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Mitochondrial RNA Polymerase Is Needed for Activation of the Origin of Light-Strand DNA Replication

Javier Miralles Fusté,[1,5] Sjoerd Wanrooij,[2,5] Elisabeth Jemt,[2] Caroline E. Granycome,[3] Tricia J. Cluett,[3] Yonghong Shi,[2] Neli Atanassova,[1] Ian J. Holt,[3] Claes M. Gustafsson,[2,4,*] and Maria Falkenberg[2,*]
[1]Division of Metabolic Diseases, Karolinska Institutet, Novum, SE-141 86 Stockholm, Sweden
[2]Department of Medical Biochemistry and Cell Biology, University of Gothenburg, P.O. Box 440, SE-405 30 Gothenburg, Sweden
[3]MRC-Mitochondrial Biology Unit, Hills Road, Cambridge CB2 0XY, UK
[4]Max-Planck-Institut für Biologie des Alterns, Gleueler Str. 50a, D-50931 Cologne, Germany
[5]These authors contributed equally to this work
*Correspondence: claes.gustafsson@medkem.gu.se (C.M.G.), maria.falkenberg@medkem.gu.se (M.F.)
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SUMMARY

Mitochondrial DNA is replicated by a unique enzymatic machinery, which is distinct from the replication apparatus used for copying the nuclear genome. We examine here the mechanisms of origin-specific initiation of lagging-strand DNA synthesis in human mitochondria. We demonstrate that the mitochondrial RNA polymerase (POLRMT) is the primase required for initiation of DNA synthesis from the light-strand origin of DNA replication (OriL). Using only purified POLRMT and DNA replication factors, we can faithfully reconstitute OriL-dependent initiation in vitro. Leading-strand DNA synthesis is initiated from the heavy-strand origin of DNA replication and passes OriL. The single-stranded OriL is exposed and adopts a stem-loop structure. At this stage, POLRMT initiates primer synthesis from a poly-dT stretch in the single-stranded loop region. After about 25 nt, POLRMT is replaced by DNA polymerase γ, and DNA synthesis commences. Our findings demonstrate that POLRMT can function as an origin-specific primase in mammalian mitochondria.

INTRODUCTION

Human mitochondria contain a double-stranded DNA genome (mtDNA) of 16.6 kb (Falkenberg et al., 2007). The genome harbors two origins of DNA replication (the heavy-strand origin, OriH, and the light-strand origin, OriL), and two alternative and partially overlapping models have been suggested for mtDNA replication (Berk and Clayton, 1974; Bogenhagen and Clayton, 2003; Clayton, 1991; Holt and Jacobs, 2003; Holt et al., 2000).

The strand-displacement model states that DNA synthesis is continuous on both strands and is performed in a strand-asymmetric manner (Figure 1). Unidirectional DNA synthesis from OriH/the noncoding region displaces the parental heavy strand (H strand), without simultaneous DNA synthesis on the lagging strand. After leading-strand synthesis has advanced two-thirds of the way around the genome, it reaches a small (30 bp) noncoding DNA region containing OriL. After the leading-strand replication fork passes OriL, the parental H strand is exposed as a single strand, and OriL adopts a stem-loop formation. How the stem-loop structure activates initiation of DNA replication is not known. It is, however, clear that OriL plays a crucial role in mtDNA metabolism, since no deleted replicating mtDNA molecules, lacking OriL, have been identified to date. It has been reported that strand-displacement replication could involve incorporation of RNA on the lagging-strand template (RITOLS model) (Yasukawa et al., 2006).

In recent years, the validity of the strand-displacement model has been questioned. Analyses of mitochondrial replication intermediates using two-dimensional neutral/neutral agarose gel electrophoresis (2DNAGE) identified conventional duplex mtDNA replication intermediates, which suggested that leading- and lagging-strand DNA syntheses may be coupled events (Holt et al., 2000). Moreover, atomic force microscopy demonstrated that initiation of lagging-strand DNA synthesis was not uniquely located to OriL, but additional initiation sites could be identified at several sites on the light strand (L strand) (Brown et al., 2005). However, individually, these other initiation sites may be used at low frequency, explaining why only OriL produces prominent free 5′ ends of DNA as predicted for initiation sites of lagging-strand mtDNA synthesis (Tapper and Clayton, 1981). An important question remaining to be addressed is whether DNA synthesis on the leading and lagging strands are coupled, i.e., physically linked via a complex containing DNA polymerases working on both the leading and lagging strand (strand-coupled), or if leading- and lagging-strand syntheses are physically unconnected processes (strand-asymmetric).

Transcription from the mitochondrial light-strand promoter (LSP) generates transcripts of almost genomic length, which are processed to yield the individual mRNA and tRNA molecules (Montoya et al., 1982). Transcription from the LSP also produces the RNA primers needed for initiation of mtDNA replication at OriH (Gillum and Clayton, 1979; Cantatore and Attardi, 1980; Montoya et al., 1982; Pham et al., 2006). RNA covalently

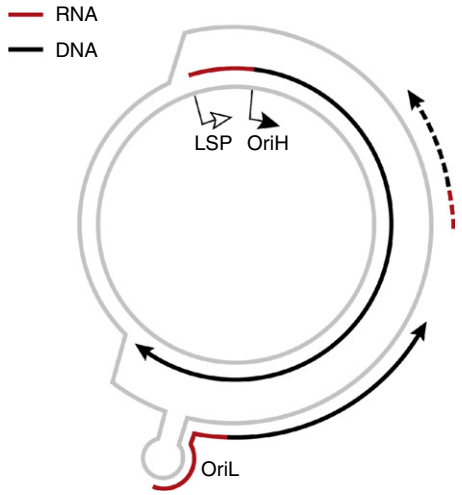

Figure 1. Origin-Specific Initiation of Strand-Displacement mtDNA Replication

Leading-strand DNA synthesis is initiated from the heavy-strand origin (OriH) of DNA replication and passes OriL. The single-stranded OriL is exposed and adopts a stem-loop structure. OriL is the major initiation site for lagging-strand mtDNA synthesis, but other minor initiation sites also exist. In contrast, initiation of strand-coupled mtDNA synthesis occurs across a broad zone of several kilobases (Bowmaker et al., 2003).

attached to the newly synthesized H strand has been detected in both mouse and human cells (Xu and Clayton, 1995, 1996).

The strand-displacement and the RITOLS models for mtDNA replication recognize OriL as a dominant site for initiating lagging-strand DNA synthesis. How DNA replication is initiated from OriL has, however, remained largely unknown. Studies of the mitochondrial DNA helicase, TWINKLE, suggest that this protein may function as a primase in some nonmetazoan cells but that this activity has been lost in mammalian TWINKLE (Shutt and Gray, 2006). An OriL-specific primase activity was also reported in extracts from human mitochondria in 1985, but the enzyme responsible was never identified (Wong and Clayton, 1985a).

Recently, we have demonstrated that the mitochondrial RNA polymerase (POLRMT) can act as lagging-strand primase in vitro (Wanrooij et al., 2008). POLRMT is highly processive on double-stranded DNA, but synthesizes RNA primers with a length of 25–75 nt on a single-stranded template. The short RNA primers synthesized by POLRMT can be used by the mitochondrial DNA polymerase γ (POLγ) to initiate DNA synthesis. When combined, POLRMT, POLγ, TWINKLE, and the mitochondrial ssDNA binding protein (mtSSB) are capable of simultaneous leading- and lagging-strand DNA synthesis in vitro (Korhonen et al., 2004; Wanrooij et al., 2008). The template used for this experiment was a small 70 bp minicircle template, which lacked an OriL sequence, and primer synthesis was not localized to any specific region. Thus, even if these findings provide evidence for a role of POLRMT as a lagging-strand primase, they fail to explain the sequence-specific initiation of lagging-strand DNA synthesis observed at OriL in vivo.

