Lagging strand DNA synthesis by calf thymus DNA polymerases α , β , δ and ϵ in the presence of auxiliary proteins

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ABSTRACT

By using a defined gapped DNA substrate that mimicks a lagging strand of 230 nucleotides and that contains a defined pause site, we have analyzed calf thymus DNA polymerases (pol) α , β , δ , and ϵ in the presence of the three auxiliary proteins proliferating cell nuclear antigen (PCNA), replication factor C (RF-C) and replication protein A (RP-A) for their ability to complete an Okazaki fragment. Pol α alone could fill the gap to near completion, but was strongly stopped by the pause site. Addition of low amounts of RP-A resulted in an increased synthesis by pol α past the pause site. In contrast, high amounts of RP-A strongly inhibited gap filling by pol α . Further inhibition was evident when the two other auxiliary proteins, PCNA and RF-C, were added in addition to RP-A. Pol β could completely fill the gap without specific pausing and also was strongly inhibited by RP-A. PCNA and RF-C had no detectable effect on pol β . Pol δ , relied as expected, on all three auxiliary proteins for complete gap filling synthesis and could, upon longer incubation, perform a limited amount of strand displacement synthesis. Pol ϵ core enzyme was able to fill the gap completely, but like pol α , essentially stopped at the pause site. This pausing could only be overcome upon addition of PCNA, RF-C and *E.coli* single-stranded DNA binding protein. Thus pol ϵ holoenzyme preferentially synthesized to the end of the gap without pausing. Ligation of the DNA products indicated that pol β core enzyme, pol δ and pol ϵ holoenzymes (but not pol α and pol ϵ core enzyme) synthesized products that were easily ligatable. Our results indicate that pol ϵ holoenzyme fills a defined lagging strand gapped template to exact completion and is able to pass a pause site. The data favour the hypothesis of Burgers (Burgers, P.M.J. (1991) *J.Biol.Chem.* 266, 22698–22706) that pol ϵ might be a candidate for the second replication enzyme at the lagging strand of the replication fork.

INTRODUCTION

Three different DNA polymerases (pol), called α , δ and ϵ , appear to be involved in DNA replication in eukaryotic cells (reviewed e.g. in [1–5]). *In vitro* replication studies using simian virus 40 (SV40) DNA as a model system, indicated that at the replication origin of SV40 DNA initiation and elongation of leading and lagging strand synthesis occurs by two different pols, namely pol α and δ , respectively [6]. At least three pol auxiliary proteins called proliferating cell nuclear antigen (PCNA) [7], replication protein A (RP-A) [8] (also called replication factor A [9] or single-stranded DNA binding protein (SSB) [10]) and replication factor C (RF-C) [11] are required to support pol action. First, unwinding of the origin of replication occurs by the combined action of T antigen and RP-A, followed by pol α /primase initiation of DNA synthesis at the viral origin. After synthesis of relatively short stretches of DNA, the two pol auxiliary proteins PCNA and RF-C form a tight complex in the presence of ATP at the 3'-OH end of the growing DNA chain. Binding of RF-C and PCNA prevents pol α /primase from further interaction with the 3'-OH terminus and instead favours pol δ binding and consequent start of leading strand DNA synthesis. The dissociated pol α /primase cycles to the next priming site on the lagging strand to resume discontinuous replication. Simultaneously, pol δ , in the presence of the auxiliary proteins, proceeds to synthesize the leading strand continuously.

A pol, first discovered in yeast [12] has now been found in many other eukaryotic cells and is termed pol ϵ [13]. This enzyme was found to be essential for viability in the yeast *Saccharomyces cerevisiae* [14,15] and an important role in DNA replication has therefore been suggested. The three pol auxiliary proteins PCNA, RF-C and RP-A are required by pol δ for replication of natural DNA [16,17]. However these three proteins also interact with pol ϵ , but in a much more complex way [18–20]. The high degree of functional similarity between pol's δ and ϵ in their capacity to form holoenzyme structure with the same auxiliary proteins and combined with the fact that both pol's are essential gene products of yeast [14, 21, 22], suggests that both pol δ and pol ϵ might function in chromosomal DNA replication in addition

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to pol α /primase [23]. Furthermore, *in vivo* studies using specific pol inhibitors have indicated that replication of the lagging strand requires two pol, the first being pol α /primase and the second either pol δ or pol ϵ [24].

