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Assembly of Simian Virus 40 Okazaki Pieces from DNA Primers Is Reversibly Arrested by ATP Depletion

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We have previously proposed that DNA polymerase α-primase provides short RNA-DNA precursors below 40 nucleotides (DNA primers), several of which assemble into an Okazaki piece after intervening RNA has been removed and the gaps have been filled by DNA polymerase δ (or ε) (T. Nethanel, S. Reisfeld, G. Dinter-Gottlieb, and G. Kaufmann, J. Virol. 62:2867–2873, 1988; T. Nethanel and G. Kaufmann, J. Virol. 64:5912–5918, 1990). In this report, we confirm and extend these conclusions by studying the effects of deoxynucleoside triphosphate (dNTP) concentrations and the presence of ATP on the occurrence, dynamics, and configuration of DNA primers in simian virus 40 replicative intermediate DNA. We first show that these parameters are not significantly affected by a 10-fold increase in dNTP precursor concentrations. We then demonstrate that Okazaki piece synthesis can be arrested at the level of DNA primers by ATP depletion. The arrested DNA primers faced short gaps of 10 to 20 nucleotides at their 3′ ends and were progressively chased into Okazaki pieces when ATP was restored. ATP could not be substituted in this process by adenosine-5′-*O*-(3-thiotriphosphate) or adenyl-imidodiphosphate. The chase was interrupted by aphidicolin but not by butylphenyl-dGTP. The results implicate an ATP-requiring factor in the switch between the two DNA polymerases engaged in Okazaki piece synthesis. They also suggest that the replication fork advances by small, DNA primer-size increments.

DNA polymerases (Pols) α, δ, and ε are implicated in replication of eukaryotic chromosomes, but the manner in which they cooperate in this process is not fully understood (2, 12, 14, 25, 26, 31). Pol α, which has an associated primase activity, lacks a proofreading 3′→5′ exonuclease. The converse holds for Pols δ and ε, which have been distinguished by their different responses to proliferating cell nuclear antigen (PCNA), shown to enhance the processivity and activity of mammalian Pol δ (3, 21).

The advent of a reconstituted simian virus 40 (SV40) DNA replication system (11) enabled investigators to discern specific roles of individual Pols and ancillary replicative factors. Thus, SV40 large T antigen unwinds the DNA at the viral origin of replication (*ori*). Assisted by the single-stranded DNA-binding protein RF-A (RP-A, HSSB), T antigen directs the permissive cell Pol α-primase to initiate DNA synthesis at *ori* (6, 9, 10, 15, 33). Yet Pol α alone may not sustain full-fledged replication, since omission of PCNA from the system selectively abolishes leading-strand synthesis (20). PCNA cooperates with another replication factor, RF-C ATPase (A1), in supplanting Pol α with Pol δ at a primed template (26, 27, 30). The absence of RF-C from the replication system also inhibits leading-strand synthesis (27). These facts underlie the proposal that Pol α-primase synthesizes Okazaki pieces, whereas Pol δ elongates the leading strands, utilizing an *ori*-Okazaki piece as a primer (20, 26). However, the findings that Pol ε is essential for nuclear DNA replication in the yeast *Saccharomyces cerevisiae* (1, 14) and may depend on RF-C/PCNA (5) render uncertain the identity of the leading strand Pol and invoke a specific role of a third replicase. It is also noteworthy that replication errors in leading- and lagging-strand DNA are equally proofread during SV40 replication in vitro (22), suggesting that a 3′→5′ exonuclease supplements Pol α-primase in Okazaki piece synthesis. This activity could be provided by Pol δ or ε.

A model consistent with specific functions of three replicases has been proposed on the basis of studies on SV40 DNA replication in isolated nuclei (17, 19). Accordingly, an Okazaki piece arises by ligation of shorter precursor chains, and its synthesis involves two DNA polymerases. Specifically, Pol α-primase deposits several initiator RNA (*i*RNA)-DNA units below 40 nucleotides (nt) (DNA primers) within the Okazaki zone. The DNA primer array is processed by removal of intervening *i*RNA, gap filling by Pol δ (or ε), and ligation of juxtaposed DNA primer units. A mature Okazaki piece joins in turn onto the growing 5′ end of a long nascent chain. This nested discontinuity model has been inferred from two principal observations. First, DNA primers synthesis can be differentiated from DNA primer assembly into Okazaki pieces by various agents. Butylphenyl-dGTP (BuP-dGTP) and Pol α-specific monoclonal antibodies preferentially inhibit the synthesis of DNA primers (17), whereas aphidicolin (17, 19) and carbonyldiphosphonate (18) selectively inhibit assembly of DNA primers into an Okazaki piece. Second, DNA primers configure in SV40 replicative intermediate (RI) DNA in a near-contiguous array, separated by short gaps of 20 nt or less. This configuration has been inferred from the limited extension of DNA primers achieved with Klenow DNA polymerase and subsequent joining of the extended DNA primers into Okazaki pieces by T4 DNA ligase (19).