In this report, we demonstrate that POLRMT can function as a lagging-strand primase on longer, double-stranded templates and that depletion of POLRMT in vivo extends the delay between initiation of leading- and lagging-strand DNA synthesis, commensurate with decreased initiation at OriL. Furthermore, we identify POLRMT as the OriL-dependent primase in human cells and reconstitute origin-dependent initiation of lagging-strand DNA synthesis in vitro. Upon passage of the leading-strand DNA replication machinery, single-stranded OriL is exposed and adopts a stem-loop structure. POLRMT initiates primer synthesis from a poly-dT stretch in the single-stranded loop region of OriL. After about 25 nt, POLRMT is replaced by POLγ, and lagging-strand DNA synthesis is initiated. Interestingly, only POLRMT is required for origin-specific primer synthesis, and the transcription factor B2 (TFB2M) found essential at mitochondrial promoters is not needed for activation of OriL (Falkenberg et al., 2002).

RESULTS

POLRMT Is Required for Initiation of Lagging-Strand DNA Synthesis In Vivo

We have previously reported that POLRMT can act as a primase in vitro and support lagging-strand DNA synthesis on a small 70 bp minicircle (Wanrooij et al., 2008). To address the role of POLRMT for initiation of lagging-strand DNA synthesis in vivo, we used RNA interference to deplete POLRMT in human cell lines. Initially, two shRNAs targeting different portions of the POLRMT message were cloned into pSuper vector (Invitrogen) and stably expressed in human osteosarcoma (HOS) cells, resulting in a 50% reduction of POLRMT expression (Figure S1). To boost the effects of RNAi, two shRP2 lines were transiently transfected with a double-stranded RNA (dsRNA365) targeting POLRMT. The combination of shRP2 and dsRNA365 RNAi produced a 75% decrease in POLRMT expression and a 50% decrease in mitochondrial transcripts.

To analyze the effects on lagging-strand DNA synthesis, we used 2DNAGE (Wanrooij et al., 2007; Yasukawa et al., 2006). In combination with restriction enzymes that cleave only duplex DNA, 2DNAGE provides a straightforward assay of delayed lagging-strand DNA synthesis. Both branches of a replication intermediate are cleaved at sites where second-strand DNA synthesis has occurred; in contrast, delayed second-strand DNA synthesis means that one branch does not comprise duplex DNA, and so the enzyme fails to cleave, resulting in products of increased mass and different shape, which can be resolved by 2DNAGE.

For our analysis, we used BclI, which cleaves nonreplicating mtDNA from HOS cells at four sites, and detected replication intermediates with Southern blotting using a DNA probe corresponding to position 4220–4665 in mtDNA (Figure 2). BclI digestion yielded a standard replication fork (Y) arc and two prominent spots resolving well above the arc of linear duplex DNAs. We clarified the identities of these two prominent spots, as discussed in detail in the Supplemental Information (Figure S2). One spot represents replication intermediates in which leading-strand synthesis has progressed past all four BclI restriction sites without any accompanying lagging-strand DNA synthesis; in this case, all four sites will be blocked, thereby yielding an arc comprising circular mtDNA molecules with two

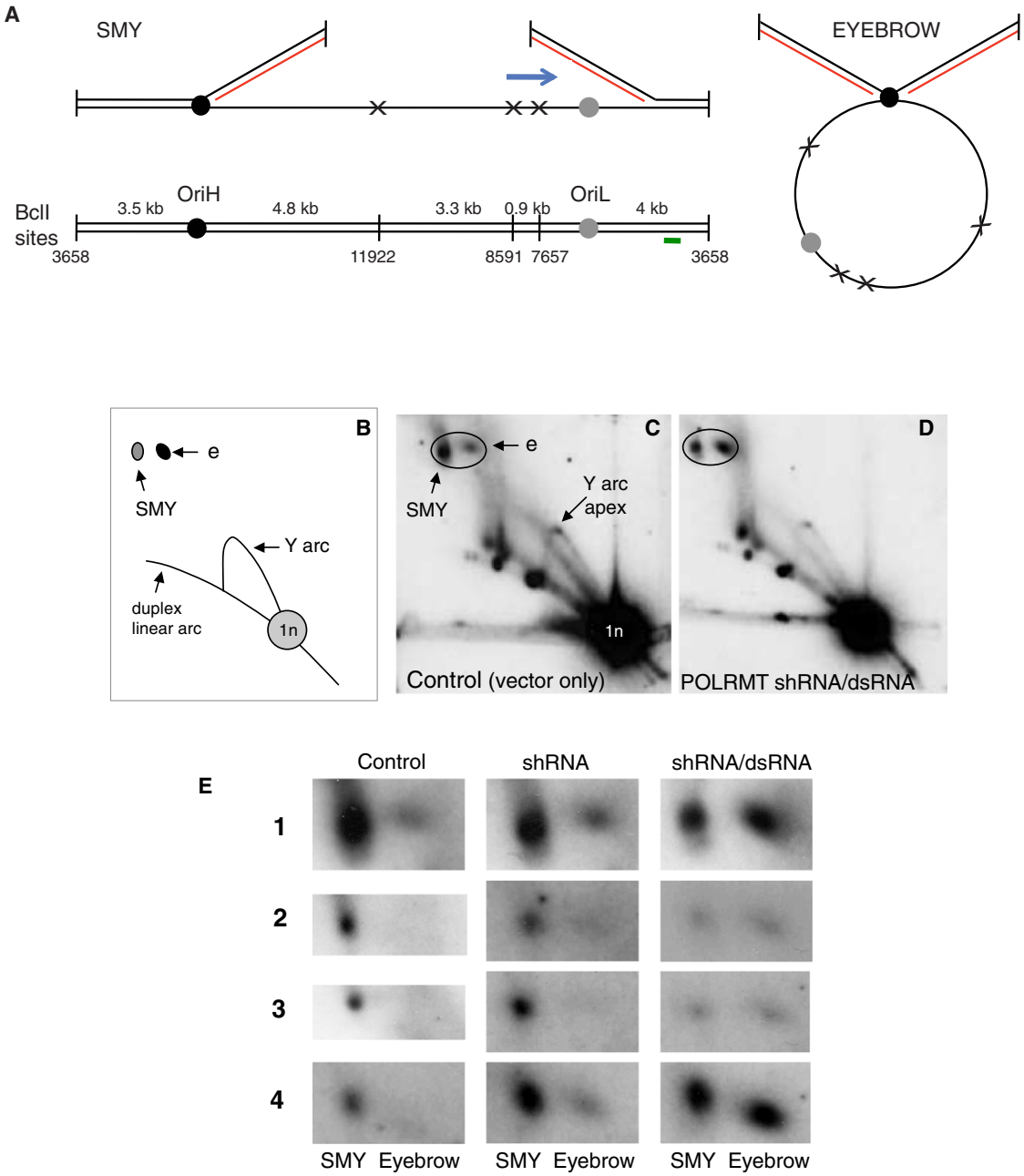

Figure 2. Replication Intermediates Associated with an Unusually Prolonged Delay in the Initiation of Second Strand DNA Synthesis Are Enhanced by Combined shRNA and dsRNA POLRMT Gene Silencing

(A) Delayed second-strand DNA synthesis during mtDNA replication will result in the formation of a series of replication intermediates that will not be cleaved on one branch by enzymes such as BclI that cut only duplex DNA. The displaced lagging-strand template has been proposed to be coated with mtSSB (Clayton, 1991) or hybridized to RNA (Yasukawa et al., 2006). In either case, BclI site blockage will produce so-called slow-moving Y (SMY) arcs, where two or more restriction fragments remain joined. BclI cleaves duplex human mtDNA at four sites (nt 3,658, 7,657, 8,591, and 11,922); if leading-strand synthesis has progressed past all four sites without any accompanying second-strand DNA synthesis, then all four sites will be blocked, thereby yielding an "eyebrow" arc comprising circular mtDNAs (see Yasukawa et al., 2006 and references therein for further details). Blue arrows indicate the direction of replication. The green bar indicates the location of probe A used for Southern blot analysis.

