest (data not shown). It is reasonable to assume that a variety of pyrimidine-rich sequences with mirror symmetry will induce DNA triplex arrest. Replication arrest in mixed sequences with a higher proportion of template cytosine residues is expected to show greater dependence on low pH.

**What Is the Mechanism for Replication Arrest in Sequences with the Potential to Form DNA Triplices?** A number of studies have documented the ability of preformed DNA triplex structures to block chain elongation when the triplex is located ahead of an advancing replication fork. It is easy to visualize how replication can be blocked by triplex structures containing oligonucleotides annealed to single-stranded DNA (28, 29) or triplex structures created during strand displacement synthesis (30). Understanding how a DNA triplex involving the nascent strand can be formed during replication is less obvious. In the original studies of replication arrest with d(CT)n sequences, Lapidot et al. (22) used the large (Klenow) fragment of DNA polymerase I and calf thymus pol α. Both of these enzymes synthesize DNA with high processivity (31). Hence, Lapidot et al. (22) suggested that these DNA polymerases dissociate in the center of d(CT)n sequences and that the DNA triplex forms following polymerase dissociation. Once the DNA triplex is formed, binding of DNA polymerase is inhibited, and continued elongation is not favored, as shown for pol γ in Fig. 8.

We cannot rule out the possibility that this model applies to our experiments on replication arrest with pol γ. However, several observations suggest that replication arrest may be driven by dynamic formation of a DNA triplex during DNA chain elongation under some circumstances. First, we have observed replication arrest for both pol γ and T7 DNA polymerase, two enzymes known to synthesize DNA with high processivity (20, 31). Second, the pattern of prematurely terminated products observed for a relatively distributive enzyme, the Klenow fragment of pol I, is qualitatively distinct from that produced by pol γ or T7 DNA pol (Fig. 5). Finally, if polymerase pausing and dissociation at an oligo(dT) sequence were required for DNA triplex formation, we would expect to observe replication arrest during DNA synthesis in the presence of 7-deaza-dATP. This is not observed. The major difficulty of this model is that hydrogen bonding to permit formation of a DNA triplex requires close contact between the nascent DNA duplex and the template strand immediately ahead of the replication fork. The single-stranded segment ahead of the polymerizing center would need to fold back to permit the template strand to contact the major groove of the nascent DNA. This interaction would need to be accommodated within the active center of the DNA polymerase. Little is known concerning the path of DNA within the “reading head” of either pol γ or T7 DNA pol, since the structures of these enzymes have not been elucidated. Beese et al. (32) have solved the structure of the large fragment of DNA polymerase I complexed with DNA, but this work
provided a model for an editing complex and did not reveal the path of the template DNA strand ahead of the polymerase site. It may be that detailed differences between the structure of pol I and the more highly processive members of the family A subset of DNA polymerases will account for different behaviors of these enzymes in elongation through template sequence capable of DNA triplex formation. Additional structural information would help to determine whether a DNA triplex can form prior to dissociation of the DNA polymerase.

What Is the Biological Significance of DNA Triplex Formation in Oligo(dT) Tracts?—It is instructive to recall that the original discovery of replication arrest at dinucleotide repeats emerged from an effort to explain in vivo termination events at these sequences (22). Whether the sort of replication arrest coupled with DNA triplex formation we have observed can occur in vivo during mtDNA replication is an open question. The oligonucleotide-primed replication of an extensive single-stranded template used in our experiments is a valid model for the synthesis of the lagging strand of mtDNA in vertebrates. DNA triplex formation could be a factor in mtDNA replication if two conditions were met: (1) sequences capable of supporting DNA triplex formation would need to occur within mtDNA; (2) DNA triplexes could block mtDNA replication only under conditions of mtSSB depletion, since addition of mtSSB allows pol γ to overcome replication arrest.

The question of whether sequences with the potential to form DNA triplexes occur in mtDNA genomes is not straightforward, since the full range of sequences capable of causing replication arrest by DNA triplex formation is not well understood. Dayn et al. (33) have found that homopyrimidine-homopurine structure and mirror symmetry are not absolutely required for DNA triplex formation. The particular dT₄₅ sequence we have studied does not occur in X. laevis mtDNA. However, pyrimidine tracts or AT-rich regions with the appropriate symmetry to form DNA triplexes may occur in mtDNA genomes. The S. cerevisiae and Drosophila melanogaster mtDNA genomes are particularly AT rich and contain sufficiently long oligo(dT) tracts to impede replication by pol γ.

The question of whether the supply of mtSSB can be a limiting factor for replication is equally uncertain. Under conditions of depletion of mtSSB, as in a rim1 (mtSSB-deficient) yeast mutant (19), pol γ would be expected to have difficulty replicating the AT-rich S. cerevisiae mtDNA genome. This would provide a sufficient explanation for the requirement for mtSSB for mtDNA maintenance apart from the need to replicate through DNA hairpin structures. Little is known regarding the regulation of the mtSSB supply in higher eukaryotic cells. It will be interesting to determine whether there are physiological or pathological conditions in higher organisms in which mtSSB might be limiting for mtDNA replication.

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