Using a replication system containing a HeLa cell extract supplemented with SV40 T antigen, Bullock et al. (4) have shown that a 34-nt, *i*RNA-containing DNA primer of the lagging-strand sense initiates replication at SV40 *ori*. Incorporation of this DNA primer into a leading chain was prevented by anti-PCNA antibodies, but excess PCNA reversed the inhibition. The antibodies also caused DNA primer accumulation away from *ori*. Thus, PCNA/RF-C ATPase may replace Pol α-primase with Pol δ (or ε) upon

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completion of a DNA primer, rather than an Okazaki piece. Moreover, this exchange may occur not only at *ori* but also throughout lagging-strand synthesis.

The present report confirms the proposed role of DNA primers as significant precursors of Okazaki pieces. We first show that the occurrence, dynamics, and configuration of DNA primers in SV40 RI DNA are not significantly affected by a 10-fold increase in deoxynucleoside triphosphate (dNTP) precursor concentrations. In addition, we show that assembly of Okazaki pieces from DNA primers could be reversibly arrested by ATP depletion, implicating an ATP-requiring factor such as RF-C in switching the two Pols during Okazaki piece synthesis. The data also suggest that the replication fork advances by small, DNA primer-size increments.

MATERIALS AND METHODS

Materials. The sources of materials were described previously (17, 19) except for adenylyl-imidodiphosphate (AMP-PNP), which was purchased from Sigma, adenosine-5'-*O*-(3-thiotriphosphate) (ATPγS), which was purchased from NEN Research Products, and Klenow DNA polymerase and yeast hexokinase, which were purchased from Boehringer.

SV40 DNA synthesis in isolated nuclei. In vitro SV40 DNA synthesis was performed in isolated nuclei supplemented with cytosol. Both fractions were isolated from infected CV-1 African monkey kidney cells essentially as described before (19). To deplete the nuclei and cytosol fractions of remaining ATP, the complete reaction mixture containing them (0.07 ml) was preincubated for 90 s at 25°C with 100 μg of hexokinase and 1 mM glucose. dNTPs were used at 2 μM each except for the α-^{32}P-labeled nucleotide, which was used at 1 μM. However, when [α-^{32}P]dGTP was the labeled nucleotide, it was used at 0.5 μM. These conditions are referred to as 1× dNTPs. Where indicated, 10-fold-higher concentrations of dNTPs (10× dNTPs) were used. During continuous labeling, ATP was used at 1 mM, whereas rNTPs other than ATP were used at 10 μM or at 1 μM when radioactively labeled to tag *i*RNA. In the latter case, all dNTPs were used at 2 μM (1×) or 20 μM (10×). In addition, α-amanitin was included at 200 μg/ml to quench transcriptional incorporation (19). In pulse-chase experiments, DNA primers were chased by addition of 100- to 150-fold molar excesses of the corresponding nonradioactive dNTP or rUTP, as indicated. In ATP depletion and restoration experiments, the DNA was pulse-labeled in the absence of ATP and chased upon addition of 2 mM ATP. Analogs replacing ATP during the chase were also at 2 mM. Aphidicolin was used at 10 μg/ml, and BuPdGTP was used at 50 μM.

Fractionation and analyses of nascent DNA. Isolated SV40 RI DNA was dissociated in formamide, and the nascent DNA was fractionated by denaturing polyacrylamide gel electrophoresis along with DNA size markers (*Msp*I restriction fragments of pBR322 DNA). The gel was autoradiographed, and the distribution of radioactivity as a function of DNA chain length was quantified by densitometry (19). Nascent DNA fractions were eluted from the gel and analyzed for replication fork polarity by hybridization to the appropriate unidirectional single-stranded DNA M13-SV40 probes immobilized as dot blots on nylon membrane filters (17, 19). The amount of DNA in the dots was evaluated by hybridization with ^{32}P-M13 DNA labeled by random priming with a hexanucleotide mixture (Boehringer). The content of *i*RNA in nascent DNA fractions was determined by the extent of size shift caused by RNase T_2 digestion (19).

Simulated synthesis of Okazaki pieces in isolated SV40 RI DNA. DNA primers were selectively labeled by tagging the *i*RNA moiety during a 1-min pulse at 30°C with [α-^{32}P]UTP. DNA primers synthesized under conditions of ATP depletion were pulse-labeled for 2 min with [α-^{32}P]dGTP. SV40 RI DNAs containing the labeled DNA primers was isolated as described above. Aliquots corresponding to 20 μl of packed nuclei were resuspended in 40 μl of 10 mM Tris-HCl–1 mM Na_2EDTA; the $MgCl_2$ concentration was then adjusted to 10 mM. Portions of 4 μl were incubated with the large fragment of *Escherichia coli* DNA polymerase I (Klenow fragment), with T4 DNA ligase, or with both enzymes. The reaction mixtures (8 μl) contained 200 to 1,000 cpm of ^{32}P-labeled RI DNA, the indicated combinations of 0.012 U of Klenow DNA polymerase and 1 or 10 U of T4 DNA ligase, 0.25 mM ATP–0.25 mM each of the four dNTPs–10 mM $MgCl_2$–0.5 mM Na_2EDTA–10 mM Tris-HCl buffer (pH 8.0), and 2.5 mM dithiothreitol. Incubation was for 12 h at 15°C, following which the DNA was isolated from the reaction mixture by ethanol precipitation, dissolved in formamide, and fractionated by polyacrylamide-urea gel electrophoresis. It should be noted that the amount of Klenow polymerase was calibrated to achieve gap filling without strand displacement. In addition, care was taken to minimize the dissociation of DNA primers by lowering the temperature in which SV40 RI DNA was isolated from 25 to 4°C. These modifications resulted in gaps smaller than those reported previously (19).