(B) 2DNAGE of BclI-digested HOS cell DNA was performed on control cells.

(C) 2DNAGE of BclI-digested HOS carrying shRP2 and transformed with dsRNA 365. POLRMT knockdown in these cells was about 75% (see Supplemental Information for details).

(D) The spot (compressed arc), designated SMY, was detected in all samples; a second spot (eyebrow) was much less prominent except in (D).

(E) The outcome of four independent experiments on the relative abundance of the SMY and eyebrow arcs.

duplex DNA branches, which we refer to as the eyebrow arc (Figure 2A) (Yasukawa et al., 2006). However, shortly after lagging-strand DNA synthesis initiates, BclI will cleave the replicating molecules, and circles with four blocked restriction sites will then be of low abundance. The second spot represents replication intermediates in which no lagging-strand synthesis has been initiated and leading-strand replication has progressed past nt 7657 (but not past the BclI site at nt 3658). These intermediates, referred to as slow-moving Y (SMY) arcs, migrate close to the eyebrow arcs and are relatively abundant (Figures 2A and 2B). The intensity of the SMY arc should not be directly affected by reduction of lagging-strand initiation, and the ratio between SMY and eyebrow arcs may therefore be used to estimate effects on lagging-strand DNA synthesis.

Having established the nature of the key replication intermediates, we examined the effect of POLRMT depletion in human cultured cells. The 2DNAGE analysis of mtDNA isolated from WT cells revealed the presence of both eyebrow and SMY arcs (Figure 2). 2DNAGE analysis revealed a weak eyebrow arc, relative to SMY, in control samples (Figure 2C). In contrast, the eyebrow arc was of similar intensity to the SMY spot when POLRMT expression was reduced to 25% of control values (Figures 2C–2E). The eyebrow arc was also more prominent compared with other intermediates, such as the spot close to the apex of the Y arc (Figures 2C and 2D and data not shown), and so it is concluded that the change reflected an increase in the abundance of the eyebrow arc rather than a decrease in the SMY arc. Thus, POLRMT depletion markedly increased the frequency of restriction site blockage at all four sites, which is commensurate with leading-strand synthesis progressing beyond nt position 3658 before second-strand DNA synthesis initiates, as would occur if lagging-strand initiation at OriL was suppressed. Therefore, these findings suggest that POLRMT is involved in the timing and location of initiation of lagging-strand DNA synthesis in vivo.

Initiation of OriL-Dependent DNA Replication In Vitro

Our previous findings strongly supported a role for POLRMT as the lagging-strand primase (Wanrooij et al., 2008). However, in vivo, primer synthesis and initiation of lagging-strand DNA synthesis is not equally distributed over the entire mtDNA molecule. Many reports have demonstrated that OriL is a preferred site for initiation of lagging-strand DNA synthesis. Indeed, the existence and partial characterization of an OriL-specific primase activity in mitochondrial lysates has previously been reported, but the corresponding enzyme has never been identified (Wong and Clayton, 1985a, 1985b).

To test if POLRMT specifically could initiate primer synthesis from OriL, we made use of our reconstituted system for leading- and lagging-strand DNA replication (Wanrooij et al., 2008). We used a template containing a replication fork for loading the replication machinery, a 3886 bp dsDNA region, and a free 3′-overhanging terminus that can act as a primer for leading-strand DNA synthesis. The template also contained a 928 bp mtDNA fragment covering the OriL region (Figure 3A). Once initiated, leading-strand DNA synthesis coupled to continuous unwinding of the double-stranded template could, in principle, progress indefinitely (Korhonen et al., 2004). We incubated the circular template with the components of the mtDNA replisome (POLγ, TWINKLE, and mtSSB) and monitored DNA synthesis using radiolabeled dNTPs in a time course experiment. We observed a linear increase in the size of the DNA products (Figure 3B, lanes 1–5).

We next added POLRMT and observed additional replication products, suggesting lagging-strand DNA synthesis (Figure 3B, lanes 9–13). The majority of the POLRMT-dependent replication products were found in a specific band of about 2100 nt. The product had the expected size for an OriL-dependent initiation event, and we hypothetically named the band "OriL product" (Figure 3A). To demonstrate that this was indeed the result of an OriL-dependent initiation event, we first verified that the product was absent when we used a template lacking the OriL-containing mtDNA fragment (Figure S3). We next did Southern blotting using strand-specific probes and demonstrated that the OriL product was a result of lagging-strand DNA synthesis (Figures 3C and 3D). We also mapped the exact location of the putative OriL product using a series of strand-specific probes complementary to regions surrounding OriL, including probes situated outside the mtDNA insert (Figures 3A and 3E). Our findings supported that the observed band was a lagging-strand DNA replication product, migrating with an apparent size of 2100 nt. The observed size was in excellent agreement with the calculated size of 2149 nt for lagging-strand synthesis initiated at OriL and terminated at the free single-stranded 5′ tail of the template. Southern blot analyzes also confirmed that the OriL product displayed a dose-dependent relationship with POLRMT concentrations (Figure S4A).

At promoters, POLRMT requires the general transcription factor TFB2M for site-specific initiation of transcription. Interestingly, we observed efficient primer synthesis at OriL in the absence of TFB2M, and increasing concentrations of the TFB2M protein did not affect leading- or lagging-strand DNA synthesis (Figure S4B).

To demonstrate that the OriL stem-loop structure was required for the observed lagging-strand initiation events, we generated two DNA replication templates identical to the template in Figure 3A, but carrying mutant versions of OriL (Figure 4A). Site-specific initiation of lagging-strand DNA replication was not observed with a construct lacking the OriL stem-loop structure but containing the surrounding mtDNA sequences (Figures 4A and 4B). In a second mutant construct, we destabilized the OriL stem-loop structure by G-to-A transitions at three positions in the middle of the stem (3G → 3A), which changed the predicted ΔG for the OriL hairpin structure from −10.09 to −4.04 kcal/mol and resulted in a severe decrease of lagging-strand replication (Figures 4A and 4B). It is interesting to note that OriL is surrounded by a number of tRNA-encoding genes, which are all expected to form various stem-loop structures in the ssDNA conformation (Ojala et al., 1980, 1981). Apparently, these structures cannot act as efficient origins of lagging-strand initiation.