In calf thymus, we have recently shown that pol δ and pol ϵ are both dependent on the three auxiliary proteins PCNA, RF-C and RP-A (or SSB) in order to form stable holoenzyme complexes [20]. However, the holoenzyme forms of pol δ and pol ϵ function somewhat different [25]. Pol ϵ holoenzyme could synthesize longer products and used less primers than the pol ϵ core enzyme (pol ϵ in the absence of the three auxiliary proteins PCNA, RF-C and SSB). Cooperation of pol α /primase with pol δ or pol ϵ to form long products was influenced by the presence of RP-A or *E. coli* SSB. Primers synthesized by pol α /primase on a ssM13 DNA could be elongated into long DNA products by both pol δ and pol ϵ holoenzymes [25]. In this communication we have used a model template with a gap size of 230 nt that allows analysis of lagging strand DNA synthesis. The data obtained support the model introduced first by Burgers [18] that pol ϵ appears to be the second lagging strand replication enzyme.

MATERIALS AND METHODS

Enzymes and proteins

Calf thymus enzymes and proteins were isolated as described: pol α [25], pol β [26], pol δ [27], pol ϵ [28], PCNA [7], RF-C [20], RP-A [29]. 1 unit of polymerase activity corresponds to the incorporation of 1 nmol of dNMP into acid-precipitable material in 60 min at 37°C. Pol α and pol β were tested in the assays containing activated calf thymus DNA ([27] and [26], correspondingly). Pol δ and pol ϵ were tested, respectively, in an assay containing poly(dA)/oligo(dT₁₂₋₁₈) in the presence or in the absence of PCNA [27]. *E. coli* SSB was purified from the overproducer RLM 727 (gift of Martine Defais, CNRS, Toulouse) according to [30]. T4 ligase, restriction endonuclease *Mvn* I, T4 polynucleotide kinase, *E. coli* pol Klenow fragment were purchased from Boehringer. The Sequenase kit was obtained from USB.

Preparation of gapped DNA substrate

Single-stranded M13(mp11) DNA (ssDNA) was prepared according [31]. The synthetic 40-mer oligodeoxynucleotide complementary to nt 7041–7080 of the M13 genome (called 'primer') and the 40-mer oligodeoxynucleotide complementary to nt 6771–6810 (called 'terminator') were synthesized at the service Department (Zoology) of the University of Zürich and purified by electrophoresis on a 5% denaturing PAGE. Primer (or terminator, if indicated) was labelled with γ -[³²P] ATP (Amersham) and polynucleotide kinase without isotopic dilution (equal amount of oligonucleotide and labelled nucleotide in the

reaction mixture) according to [31]. The labelled oligonucleotide (2-fold molar excess over template) and unlabelled oligonucleotide (10-fold excess) were annealed to M13 DNA as described [20]. Further details can be found in Results and Figure 1.

Gap filling assay

A final volume of 25 μ l contained the following components: 40 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 10 mM MgCl₂, 1 mM ATP, dATP, dGTP, dCTP, dTTP each at 50 mM, 100 ng of gapped [³²P]M13 DNA (labeled primer), 100 ng of PCNA, 6 ng of RF-C, 350 ng of *E. coli* SSB or 600 ng of RP-A and amounts of pol as indicated in the appropriate figure. The mixture was incubated for the indicated amount of time at 37°C, then the reaction was terminated by addition of 13 μ l of 95% (v/v) formamide, 20 mM EDTA, pH 8). Samples were heated for 5 min at 95°C before loading onto a 5% PAGE, containing 7 M urea (17cm×21 cm×0.4 mm). Electrophoresis was performed at 30 V/cm until the bromophenol blue dye reached the bottom. The gel was then fixed in 10% acetic acid, 12% methanol, washed with distilled water, dried at 80°C and exposed to a X-ray film.

Ligation after gap filling was performed by adding 0.5 U T4 DNA ligase to the gap filling assay (labeled terminator) described above.

RESULTS

A defined gapped template as a model for the lagging strand

A gapped DNA substrate was prepared that should mimic the lagging strand of chromosomal DNA replication. For this a primer and a terminator were synthesized that created a gap of 230 nucleotides when annealed to M13 plus strand DNA (Fig. 1). This gap approximates that of an Okazaki fragment [32]. For measurements of gap filling, the primer (nt 1–40) was labeled before annealing and for the determination of ligation after gap filling, the terminator oligonucleotide (nt 270–310) was labeled. In addition, at position nt 160 (corresponding to nt 6920 of M13 DNA) a pause site was present that could show whether a particular pol was able to pass such a site.