RESULTS

Occurrence, dynamics, and configuration of DNA primers synthesized at different dNTP concentrations. Assembly of DNA primers into an Okazaki piece has been inferred from studies on SV40 DNA replication in isolated nuclei at dNTP levels of 0.5 to 2 μM (17, 19). However, these levels are by about an order of magnitude lower than the optimal dNTP concentrations reported for papovavirus in vitro replication systems (8). Therefore, it could be argued that the observed clustering of DNA primers reflects an artificial increase in RNA priming frequency due to suboptimal dNTP concentrations. In fact, increased RNA primer utilization at low dNTP concentrations has been observed in Okazaki piece synthesis during rolling-circle replication mediated by *E. coli* proteins in vitro (34). To address this problem, we investigated the effect of dNTP concentrations on properties of nascent SV40 DNA.

SV40 DNA pulse-labeled in isolated nuclei with 10 μM [α-^{32}P]dATP and 20 μM each of the other dNTPs (10× dNTPs) featured the typical DNA primer band migrating with the 34-nt marker (34-mer). However, an additional band migrating with the 26-nt marker (26-mer), usually seen with DNA primers pulse-labeled at 10-fold-lower concentrations of dNTPs (1× dNTPs), was barely detectable even during relatively short incubation times (Fig. 1). The low proportion of DNA primer 26-mers at 10× dNTPs could be due to their faster maturation into the 34-mers under these conditions. In agreement, DNA primer 26-mers could be detected at 10× dNTPs by monitoring their de novo synthesis with a radioactive precursor of *i*RNA. As shown, brief pulse-labeling with [α-^{32}P]rUTP at 20 μM each dNTP yielded both DNA primer 26-mers and 34-mers (Fig. 2A). The ensuing chase with nonlabeled UTP gradually shifted the radioactivity into the range of Okazaki pieces (Fig. 2B). As expected, the label decreased during the chase, in keeping with the transient nature of *i*RNA. A 26-mer→34-mer→Okazaki piece precur-

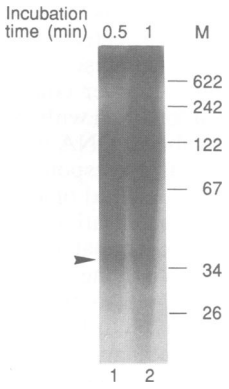

FIG. 1. Size distribution of nascent SV40 DNA synthesized in isolated nuclei. SV40 RI DNA was synthesized for the indicated times in isolated nuclei in the presence of 10 μM [α-^{32}P]dATP and 20 μM each of the other dNTPs. The nascent DNA was isolated as described in Materials and Methods, dissociated in formamide, and fractionated by 12% polyacrylamide–7 M urea gel electrophoresis. In all figures except Fig. 5B and 10, M indicates DNA size markers (*Msp*I restriction fragments of pBR322 DNA) in nucleotides.

sor-product relationship has been observed before at 1× dNTPs (19).

To compare the configurations of DNA primers synthesized at 1× and 10× dNTPs, we simulated their assembly into Okazaki pieces. Isolated SV40 RI DNAs containing the *i*RNA-tagged DNA primers were incubated with Klenow DNA polymerase, with T4 DNA ligase, or with both enzymes. A similar outcome was obtained in each case, whether DNA primers had been labeled at the low or high dNTP concentration. Klenow polymerase extended DNA primers by 12 nt on the average (compare lanes 1 and 2 and lanes 7 and 8 in Fig. 3). The extended chains still featured a periodic pattern with bands spaced about 10 nt apart. Hence, most DNA primers gained about one such repeat. Exhaustive treatment with T4 DNA ligase converted the extended DNA primers into products resembling Okazaki pieces (Fig. 3, lanes 4 and 10). Hence, the extension was a measure of the