OriL Is a Preferred Initiation Site for POLRMT Primer Synthesis on ssDNA

OriL does not function as a promoter in its double-stranded conformation (data not shown), but our data suggested that

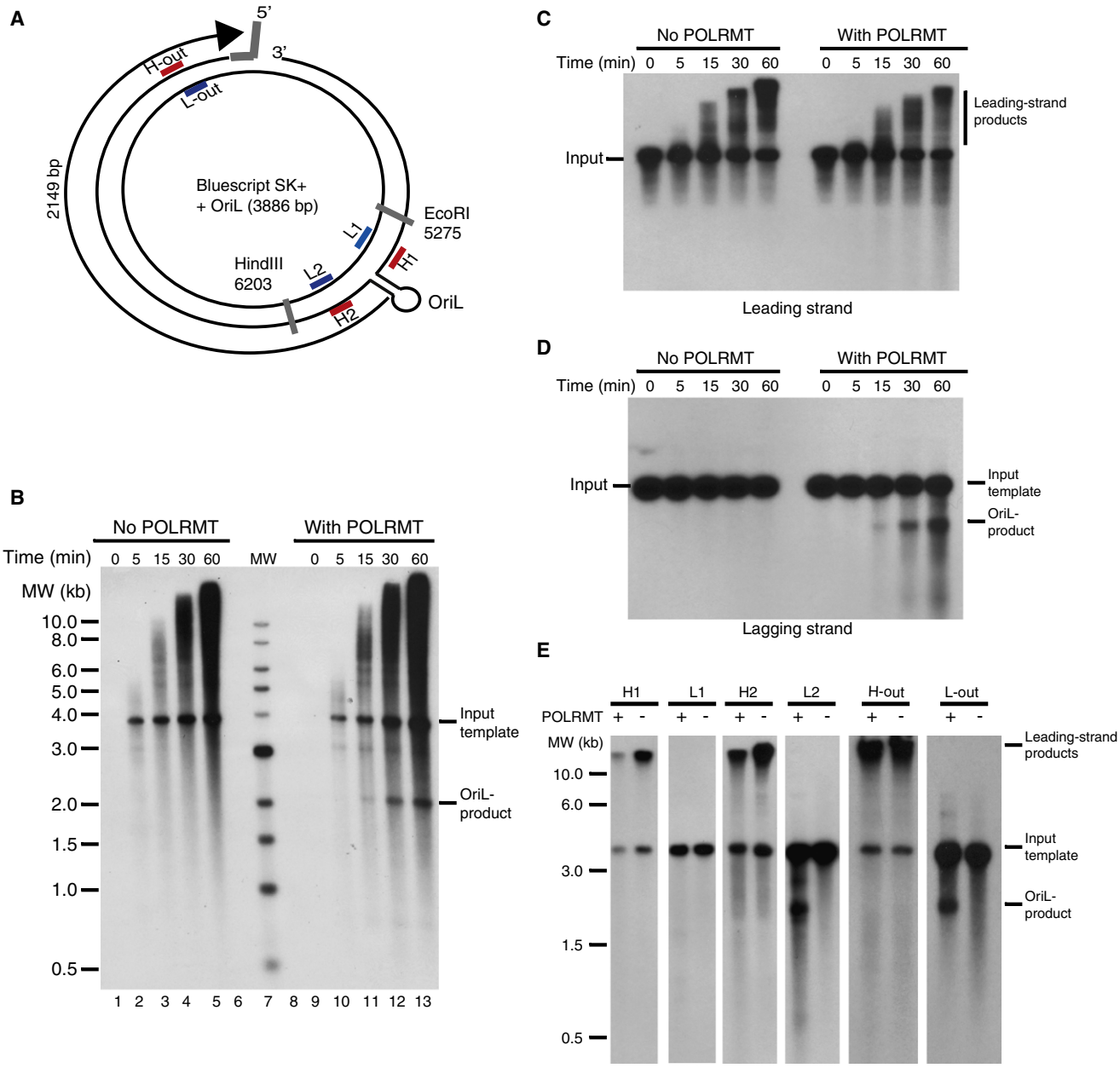

Figure 3. OriL-Dependent Initiation of DNA Replication In Vitro

(A) The template used for rolling circle DNA replication contains OriL and surrounding sequences cloned in between the HindIII and EcoRI sites in pBluescript SK(+). The arrow indicates the lagging-strand DNA product expected to be synthesized if DNA replication is initiated at OriL. Locations of the DNA probes (H1, H2, H-out, L1, L2, and L-out) used to detect replication products by Southern blotting are indicated. Please note that OriL is depicted as a stem-loop structure for clarity, but in reality the sequence adopts this structure only in its ssDNA conformation.

(B) DNA replication was performed in the presence of [α-^{32}P]dCTP in order to label newly synthesized DNA. A time course experiment was done in the absence (lanes 1–5) or presence of POLRMT (lanes 9–13). Lanes 6 and 8 were empty. At the times indicated, 25 μl reactions were removed for analysis by electrophoresis on a 0.7% denaturating agarose gel as described in Experimental Procedures. A specific product of about 2100 nt appears in the presence of POLRMT.

(C) DNA replication was performed as described in Experimental Procedures. Reactions were separated by electrophoresis as in (B), but replication products were analyzed by Southern blotting using the H-out probe to detect leading-strand DNA synthesis. The experiment was performed in the absence or presence of POLRMT.

(D) As in (C), but Southern blotting was performed with the L-out probe to detect lagging-strand DNA synthesis products.

(E) Southern analysis using upstream and downstream probes confirms lagging-strand initiation from OriL. The localizations of the probes used are indicated in Figure 3A. All reactions were incubated for 60 min, but otherwise analyzed as in (C).

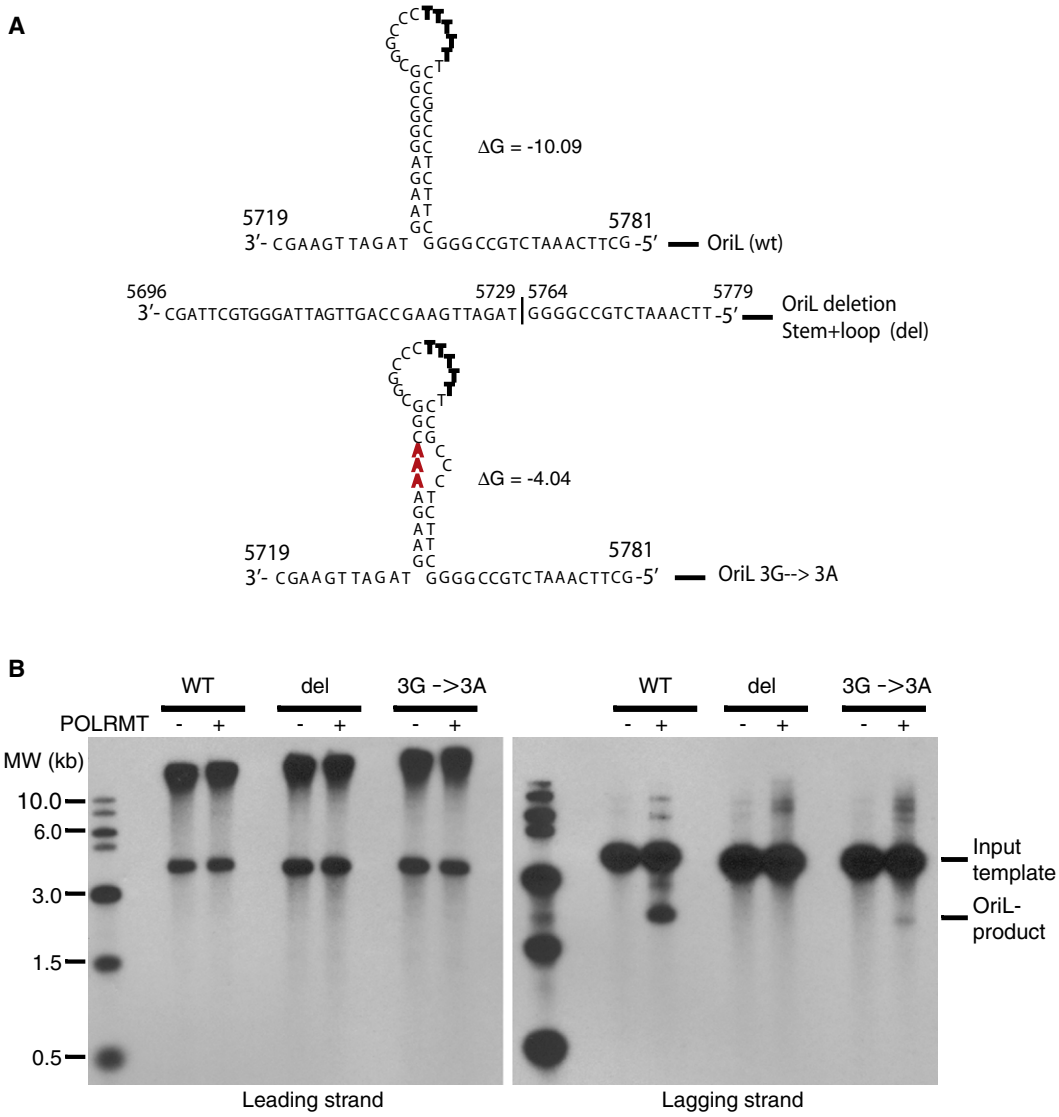

Figure 4. The OriL Stem-Loop Structure Is Required for Site-Specific Initiation of Lagging-Strand DNA Synthesis

(A) The template in Figure 3A was mutated either by deleting the OriL stem-loop structure completely or by destabilizing the stem (3G → 3A).