Lagging strand DNA synthesis by pol α

High amounts of pol α (2.5–5 U) were sufficient to synthesize a nearly full length product of 270 nucleotides (40 nt of the primer and 230 nt newly synthesized DNA, Fig. 2, lanes 1–6) using the above described substrate. The pause site could only be passed at very high amounts of pol α and then only after 30 min of incubation. In low amounts (250–500 ng), RP-A stimulated pol α synthesis through the pause site (Fig. 2, lanes 7–10). High amounts of RP-A on the other hand, dramatically inhibited pol α (Fig. 2, lanes 11 and 12). PCNA and RF-C alone

Figure 1. Gapped DNA substrate as a model for a lagging strand. Gapped DNA substrate was prepared as described in Materials and Methods by the annealing two synthetic oligonucleotides thus leaving the 230 nt gap. For further details see text.

had no influence on pol α (Fig. 2, compare lanes 5, 6 and 13, 14), but all three auxiliary proteins together showed a dramatic inhibition of pol α (Fig. 2, lanes 15 and 16). In sum, pol α does not require the auxiliary proteins PCNA, RF-C and RP-A for DNA synthesis on a lagging strand template.

Lagging strand DNA synthesis by pol β

An analogous experiment was carried out with pol β . Here addition of 2.5 U of pol β for 30 min or 5 U for 10 min resulted in complete gap filling while the pause site at nt 160 was passed without difficulty (Fig. 3, lanes 1–6). Upon longer incubation, it appeared that pol β was capable of limited strand displacement synthesis (see also Fig. 5). RP-A, at all concentrations tested, inhibited DNA synthesis by pol β significantly (Fig. 3, lanes 7–12). PCNA and RF-C had no effect on pol β synthesis (Figure 3, compare lanes 5, 6 and 13, 14) and the effect of all three auxiliary proteins together was similar to the inhibitory effect of RP-A alone (Figure 3, compare lanes 9, 10 and 15, 16). In sum, pol β , like pol α , does not require the auxiliary proteins PCNA, RF-C and RP-A for DNA synthesis on a model lagging strand template. However, pol β is not effected by the pausing site and does not exactly stop at a terminator.

Lagging strand DNA synthesis by pol δ and ϵ

Since calf thymus PCNA and RF-C can form stable holoenzyme complexes with pol δ and pol ϵ [20], we were interested in the synthetic characteristics of these complexes on a model lagging

strand substrate. Pol δ core alone was completely unable to act on the the gapped template (Fig. 4A, lanes 1 and 2) and was fully active only in the presence of PCNA, RF-C and RP-A (Fig. 4A, lanes 7–12). Upon longer incubation (30 min), pol δ holoenzyme similarly to pol β clearly showed limited strand displacement synthesis (Fig. 4A, lanes 8, 10 and 12, and see also Fig. 5). Pol δ holoenzyme was not effected by the pause site. As expected, (see [25] for the rationale) the situation with pol ϵ was more complex. Low levels (0.08–0.25 U) of pol ϵ core alone were capable of filling the gap but were also stopped by the pause site (Fig. 4B, lanes 1–6). The pause site could be overcome by addition of PCNA, RF-C and *E. coli* SSB (Fig. 4C, for the dependence of gap filling by pol ϵ on the concentration of *E. coli* SSB in the presence of PCNA and RF-C see [25]). A weaker effect was obtained if RP-A replaced SSB. We have no explanation for this phenomenon, but similar data have been obtained with measurements on primed or unprimed M13 DNA, the latter previously primed with pol α /primase [25]. In contrast to pol δ holoenzyme, pol ϵ either in the form of the core- or the holoenzyme, stopped at the termination point (see also Fig. 5).

Sequence determination of the region where pol α , pol β , pol δ holoenzyme, pol ϵ core enzyme and pol ϵ holoenzyme stop DNA synthesis

From Fig. 5 it can be seen that pol α , pol ϵ core and pol ϵ holoenzyme stop within 1–3 nt of the precise junction between product DNA and the terminator 5' end. The three nucleotides that were synthesized into the terminator might be due to the long incubation time (30 min) at 37°C, where slight 'breathing' of

Figure 2. Lagging strand DNA synthesis by pol α . Reaction mixtures with gapped DNA (labelled primer, unlabelled terminator) were incubated with pol α (different amounts as indicated on top of the autoradiogram) and auxiliary proteins (100 ng PCNA, 6 ng RF-C and different amounts of RP-A as indicated on top of the autoradiogram), aliquots were taken at 10 and 30 min and analyzed in a 5% denaturing PAGE as described in Materials and Methods. Markers were: positions 270 nt and 160 nt were determined by sequencing of the *in vitro* replication products (e.g. for 270 nt see Fig. 5), 40 nt corresponds to initial labeled primer.