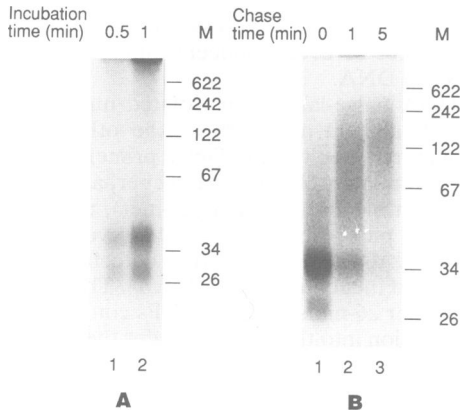

FIG. 2. Pulse-chase kinetics of *i*RNA-tagged DNA primers. (A) SV40 RI DNA was pulse-labeled for the indicated times with [α-^{32}P]UTP at 20 μM each dNTP and in the presence of 200 μg of α-amanitin per ml (17). (B) SV40 RI DNA pulse-labeled for 1 min was chased for the indicated times with a 100-fold molar excess of nonlabeled UTP. Nascent DNA was fractionated as described in the legend to Fig. 1.

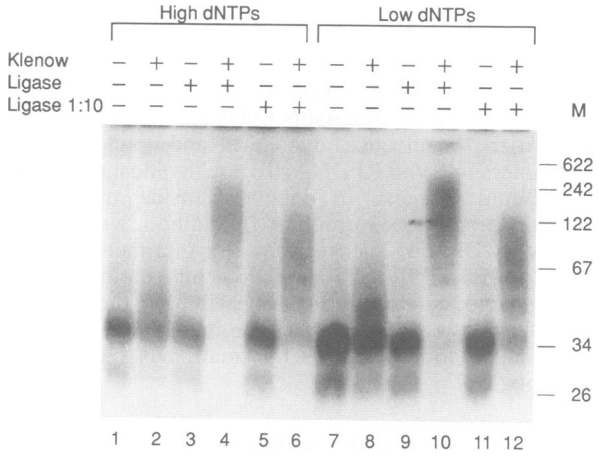

FIG. 3. Configuration of DNA primers synthesized in the presence of different concentrations of dNTPs. SV40 RI DNA was pulse-labeled with [α-^{32}P]UTP for 1 min in the presence of 2 μM (low) or 20 μM (high) each dNTP essentially as described in the legend to Fig. 2B. The RI DNA was purified and subjected to the indicated enzymatic treatments (see Materials and Methods). Subsequently, the DNA was precipitated in ethanol, dissociated in formamide and fractionated by 12% polyacrylamide–7 M urea gel electrophoresis.

gap separating the labeled DNA primer from a downstream DNA chain. A lower amount of DNA ligase shifted the label into products of intermediate sizes with a periodicity of about 10 nt (Fig. 3, lanes 6 and 12). The gradual ligation indicated that Okazaki pieces were assembled from several defined units. DNA ligase alone did not shift detectable DNA primer label into longer chains (Fig. 3, lanes 3, 5, 9, and 11), indicating that most, if not all, DNA primers faced gaps at their 3′ ends. In sum, the occurrence, dynamics, and configuration of DNA primers synthesized at the low and high levels of dNTPs were similar.

ATP depletion arrests lagging-strand synthesis at the level of DNA primers. Previously we used specific inhibitors and monoclonal antibodies to separate the synthesis of DNA primers from their assembly into an Okazaki piece (17, 19). In this study, we show that ATP depletion can serve this purpose as well. When ATP was merely omitted from the replication system (at 1× dNTPs), lagging-strand synthesis during a brief pulse was reduced to 20% of the control value. The residual products consisted mainly of DNA primers and short Okazaki pieces (Fig. 4; compare lanes 1 and 2). More severe inhibition was observed when remaining ATP was removed from the nuclei and cytosol by preincubating the reaction mixture with hexokinase and glucose (see Materials and Methods). Lagging-strand DNA synthesis dropped under these conditions to 2.5% of the control value and was confined to DNA primers distributed between 26-mers and 34-mers (Fig. 4, lane 3). Prolonged incubation without ATP did not alter this pattern (not shown). Although DNA primers incorporated dNMPs in the absence of ATP, de novo synthesis of DNA primers (monitored by tagging the *i*RNA moiety) could not be detected under these conditions. Thus, incorporation of [α-^{32}P]UTP into SV40 RI DNA dropped to less than 0.5% of the control value, and no DNA primer bands could be distinguished (not shown). Nevertheless, a majority of DNA primers that incorporated radioactive dNMPs in the absence of ATP contained *i*RNA, as evidenced by the reduction in their size by about 10 nt

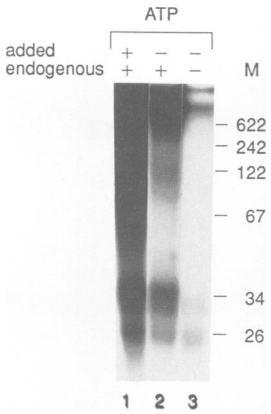

FIG. 4. Effect of ATP depletion on the distribution of nascent SV40 DNA. SV40 RI DNA was pulse-labeled with [α-^{32}P]dGTP under standard conditions (lane 1), without addition of ATP to the reaction mixture (lane 2), or under conditions of ATP depletion with hexokinase-glucose as detailed in Materials and Methods (lane 3). SV40 RI DNA was purified, and the nascent chains were dissociated and separated as for Fig. 1.