(B) Reaction mixtures containing the replisome and the mutant versions of OriL were incubated in the presence or absence of POLRMT. Reaction products were analyzed by Southern blotting using probes for leading-strand (H2) and lagging-strand (L2) DNA synthesis products as described in Experimental Procedures.

POLRMT could specifically initiate primer synthesis from the single-stranded OriL sequence. To directly demonstrate this, we monitored transcription initiation on an ssDNA template containing OriL and flanking sequences (Figure 5A). We used S1 nuclease protection assays to identify transcription products. One probe hybridized to a control region present at about 2000 nt from OriL (Control probe), and with this probe we observed a weak transcription product, which was completely abolished upon addition of mtSSB (Figure 5B). In contrast, using an oligonucleotide covering OriL, we observed a strong transcription product, which was unaffected by addition of the mtSSB protein (Figure 5B). Apparently, mtSSB represses non-sequence-specific primer synthesis and thereby causes a relative increase of OriL-specific initiation events.

To map the exact initiation sites for primer synthesis at OriL, we used two different ssDNA probes covering OriL (Figure 5C). In S1 nuclease protection assays, both probes mapped the initiation site to the poly-dT stretch in the loop region of OriL (nt 5747–5751) (Figures 5C and 5D). The observed initiation sites corresponded precisely with those observed for OriL-dependent primer synthesis in vivo (Wong and Clayton, 1985a).

The Poly-dT Repeat Region of OriL Is an Essential Element for Primer Synthesis

To define sequence motifs required for initiation of primer synthesis, we analyzed how mutations in OriL affect POLRMT activity (Figure 6A). As template, we used oligonucleotides corresponding to a 68 nt mtDNA region spanning OriL and detected

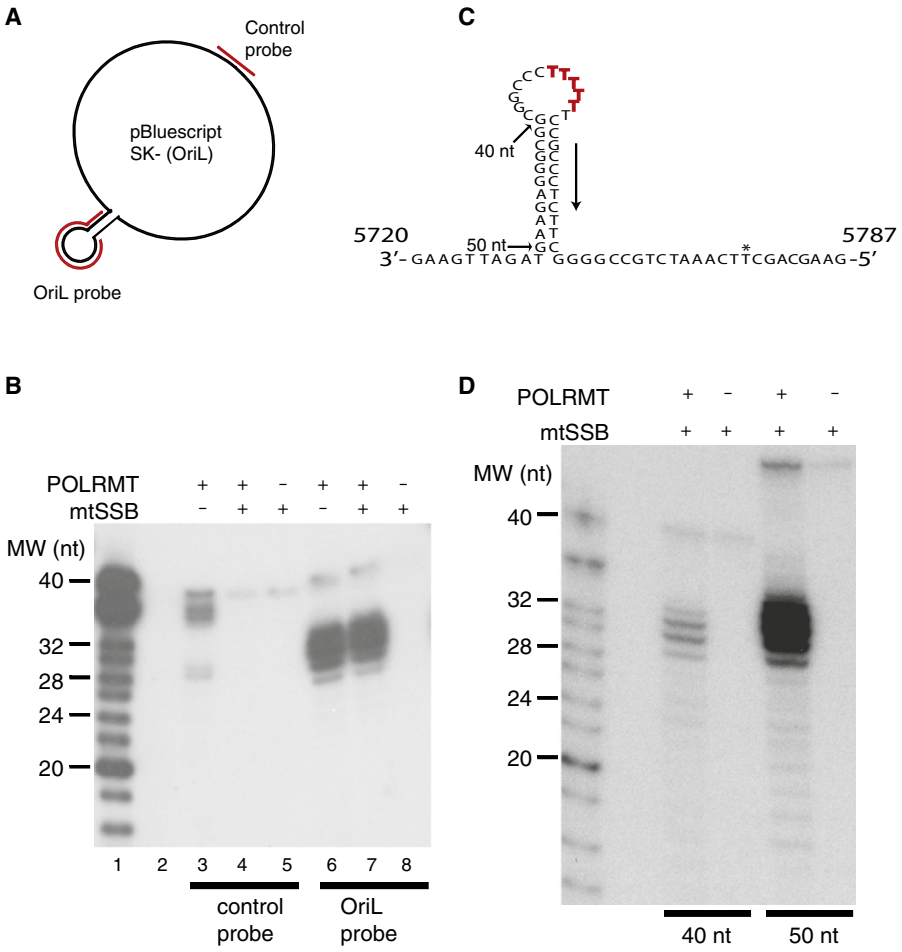

Figure 5. MtSSB Cannot Repress Site-Specific Primer Synthesis at OriL

(A) Schematic picture of the ssDNA template. The locations of probes used for S1 nuclease protection assays are indicated.

(B) In the absence of mtSSB, POLRMT can initiate transcription from both OriL and the nonspecific control region (lanes 3 and 6). Addition of mtSSB abolishes RNA synthesis from the control region, but does not affect OriL-dependent primer synthesis (lanes 4 and 7). S1 nuclease assays were performed using a nonspecific probe or an OriL-specific probe as indicated in (A).

(C) Schematic picture indicating the common 5′ end (*) and the two different 3′ ends of the 40 nt and 50 nt probes used for the S1 nuclease protection assay in (D). The probes were radioactively labeled on the 5′ ends.

(D) The template in (A) was incubated with mtSSB in the presence or absence of POLRMT as indicated. S1 nuclease protection analysis using the 40 nt or 50 nt probes revealed that initiation of primer synthesis was located to the poly-dT stretch in the loop region of OriL (indicated in red in [C]).

transcription by S1 protection analyses. On this template, primer synthesis started at the poly-dT stretch in the loop region, identical to the results obtained using the OriL-containing circular ssDNA template (Figures 5 and 6 and data not shown). Deletion of the entire stem-loop structure (OriL-del) abolished OriL-dependent primer synthesis (Figure 6B, lane 5).

Replacement of the poly-dT stretch (5747–5751) with a poly-dA sequence also resulted in a total loss of primer synthesis (Figure 6B, lane 7). The crucial importance of the poly-dT stretch was further evident from an experiment with the OriL complement sequence. This structure is not expected to exist in a single-stranded conformation in vivo, since leading-strand DNA replication will use the L strand as a template for DNA synthesis, creating a dsDNA product. The OriL-complement template (OriL-compl) failed to support primer synthesis in the presence of POLRMT (Figures 6A and 6C, lane 3). However, by simply replacing the naturally occurring poly-dA stretch with poly-dT, we observed primer synthesis (Figure 6C, lane 5).