Figure 3. Lagging strand DNA synthesis by pol β . Reaction mixtures with gapped DNA (labelled primer, unlabelled terminator) were incubated with pol β (different amounts as indicated on top of the autoradiogram) and auxiliary proteins (100 ng PCNA, 6 ng RF-C and different amounts of RP-A as indicated on top of the autoradiogram). Aliquots were taken at 10 and 30 min and analyzed in 5% PAGE as described in Materials and Methods. Markers were as in Fig. 2.

Figure 4. Lagging strand DNA synthesis by pol δ or ϵ . Reaction mixtures with gapped DNA (labelled primer, unlabelled terminator) were incubated with pol δ or ϵ (different amounts as indicated on top of the autoradiogram) and auxiliary proteins (100 ng PCNA, 6 ng RF-C and different amounts of RP-A as indicated on top of the autoradiogram) as described in Materials and Methods. Markers were as in Fig. 2. (A): dependence of pol δ on the auxiliary proteins to perform gap completion. (B): gap completion by pol ϵ in the absence of auxiliary proteins (C): effect of bovine RP-A and *E. coli* SSB on pol δ and pol ϵ to perform gap completion. Incubation was for 30 min.

the A-T stretch might occur. Pol β and especially pol δ holoenzyme, in contrast, performed limited strand displacement synthesis, especially at longer incubation times. Since we were unable to detect any nonspecific nuclease, nor a 5'→3' exonuclease in both enzyme preparations (data not shown), a simple strand replacement DNA synthesis mechanism is very unlikely.

Ligation of elongated primers to the terminator after lagging strand DNA synthesis by pol α , pol β , pol δ holoenzyme, pol ϵ core enzyme and pol ϵ holoenzyme

Figure 6B shows the rationale used to measure ligation after lagging strand DNA synthesis. The four pol's were present in amounts that filled the gap within 30 min (Fig. 2–4). Since now the terminator was labeled, all the pol's could also convert it into a visible long DNA product. With the exception of pol ϵ core, all pol's synthesized long polynucleotides from 3'-end of the terminator, that could be converted by *Mvn I* into a 126 nt long product. Pol ϵ core, under the same conditions, elongated the terminator only with 10–17 nt and thus only a small amount of 126 nt long product was obtained after restriction (Fig. 6, lanes 7, 8). When T4 DNA ligase and *Mvn I* were added together in the presence of all pol's tested, the labelled 126 nt product was ligated to the 270 nt gap filling product. As a control, *E. coli* pol I Klenow fragment was used and DNA synthesis was measured from the labeled primer in the absence of the terminator, resulting in a total product of 396 nt (Fig. 6, lane 16). For pol α , few detected products were ligated (Fig. 6, lane 3, compare spots at 126 and 396 nt), probably due to the fact that pol α left a 1–3

nt gap not completed (Fig. 5). Pol β and pol δ holoenzyme were very efficient in synthesizing ligatable products (Fig. 6, lanes 6 and 15, respectively). The pol ϵ core product was as inefficient in the ligation assay as pol α (Fig. 6, lane 9). The main product was shorter than 396 nt and was a result of ligation of the 270 bases polynucleotide to the terminator (40 nt) elongated by 10–17 nt only. Pol ϵ holoenzyme yielded notably more ligated products, when compared with its core enzyme. Two main products were obtained after ligation: the upper one corresponded to the ligation of 270 nt to 126 nt and lower one resulted from the ligation of 270 nt to the not-elongated primer (Fig. 6, lane 12).

DISCUSSION

The four nuclear pol's α , β , δ , and ϵ were tested in the presence of the three auxiliary proteins PCNA, RF-C and RP-A for their ability to perform DNA synthesis on a model lagging strand template. The substrate has deliberately been designed without an RNA primer at the downstream oligonucleotide in order to address the question of which pol is a likely candidate for precise gap filling. The results obtained indicated that under certain conditions all four pol's were able to fill the Okazaki sized gap.