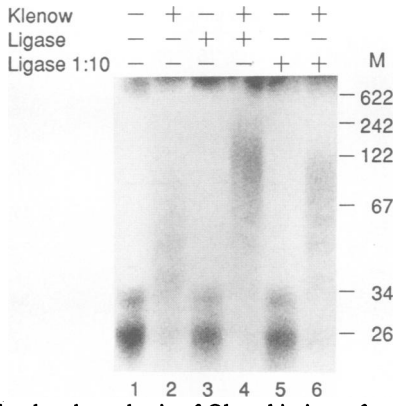

FIG. 6. Simulated synthesis of Okazaki pieces from DNA primers arrested by ATP depletion. DNA primers were pulse-labeled under conditions of ATP depletion (see Materials and Methods and the legend to Fig. 4). SV40 RI DNA containing them was purified and subjected to the indicated enzymatic treatments. The treated DNA was isolated, denatured, and fractionated as detailed in Materials and Methods and the legend to Fig. 3.

following exhaustive digestion with RNase T_2 (Fig. 5A). Hence, the DNA primers synthesized in vitro in the absence of ATP arose by extension of in vivo-initiated chains. The arrested DNA primers hybridized asymmetrically to lagging-strand template probes devoid of *ori* and terminal sequences (Fig. 5B). Taken together, these properties suggest that DNA primers arrested by ATP depletion are genuine precursors of Okazaki pieces, similar to the steady-state and aphidicolin-arrested counterparts (17, 19).

DNA primers arrested by ATP depletion configure in a near-contiguous array. To determine whether ATP depletion arrested DNA primers before or after the gap-filling step, we simulated their assembly into Okazaki pieces essentially as shown in Fig. 2. However, since ATP depletion abolished Okazaki piece synthesis, DNA primers could be selectively labeled with a radioactive dNTP. This analysis was carried out with DNA primers synthesized at 1× dNTPs. Incubation with Klenow polymerase extended these products by 17 nt on the average (compare lanes 1 and 2 in Fig. 6), adding one or two repeat units of about 10 nt to most chains. Incubation with T4 DNA ligase converted the extended chains into Okazaki piece-like products (Fig. 6, lane 4) through the typical intermediates spaced about 10 nt apart (Fig. 6, lane 6). Incubation with DNA ligase alone had no effect on the length of the original chains (Fig. 6, lanes 3 and 5), indicating that most if not all of them faced gaps downstream. A considerable fraction of these DNA primers were arrested as 26-mers and faced gaps longer than those seen with counterparts synthesized in the presence of ATP (Fig. 3). These attributes could be taken to indicate that ATP depletion also inhibits the 26-mer→34-mer conversion.

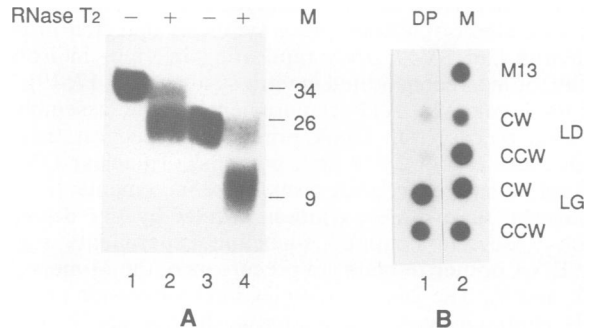

FIG. 5. Initiator RNA content and replication fork polarity of DNA primers synthesized in the absence of ATP. DNA primers were extracted from the 36- and 25 nt-bands of Fig. 4, lane 3, and analyzed as follows. (A) The chains were digested with RNase T_2 and separated on a 20% denaturing polyacrylamide gel along with parent chains. (B) The chains were hybridized to approximately 1.5-μg dots of single-stranded DNA of M13-SV40 clones serving as unidirectional probes (17). M13, M13mp18 single-stranded DNA; ccw and cw, counterclockwise and clockwise forks, respectively; LD and LG, leading and lagging templates, respectively; DP, ^{32}P-labeled DNA primers; M, ^{32}P-labeled M13 DNA probe.

The arrest of DNA primers by ATP depletion can be reversed. Pulse-chase experiments indicated that inhibition of DNA primer assembly by ATP depletion is reversible. DNA primers were pulse-labeled with [α-^{32}P]dTTP in the absence of ATP. Subsequently, ATP was restored and the nascent DNA was chased with excess nonlabeled dTTP. During the chase, the label shifted gradually from 26-mers into 34-mers and further into Okazaki pieces (Fig. 7A). In the absence of ATP, the chasing nucleotide sustained partial maturation, mainly from the 26-mers into 34-mers (Fig. 7B). Thus, an individual dNTP used at high concentration replaced ATP in the 26-mer→34-mer transition but was ineffective in driving the ensuing assembly of DNA primers into Okazaki pieces. A similar result was obtained when dGTP was used as the labeling nucleotide in this pulse-chase regimen (Fig. 8A). When ATP was substituted with the nonhydrolyzable analog AMP-PNP (Fig. 8A) or with ATPγS (Fig. 8B), the arrested DNA primers were not chased into Okazaki pieces. The partial maturation of 26-mers to 34-mers driven by the chasing dNTP was also inhibited under these conditions, AMP-PNP being more potent in this regard.