A GGCGG sequence (from −2 to −6 of the promoter start site) has previously been shown to be essential for transcription initiation from human LSP (Gaspari et al., 2004). Interestingly, OriL contains two identical GGCGG sequences just upstream of the poly-dT stretch (positions 5739–5743 and 5736–5740). This observation provoked us to test if OriL, when double stranded, could function as transcription start site in the presence of TFB2M and TFAM. However, no transcription activity was observed using a double-stranded LSP-containing template, and alterations of the GGCGG sequence motifs did not affect POLRMT-dependent priming at OriL (data not shown).

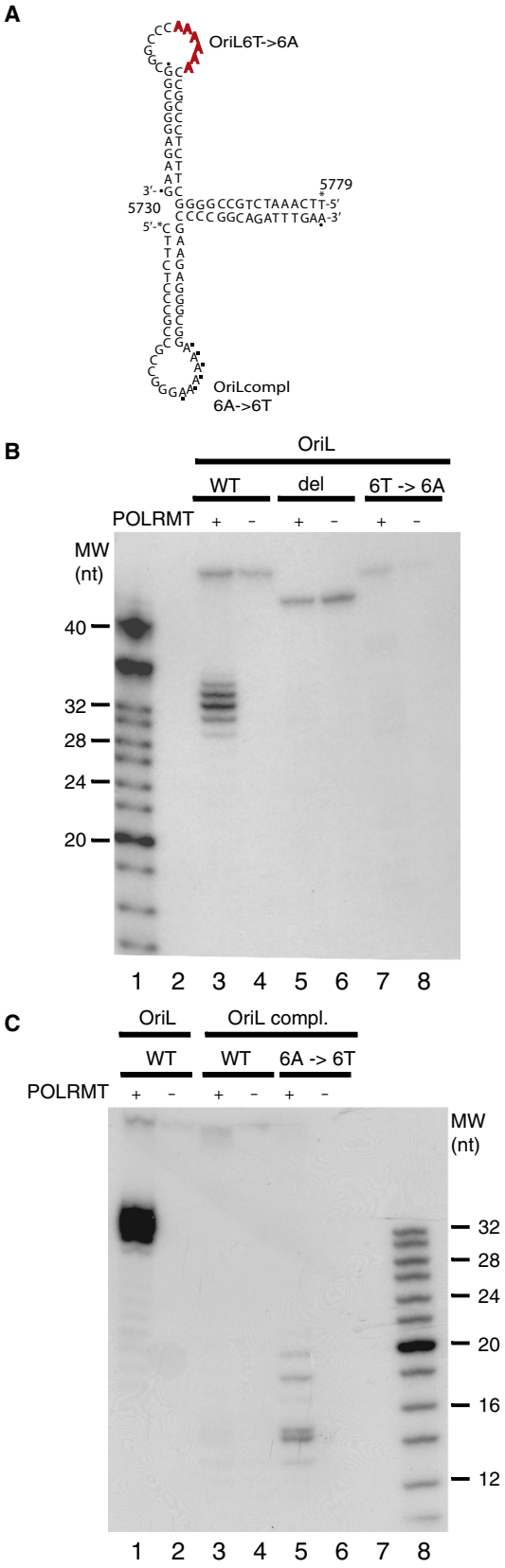

Figure 6. Primer Synthesis Is Dependent on the Poly-dT Stretch in the Loop Region of OriL

(A) Oligonucleotides corresponding to WT and mutant OriL were used as templates for POLRMT-dependent primer synthesis. In the 6T → 6A construct, the poly-dT stretch had been replaced by a poly-dA stretch. In the OriL complement 6A → 6T construct, a poly-dT stretch replaced the indicated dA residues. The OriL-del construct lacked the OriL stem-loop sequence but contained 34 nt upstream (5696–5729) and 16 nt downstream (5764–5779) of the origin.

(B) The poly-dT stretch is required for OriL-dependent primer synthesis. Mutant versions of OriL were incubated in the presence or absence of POLRMT as described in Experimental Procedures. The S1 nuclease protection assays were performed using 50 nt probes complementary to the specific template.

(C) The OriL complement sequence cannot support POLRMT-dependent primer synthesis. Replacing the poly-dA stretch with a poly-dT stretch in the OriL complement leads to POLRMT-dependent initiation of transcription. S1 nuclease protection assays were performed as in (B).

Taken together, our results indicate that it is the overall structure of OriL in combination with the poly-dT repeat region that is essential for POLRMT activity.

Origin-Specific Initiation of DNA Synthesis

The RNA-to-DNA transitions at OriL have been mapped in vivo (Kang et al., 1997). We wanted to see if our reconstituted system could faithfully reproduce this step in origin activation, and we therefore mapped RNA-to-DNA transition sites (Figure 7). We initiated DNA synthesis by adding POLRMT and POLγ to an OriL-containing ssDNA template.

To map the RNA-to-DNA transition sites, we performed a primer-extension experiment. We annealed a radioactively labeled oligonucleotide complementary to a region located 200 nt downstream of the OriL region (position 5947–5928). The primer was extended by incubation with POLγ (Figures 7A and 7B) or a thermophilic DNA polymerase (Taq DNA polymerase [data not shown]). DNA polymerases can copy a certain stretch of RNA templates when they get a jumpstart on DNA covalently linked to RNA (Mosig et al., 1995; Pham et al., 2006). To overcome this problem, we performed primer elongation before and after RNase H treatment. *E. coli* RNase H will specifically degrade the RNA part of the nucleic acid products and thereby allow us to exactly map the RNA-to-DNA transition sites. Without RNase H treatment, we detected a series of bands located all the way up to the poly-dT region (Figure 7A, lane 5), which is the expected location for the 5′ end of the primer starting at OriL. However, a significant signal was also detected on both templates at positions 5764 and 5765, located precisely at the beginning of the stem region (Figure 7A, indicated with an asterisk). This finding could indicate that POLγ has problems reading through the stem-loop region in the absence of mtSSB. After RNase H treatment, the signal from the poly-dT stretch and the positions 5764 and 5765 was diminished, and we instead observed a series of bands located in the region between 5770 and 5781, revealing RNA-to-DNA transitions at these locations (Figure 7A, lane 6). These transitions coincided exactly with the region previously mapped by others as the initiation site for OriL-dependent lagging-strand DNA synthesis in vivo (Figure 7C) (Kang et al., 1997). As a control for this experimental approach, we omitted POLRMT and instead used a synthetic 25 nt RNA