At this point an important question arises: how is the lagging strand organized *in vivo*? An *in vitro* DNA repair system showed that PCNA [33, 34] and RP-A [35, 36] are essential components for excision repair. No evidence has yet been presented about the role of RF-C in repair DNA synthesis. However the interaction of PCNA and RF-C was demonstrated on RP-A- or

Figure 5. Sequence data of lagging strand DNA synthesis by pol α , β , δ and ϵ in the absence (—) or presence (+) of the three auxiliary proteins PCNA, RF-C and *E. coli* SSB. (A): To prepare M13 DNA sequences only [32 P]-labelled primer was annealed to the template. M13 DNA was sequenced with Sequenase T7 DNA polymerase (USB) according to the manufacturer's protocol. Left lanes of the autoradiograph show the patterns with all four ddNTP. Markers are shown as the length of polynucleotides beginning from the 5'-end of the 40 nt long primer. The arrow corresponds to last nucleotide of the gap. Reaction mixtures contained gapped DNA (labelled primer, unlabelled terminator), pol α (2.5 U), pol β (2.5 U), pol δ (0.25 U) or pol ϵ (0.25 U) and PCNA (100 ng), RF-C (6 ng) and SSB (350 ng) (if indicated) were incubated for 30 min at 37°C and analyzed on a 5% PAGE. together with the sequence samples. (B) Square brackets for each polymerase show the region where DNA synthesis was mainly completed (the last nt of gap and first nt of terminator-template junction).

SSB-covered singly-primed DNA [17–20]. The absolute and simultaneous requirement of PCNA, RF-C and RP-A for *in vitro* SV40 DNA replication [6] is further strong evidence that all three auxiliary proteins can act everywhere at template-primer junctions available in nuclei. Thus if auxiliary proteins act at the lagging strand, then our data exclude the possibility that pol α /primase synthesizes the lagging strand alone. In contrast, PCNA, RF-C and RP-A (SSB) form with template-primer the specific complex that is essential for pol δ and pol ϵ action at the 3'-terminus. Pol ϵ holoenzyme appears to be a good candidate for gap filling of an Okazaki fragment since (i) the enzyme could exactly fill the gap to the 5' end of the DNA fragment downstream. (ii) A strong pause site for pol α /primase was no obstacle for pol ϵ holoenzyme. (iii) The product synthesized by pol ϵ holoenzyme could be ligated to the downstream DNA fragment.

Recently a 5'→3' exonuclease has been found to copurify with pol ϵ [37]. This activity is a likely candidate for an enzyme that can remove primers from Okazaki fragments [38]. Since this activity interacted with pol ϵ , the suggestion was made that pol ϵ might act as a lagging strand replication enzyme [37]. The pol ϵ used in the present work does not contain detectable 5'→3' exonuclease activity [27]. Our next step in this investigation of

Figure 6. Ligation of elongated primer to the terminator after leading strand DNA synthesis by various DNA polymerases. A: Reaction mixtures containing gapped DNA (unlabelled primer, [32 P]-labelled terminator), pol α (2.5 U), pol β (2.5 U), pol δ (0.25 U) or pol ϵ (0.25 U), PCNA (100 ng), RF-C (6 ng) and SSB (350 ng) (if indicated), restriction endonuclease *Mvn I* (3 U) and T4 ligase (0.5 U) (if indicated) were incubated 30 min and analyzed by 5% PAGE as described in Materials and Methods. Markers were: 40 nt correspond to the initial labelled terminator and 126 nt to the GC/GC restriction site downstream the template strand from 5'-end of the terminator. 396 nt marker was polymerized on primed M13 DNA (labelled primer, no terminator) with pol I Klenow fragment (KF, 0.5 U) with simultaneous *Mvn I* restriction and corresponds to the length from 5'-end of the primer to GC/GC restriction site downstream of the template.

Okazaki gap filling will be to test a substrate, that contains RNA at the 5' end of the downstream fragment and to look into the interaction of pol ϵ holoenzyme with 5'→3' exonuclease [37] and RNase H [39].

Why is pol ϵ not required for *in vitro* SV40 replication in general and for Okazaki fragment processing in particular? Our data clearly indicate that pol α /primase at high concentrations and upon long incubation, can completely fill an Okazaki size gap (Fig. 2) and that it can produce a ligatable product (Fig. 6). Furthermore pol δ holoenzyme (pol δ , PCNA, RF-C) together with RP-A in the *in vitro* SV40 replication system, can also perform gap filling (Fig. 4). The biochemical data on singly DNA-primed M13 DNA and on ssM13 DNA that had been primed with pol α /primase suggested a cooperation between pol α /primase and pol ϵ holoenzyme [25]. Therefore, the data presented in this paper favour pol ϵ as a gap filling enzyme. In summary our results are in accordance with the model proposed first by Burgers [18] predicting that pol δ might be the leading and pol ϵ the second lagging strand replication enzyme.

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