The ATP-dependent assembly of DNA primers into an Okazaki piece is aphidicolin sensitive and BuPdGTP resistant. Previously we showed that aphidicolin is more inhibitory to the synthesis of Okazaki pieces than to that of the DNA primers, whereas the converse holds for BuPdGTP (17, 19). The reversible arrest of DNA primer assembly into Okazaki

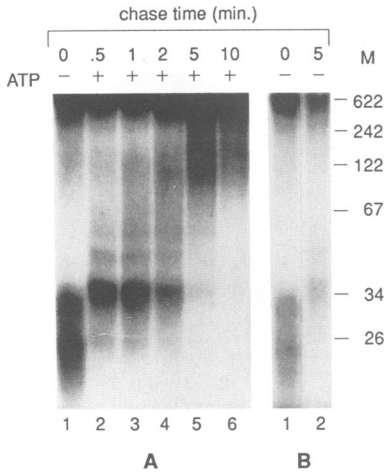

FIG. 7. Reversible arrest of DNA primers by depletion and restoration of ATP. DNA primers were pulse-labeled for 2 min with [α-^{32}P]dTTP under conditions of ATP depletion (see Materials and Methods and the legend to Fig. 4). The labeled DNA was chased for the indicated times with a 150-fold molar excess of nonlabeled dTTP in the presence of 2 mM added ATP (A) or without addition of ATP (B). SV40 RI DNA was purified, and the nascent chains were dissociated and separated as for Fig. 1.

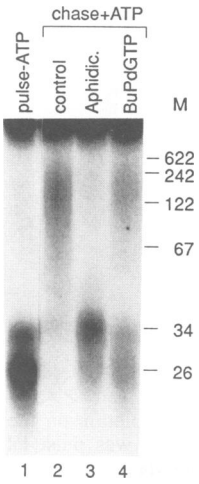

FIG. 9. Effects of aphidicolin and BuPdGTP on the ATP-dependent assembly of DNA primers into Okazaki pieces. DNA primers were pulse-labeled under conditions of ATP depletion (see Materials and Methods); 2 mM of ATP was added, and the DNA was chased with a 100-fold molar excess of nonlabeled dGTP for 5 min without or with inhibitors, as indicated. Aphidicolin was used at 10 μg/ml, and BuPdGTP was used at 50 μM.

pieces by depleting ATP (Fig. 7A) allowed us to examine more closely the effects of the two inhibitors on these partial reactions. When aphidicolin was added to the ATP-containing chase mixture, assembly of DNA primers into Okazaki pieces was blocked, but maturation of the 26-mers into 34-mers proceeded to completion (Fig. 9, lane 3). In contrast, BuPdGTP permitted a fraction of DNA primers to assemble into Okazaki pieces, while another fraction remained distributed between 26-mers and 34-mers (Fig. 9, lane 4). Presumably, DNA primers synthesized in the absence of ATP contained a subpopulation of mature units, ready to be converted into an Okazaki piece by the BuP dGTP-resistant Pol (δ of ε). In addition or alternatively, some DNA primers could mature during the chase as a result of incomplete inhibition of Pol α by BuPdGTP. That BuPdGTP inhibited Pol α under these conditions, at least in part, is indicated by the failure of the remaining DNA primers to reach the 34-nt mark in the presence of BuPdGTP, in contrast to the 26-mer→34-mer shift observed with aphidicolin.

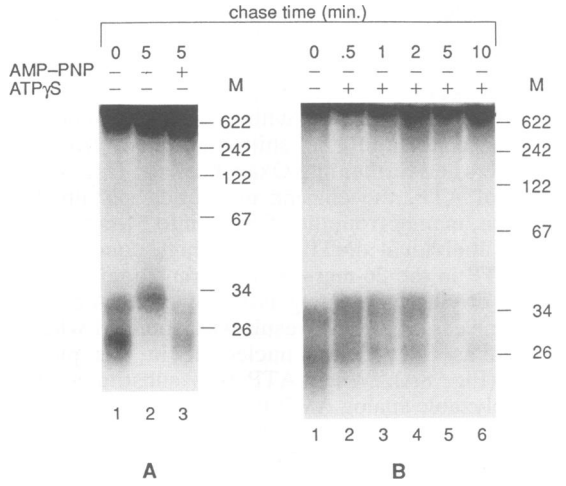

FIG. 8. Effects of dNTPs and ATP analogs on the assembly of DNA primers into Okazaki pieces. (A) DNA primers were pulse-labeled under conditions of ATP depletion as detailed in Materials and Methods and the legend to Fig. 4 and chased for 5 min with a 100-fold molar excess of dGTP. Where indicated, 2 mM AMP-PNP was included during the chase. (B) DNA primers were pulse-labeled and chased as for Fig. 7A except that ATP was substituted with 2 mM ATPγS during the chase. SV40 RI DNA was purified, and the nascent chains were dissociated and separated as for Fig. 1.