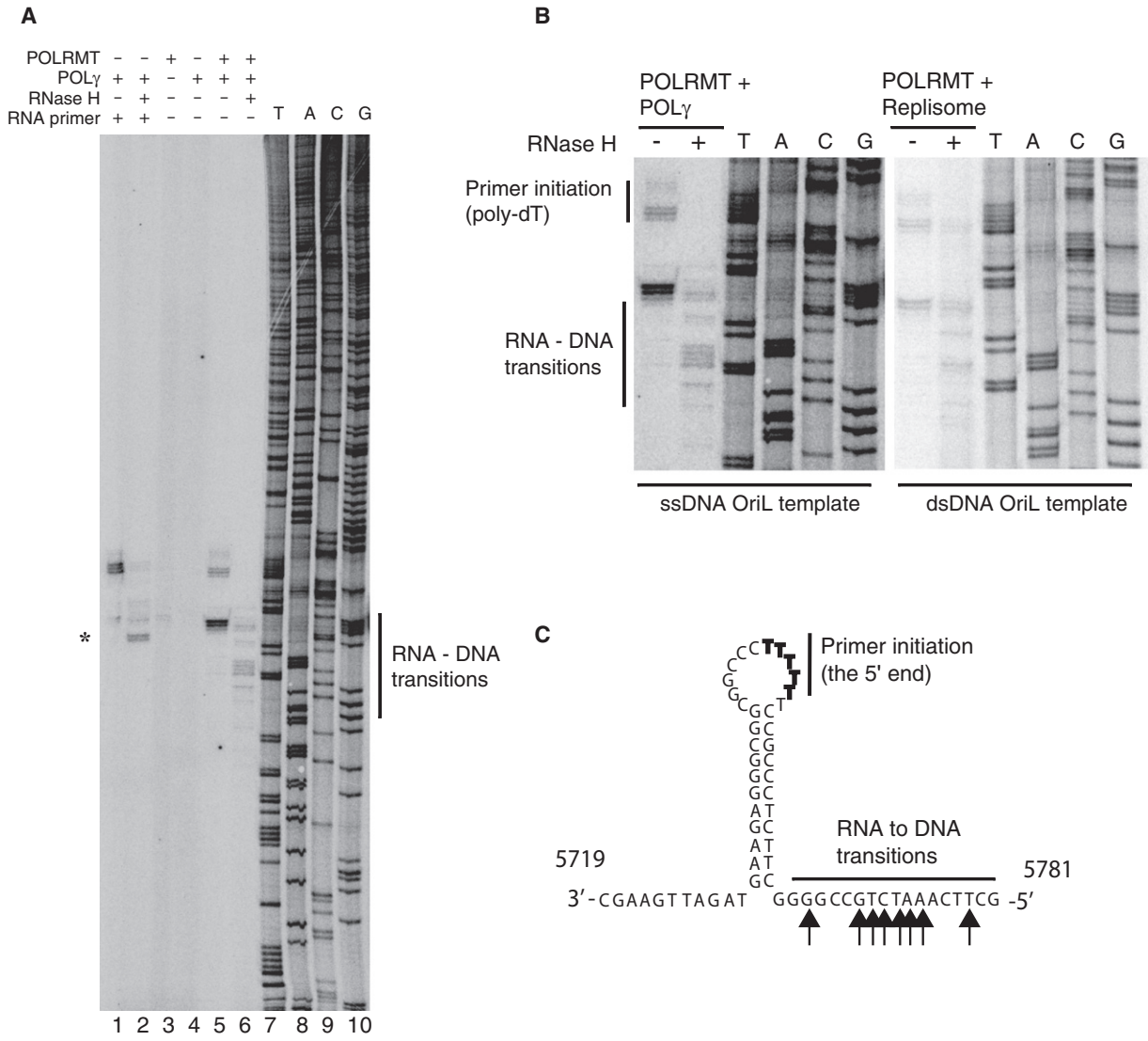

Figure 7. RNA-to-DNA Transitions Take Place in the Region between 5770 and 5781 Just Downstream of the Stem Loop

(A) The template in Figure 5A was incubated with POLRMT and POLγ as indicated. As a positive control, an RNA primer was annealed to the stem-loop region and elongated by POLγ (lanes 1 and 2) as described in Experimental Procedures. The products in lane 2 and 6 were treated with RNase H. Primer extension assays were performed using POLγ. Lanes 7–10: DNA sequencing reactions using the same template and the primer used in the primer extension assays.

(B) DNA replication was performed using the mitochondrial replisome and POLRMT as described in Experimental Procedures. The double-stranded construct depicted in Figure 3A was used as template, and primer extension analysis was performed as in (A). For comparison, details of Figure 7A are included.

(C) The OriL region. The RNA-to-DNA transition region is indicated. The arrowheads indicate initiation sites for lagging-strand DNA synthesis that have been mapped previously in vivo (Kang et al., 1997).

molecule, annealing to a region spanning from the first T in the poly-dT stretch (5747) to position 5771, hence similar to a typical primer at OriL. After annealing to the OriL-containing ssDNA template, the RNA primer was used for initiation of DNA synthesis by POLγ. Without RNase H treatment, primer extension continued to the poly-dT stretch, demonstrating that POLγ can indeed use RNA as template for DNA synthesis in the primer extension assay (Figure 7A, lane 1). After RNase H treatment, the RNA-to-DNA transition was mapped to position 5770 (Figure 7A, lane 2), which correlates closely with the expected RNA-to-DNA transition point (position 5771).

To further demonstrate that our system could faithfully reconstitute OriL activation, we repeated the experiment, but now with the complete replisome and POLRMT using the 3886 bp dsDNA template with a preformed replication fork, described in Figure 3A. We wanted to see if the positions of RNA-to-DNA transitions are affected in a reaction more similar to the in vivo situation, i.e., when initiation at OriL requires concomitant leading-strand DNA synthesis. Simultaneous leading-strand DNA synthesis did not affect the DNA-to-RNA transitions, and again, the RNA-to-DNA transition sites corresponded exactly to those mapped in vivo (Figure 7C).

DISCUSSION

We demonstrate here that POLRMT can function as an OriL-specific primase and help to initiate lagging-strand DNA synthesis. Leading-strand DNA replication is required to expose OriL in its single-stranded conformation, and the origin adopts a stem-loop structure. RNA synthesis is initiated from a poly-dT stretch in the loop region, and after about 25 nt, POLRMT is replaced by POLγ and lagging-strand DNA synthesis can commence.

Some years ago, we established an in vitro DNA replication system containing only purified POLγ, mtSSB, and TWINKLE in recombinant form. These factors form a processive replication machinery, which can use dsDNA as template to synthesize long single-stranded DNA (ssDNA) molecules (Korhonen et al., 2004). Using this system, we could recently demonstrate that POLRMT also can act as primase for initiation of lagging-strand DNA synthesis in vitro (Wanrooij et al., 2008). POLRMT could produce RNA primers with a length of 25–75 nt, which were used by POLγ to initiate lagging-strand DNA synthesis. From these findings, we concluded that POLRMT can prime lagging-strand DNA synthesis in vitro. To explore the in vivo relevance of the previous findings, we here used 2DNAGE analysis in combination with POLRMT knockdown in cell lines. The changes in the patterns of mitochondrial replication intermediates produced were consistent with a prolonged delay in the initiation of lagging-strand DNA synthesis, thereby lending support to the idea that POLRMT is required to prime lagging-strand DNA synthesis. However, we cannot rule out the possibility that depletion of POLRMT also has indirect effects on mtDNA replication. Lower levels of transcription due to loss of POLRMT may, e.g., influence the biophysical state of mtDNA, which in turn may influence DNA synthesis patterns in vivo. Alternatively, the loss of RNA-containing replication intermediates may promote different modes of DNA replication.

In vivo, lagging-strand DNA synthesis is not randomly distributed, but is primarily initiated from a specific DNA sequence, OriL. Other initiation sites have been identified by atomic force analysis and by 2D agarose gel electrophoresis of mtDNA replicative intermediates (Bowmaker et al., 2003; Brown et al., 2005), but OriL is clearly a dominant site for initiation of lagging-strand DNA synthesis. Using a large template for DNA synthesis (3886 bp), which contained the OriL region and flanking mtDNA sequences, we could indeed observe OriL-dependent initiation of lagging-strand DNA synthesis in vitro. The reaction was dependent on POLRMT, and the sites for primer initiation and RNA-to-DNA transition were exactly the same as those observed in vivo (Kang et al., 1997). The in vivo observations thus corroborate our mechanistic model, even if we cannot rule out that the specific mechanisms of OriL-dependent initiations may differ between in vivo and in vitro.