DISCUSSION

ATP depletion reversibly arrested SV40 lagging-strand synthesis at the level of DNA primers. These products were similar in size, *i*RNA content, and replication fork polarity to intermediates of Okazaki piece synthesis detected in polyomavirus and SV40 DNA replicating in vivo, in isolated nuclei, or in a reconstituted in vitro system (4, 8, 17, 19). The results invoke an ATP requirement in the assembly of Okazaki pieces from DNA primers and suggest that the replication fork of SV40 and, perhaps, of nuclear DNA in general advances by DNA primer-size increments.

Steps in Okazaki piece synthesis affected by ATP depletion. Pulse-chase and continuous-labeling experiments suggest that DNA primer 26-mers are precursors of the 34-mers (Fig. 3, 7, and 8). The 26-mer→34-mer interconversion was partially inhibited by ATP depletion at the low dNTP concentration. ATP could be replaced in this transition by raising the concentration of a single dNTP, but AMP-PNP antagonized the effect of the dNTP. These results suggest that ATP plays a specific role in the 26-mer→34-mer transition. However, ATP depletion arrested only a fraction of DNA primers as 26-mers, allowing another fraction to mature into 34-mers. Therefore, it may be further argued that ATP is needed only at the onset of the 26-mer→34-mer transition, for example to translocate the Pol relative to the template. Accordingly, DNA primers found beyond the 26-nt execution point could

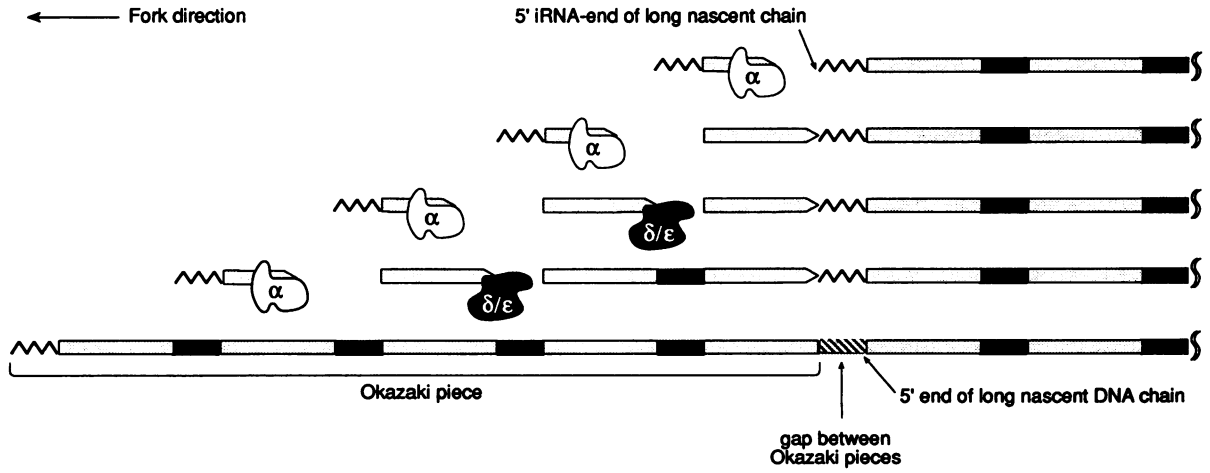

FIG. 10. Model of nested discontinuity and involvement of two replicases in SV40 lagging-strand synthesis. Pol α-primase deposits several DNA primers, moving in an overall 5′-to-3′ direction, followed closely by an undefined activity which removes *i*RNA from the nascent DNA primer. The *i*RNA-size gap is filled by Pol δ or ε, and adjacent DNA primer units are joined. Eventually, when the Okazaki zone has been filled, this process comes to a halt. The *i*RNA moiety at the growing 5′ end of the long chain (the previous Okazaki piece) is removed, the gap is filled (by an undetermined activity), and the RNA moiety becomes the new 5′-end group of the growing long chain. *i*RNA, ; DNA primer, ; gap between DNA primers, gap between Okazaki pieces, ; Pol α-primase, ; pol δ or ε, .

be completed in the absence of ATP, whereas younger chains which had not passed this point were blocked as 26-mers.

Assembly of DNA primers into Okazaki pieces exhibited a more clear-cut dependence on ATP. Neither dTTP, dGTP, AMP-PNP, nor ATPγS could replace ATP in this step (Fig. 7 and 8). The target of this inhibition could be the gap filling between adjacent DNA primers or an earlier step. These possibilities are implied by the failure to ligate the arrested DNA primers in isolated SV40 RI DNA without prior gap-filling by Klenow polymerase (Fig. 6). Moreover, conversion of the arrested DNA primers into Okazaki pieces upon restoration of ATP was blocked by aphidicolin (Fig. 9), indicating that the conversion depended on DNA synthesis.

Do specific ATP-requiring factors mediate intermediate steps in Okazaki piece synthesis? ATP is utilized by a number of proteins participating in SV40 DNA replication such as DNA ligase, the multifunctional T antigen, DNA primase, and RF-C ATPase. Hence, the primary cause for inhibition of Okazaki piece synthesis by ATP depletion is not obvious. Inhibition of DNA ligase can be ruled out as a primary cause, since the arrested DNA primers faced gaps at their 3′ ends (Fig. 6). Yet preliminary data suggest that ligation of DNA primers may be selectively inhibited by less severe limitation of ATP. In a replication system containing endogenous ATP (remaining in nuclei and cytosol) but no added ATP, DNA primers are converted into Okazaki pieces upon addition of ATP, even in the presence of aphidicolin. This result suggests that reduction in ATP concentration is more inhibitory to ligation of DNA primers than to preceding reactions (16).

Inhibition of SV40 T-antigen helicase prevents unwinding of parental DNA but not necessarily growth of Okazaki pieces on the unwound template. However, the ATP-binding T antigen also interacts with Pol α (7, 24) and stimulates its activity (6). It is therefore conceivable that ATP depletion could affect this interaction and consequently inhibit steps in DNA primer synthesis such as the 26-mer→34-mer shift. Perhaps relevant here is that ATP-binding sites of T antigen accept individual dNTPs instead of ATP (32), in keeping with the ability of individual dNTPs to drive the 26-mer→34-mer shift. More difficult is to account for inhibition of the second lagging-strand Pol through T antigen, unless coupling of the two entities is invoked. Similar arguments can be mentioned to explain how inhibition of DNA primase by ATP depletion could influence the 26-mer→34-mer transition but not the gap-filling reaction.

RF-C ATPase/PCNA could switch DNA primer synthesis by Pol α to gap filling catalyzed by Pol δ (or ε), analogous to the switch between Pols α and δ in leading-strand DNA synthesis (26). This possibility is suggested by the requirement for ATP in the assembly of DNA primers into Okazaki pieces and the failure of the nonhydrolyzable analogs ATPγS and AMP-PNP to replace ATP in this reaction (Fig. 7 and 8). These properties match requirements of reactions mediated by RF-C/PCNA (5, 30). Further support to this notion is lent by the arrest of Okazaki piece synthesis at the level of DNA primer 34-mers by anti-PCNA antibodies (4). Interestingly, DNA footprinting analysis has indicated that RF-C/PCNA protects a duplex portion of 24 bp in a model primer-template (29). This portion may correspond to the duplex formed between the DNA moiety of the DNA primer 34-mer and the lagging template.

Direction of DNA primer deposition. Most DNA primer chains synthesized in the absence of ATP contained *i*RNA (Fig. 5A), although synthesis of *i*RNA could not be detected under these conditions. It follows that DNA primer synthesis was limited to extension of preexisting *i*RNA or *i*RNA-DNA chains. If the replication fork accommodates only one molecule of Pol α-primase, it can be further assumed that only a single DNA primer chain was extended per fork. Since this newly synthesized DNA primer faced a gap of 10 to 20 nt (Fig. 3 and 6), the overall direction in which DNA primers are deposited on the lagging template must be 3′→5′. Namely, successive deposition of DNA primers in the opposite direction or growth of an Okazaki piece by synthesis and extension of a single DNA primer chain would be incompatible with such a short gap. Further support for this conclusion is lent by the reported physical (7, 24) and functional (6) interaction of Pol α-primase with T antigen at the fork (33). Thus, T antigen may translocate Pol α repeatedly, making available to it DNA primer-size portions of the lagging template. The two entities may resemble in this regard a prokaryotic primosome (13). Successive deposition of DNA primers may be closely followed by removal of intervening *i*RNA moieties. A second

lagging-strand Pol (δ or ε) may fill the resulting gaps and permit DNA ligase to join adjacent DNA primer units (Fig. 10). However, on the basis of existing data, we cannot exclude an alternative scheme in which the second Pol extends selected DNA primers, displacing others.

Prokaryotic replicases share features qualifying them for concurrent synthesis of both DNA strands. Their leading and lagging catalytic subunits remain associated, yet they translocate in opposite directions relative to DNA. Sinha et al. (23) suggested that this feat may be achieved in phage T4 replication if a portion of the lagging template is pooled back as a loop. This model has been adapted to describe the coordination of distinct leading and lagging Pols at the SV40 replication fork (28). However, if the lagging strand grows by DNA primer-size increments, then the opposite translocation of the two Pols may be accommodated merely by flexibility of the replication complex rather than by a back-pooled loop of lagging template DNA.

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