How does POLRMT recognize OriL and initiate primer synthesis? Our data suggest that initiation of primer synthesis depends on the overall structure of OriL, and that is not primarily governed by sequence-specific protein-DNA interactions. In fact, besides the stem-loop structure, the poly-dT stretch in the loop region was the only sequence element required for primer synthesis. We could even observe initiation of primer synthesis using the complement sequence to OriL, when we introduced a poly-dT stretch instead of the naturally occurring poly-dA sequence. A preference for purines over pyrimidines as initiation nucleotides has been demonstrated with many different RNA polymerases (RNAPs) (Chamberlin and Ring, 1973; Selisko et al., 2006; Wu and Goldthwait, 1969a, 1969b). The molecular basis for this phenomenon is not always clear, but the *E. coli* RNAP has a greater affinity for purine at its initiation nucleotide binding site (Naryshkina et al., 2001). In a related fashion, the T7 RNAP preferentially initiates transcription with GTP, and no less than 15 of the 17 promoters in the bacteriophage genome initiate with GTP. Structural studies have demonstrated that the initiating nucleotide binds the T7 RNAP in a location separate from the nucleotide binding sites in the elongating polymerase (Kennedy et al., 2007). Whether POLRMT has a higher affinity for ATP compared to the other NTPs remains to be investigated.

There are other examples of replicons that use RNAPs rather than specialized primases to prime DNA synthesis (Zenkin and Severinov, 2008). The *E. coli* RNAP is required for replication of the single-stranded form of the M13 phage (Brutlag et al., 1971; Wickner et al., 1972). In this system, DNA synthesis is initiated from a 17 bp long imperfect hairpin. RNAP has a high non-sequence-specific affinity to ssDNA, and to achieve origin-specific initiation, the *E. coli* ssDNA binding (*Eco* SSB) protein covers most of the single-stranded genome. In this way, *Eco* SSB excludes RNAP from ssDNA while leaving the mainly double-stranded origin region available for complex formation (Geider et al., 1978; Geider and Kornberg, 1974). As demonstrated here, mtSSB may play a similar role in OriL-dependent initiation of mitochondrial DNA replication. The protein suppresses nonspecific primer synthesis on ssDNA, but stimulates initiation from the partially double-stranded OriL region.

In coliphage N4, RNA synthesis is also initiated from hairpin-like structures (Davydova et al., 2007). The single-stranded phage contains hairpin-form promoters, which are recognized by the N4 virion-encapsidated DNA-dependent RNAP (vRNAP). Recently, a crystal structure of vRNAP in complex with promoter DNA revealed specific interactions between the polymerase and DNA sequences at the hairpin loop and stem (Gleghorn et al., 2008). In vivo, transcription initiation requires *Eco* SSB, which promotes sequence-specific initiation at the N4 promoters (Davydova and Rothman-Denes, 2003). Whereas other SSB proteins (T7 gp 2.5, T4 gp 32, and N4 SSB) cannot substitute for *Eco* SSB, since they destabilize the promoter hairpin, mtSSB did not inhibit initiation of N4 transcription, and DNase I cleavage analysis demonstrated that similar to *Eco* SSB, the mtSSB protein does not destabilize the N4 promoter hairpin (Davydova and Rothman-Denes, 2003). The mtSSB protein is structurally related to *Eco* SSB, and the mitochondrial protein also forms tetramers in vitro (Curth et al., 1994; Tiranti et al., 1993; Webster et al., 1997). Therefore, this finding could be potentially interesting to explain the ability of mtSSB to promote OriL-specific initiation of primer synthesis on a ssDNA template. We find it likely that the partially double-stranded structure of OriL prevents mtSSB binding, in a manner much similar to what has been previously observed at the M13 ori and at N4 promoters.

In support of this notion, mutations that lower the stability of the OriL hairpin structure resulted in a severe decrease of lagging-strand replication (Figure 4). In addition, the size of the single-stranded OriL loop region (12 nt) is too short to allow interactions with mtSSB (Kaguni, 2004).

The establishment of a robust in vitro system for studies of POLRMT-dependent priming of mitochondrial DNA synthesis opens interesting possibilities. A question not yet addressed is how the primer 3′ end is transferred from POLRMT to POLγ. Is POLRMT a part of the priming complex, and if yes, how does DNA polymerase gain access to the primer's 3′ end? The minimal system described here should allow us to investigate these important questions in the future.

One class of replication intermediates identified by 2DNAGE initiates exclusively within the noncoding region and incorporates RNA on the lagging strand across virtually the entire genome. These so-called RITOLS (ribonucleotide incorporation throughout the lagging strand) intermediates have been attributed to a distinct mode of DNA replication (Yasukawa et al., 2006). Others have confirmed the presence of stable and partially hybridized RNA (R-loops) on mtDNA, but have questioned their role as intermediates in DNA replication (Brown et al., 2008). A molecular mechanism that could potentially be in agreement with these observations was recently suggested by experiments in *E. coli* (Pomerantz and O'Donnell, 2008). The authors studied codirectional collisions between RNAP and the *E. coli* replisome and could demonstrate that the replisome uses the RNA transcript as a primer to continue leading-strand synthesis after the collision with RNAP that is displaced from the DNA. This action results in a discontinuity in the leading strand, yet the replisome remains intact and bound to DNA during the entire process. It is possible that something similar is happening during human mtDNA replication, and we will address this possibility in future experiments. How our studies of OriL-dependent activation in vitro relate to the RITOLS model remains to be established.

EXPERIMENTAL PROCEDURES

Recombinant Proteins

TWINKLE, mtSSB, POLγA, POLγB, POLRMT, TFB2M, and TFAM were expressed and purified as described previously (Korhonen et al., 2003, 2004; Wanrooij et al., 2008).

Template Preparation

We cloned a DNA fragment corresponding to nt 5275–6203 of the mitochondrial human genome between the HindIII and EcoRI sites in the pBluescript SK(+) and pBluescript SK(−) vectors (Agilent Technologies; La Jolla, CA). The pBluescript SK(+)OriL construct was used as a template in site-directed PCR mutagenesis reactions to generate the mutant variants of OriL. All constructs were confirmed by sequencing and used to isolate ssDNA following the manufacturer's protocol (Stratagene). The WT pBluescript SK(−)OriL ssDNA template was used for the S1 nuclease protection experiments. The pBluescript(+)OriL ssDNA templates (WT and mutant versions) were used for the rolling-circle DNA in vitro synthesis experiments. To produce the rolling-circle DNA replication template, we annealed a 70-mer oligonucleotide (5′-42[T]-ATCTCAGCGATCTGTCTATTTCGTTCAT-3′) to the pBluescript SK(+)OriL ssDNA (7.5 pmol) and synthesized the second strand by using a reaction mixture (100 μl) containing 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 25 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 1 mM DTT, 100 μg/ml BSA, 4 mM ATP, 2.8 pmol POLγA, 11.2 pmol POLγB, and 600 pmol mtSSB. After 45 min at 37°C, we stopped the reaction by adding 200 μl of stop buffer (10 mM Tris-HCl [pH 8.0], 0.2 mM NaCl, 1 mM EDTA, and 0.1 mg/ml glycogen). The samples were treated with 0.5% SDS and 100 μg/ml Proteinase K for 45 min at 42°C and then finally purified using the QIAquick PCR Purification Kit (QIAGEN). The different OriL oligos used in the S1 nuclease experiments was ordered from Eurofins (Ebersberg, Germany).

In Vitro Transcription on dsDNA

For in vitro transcription on dsDNA, we used DNA fragments corresponding to bp 1–512 (LSP) or 5275–6203 (OriL) of the human mtDNA as templates. Transcription in vitro was performed as described (Falkenberg et al., 2002).

Calculations of the Thermodynamic Stability of the OriL Hairpin-Loop Structure

ΔG for WT and mutant versions of OriL was calculated at 37°C and 150 mM NaCl using the Mfold software (http://mfold.bioinfo.rpi.edu).

Analysis of In Vitro Transcription and Rolling-Circle mtDNA Replication

Detailed protocols for in vitro transcription, rolling-circle mtDNA replication, Southern blot analysis, S1 nuclease protection, and primer extension analysis are provided in the Supplemental Information section.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at doi:10.1016/j.molcel.2009.12.021.

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