

# Processive proofreading by the adenovirus DNA polymerase. Association with the priming protein reduces exonucleolytic degradation

Audrey J. King, Wieke R. Teertstra, Luis Blanco<sup>1</sup>, Margarita Salas<sup>1</sup> and Peter C. van der Vliet\*

Laboratory for Physiological Chemistry, University of Utrecht, PO Box 80042, 3508 TA Utrecht, The Netherlands and <sup>1</sup>Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

Received January 22, 1997; Revised and Accepted March 13, 1997

## ABSTRACT

**By using a baculovirus expression system, the adenovirus (Ad) DNA polymerase was purified to homogeneity and shown to display a 3'→5' exonuclease activity which is coupled to the polymerase activity. On a partial duplex structure the exonuclease activity had a marked preference for excision of a mismatched versus a matched 3'-terminus, which enables the Ad DNA polymerase to act as a proofreading enzyme. On single-stranded DNA the exonuclease action is distributive, but during replication removal of mismatched nucleotides and the switch to synthesis occurs without dissociation of the polymerase from the template. When the Ad DNA polymerase is bound to the precursor terminal protein, the rate of exonucleolysis was four times slower. Moreover, degradation could not proceed as far as with the free Ad polymerase, indicating also a qualitative difference. These results suggest a reduced proofreading capacity of the precursor terminal protein–polymerase complex, which might affect the initial stages of DNA replication.**

## INTRODUCTION

For all DNA-dependent DNA polymerases it is an absolute requirement to copy DNA with high fidelity. One of several mechanisms that enhances the fidelity of DNA replication is proofreading. DNA polymerases performing proofreading have a 3'→5' exonucleolytic activity which is able to release dNMPs from the 3'-terminus of the growing strand. During replication this activity is used for the preferential excision of mismatched bases originating from errors made in DNA synthesis. Exonucleases are said to have proofreading activity if they satisfy the following criteria (for a review see 1): (i) they prefer a single-stranded (ss) to a double-stranded (ds) DNA substrate; (ii) they preferentially excise a mispaired rather than a correctly paired primer terminus; (iii) they are physically associated with the polymerase, either as a part of the same polypeptide or as an associated subunit; (iv) the exonucleases act coordinately with the polymerase to enhance the

fidelity of DNA synthesis. For coordination of the polymerase and exonuclease activities, displacement of the primer terminus is required, since the polymerase and exonuclease active sites are distantly located (2).

The linear genome of adenovirus contains two origins of replication, which are located in the inverted terminal repeats. Both initiation and polymerization are catalyzed by a viral DNA polymerase. In infected cells the adenovirus (Ad) DNA polymerase (pol) is present as a complex with the precursor terminal protein (pTP). For initiation the Ad DNA polymerase uses the pTP as primer and catalyzes the formation of a phosphodiester bond between the β-hydroxyl group of Ser580 in pTP (Ad2/5) and the α-phosphoryl group of a dCTP molecule. During this protein priming event the DNA polymerase employs a jumping-back mechanism, which makes use of a triplet repeat at the ends, to maintain the correct sequences at the termini. This implies that replication starts by the formation of a pTP–CAT intermediate guided by template positions 4–6. Subsequently, this pTP–CAT intermediate jumps back to become paired with template positions 1–3 before elongation starts (3). The pTP–CAT intermediate is further elongated by Ad DNA polymerase, requiring the Ad DNA binding protein (DBP) as the third viral protein (for a review see 4,5). Similar end recovery mechanisms for initiation have been found in other protein-primed DNA replication systems (6–8). The existence of such a correction mechanism suggests that maintenance of DNA ends is sensitive to errors produced during initiation. Moreover, Esteban *et al.* (9) found that the exonuclease activity of φ29 DNA polymerase is not able to act on the initiation product, suggesting that polymerase-coupled proofreading cannot correct errors during the initiation reaction.

The presence of exonucleolytic activity in a partially purified Ad DNA polymerase preparation has been reported (10,11), but any link between exonuclease activity and proofreading was not determined. In the present study we overexpressed Ad DNA polymerase employing a recombinant baculovirus, purified the enzyme to homogeneity and set out to characterize its exonucleolytic activity. We show here that this activity is intrinsic to Ad DNA polymerase and satisfies several criteria for proofreading enzymes,

\*To whom correspondence should be addressed. Tel: +31 30 253 8989; Fax: +31 30 253 9035; Email: p.c.vandervliet@med.ruu.nl

strongly suggesting that it is involved in proofreading. The processivity of both the polymerase and exonuclease activities as well as the processivity during proofreading were measured. Furthermore, we show that the exonuclease activity of complexed Ad DNA polymerase (the pTP-pol complex) was clearly lower compared with the free form of Ad DNA polymerase. Because this pTP-pol complex is only present during the first steps of replication (King *et al.*, submitted), this suggests that exonucleolytic proofreading is diminished during initiation of Ad DNA replication.

## MATERIALS AND METHODS

### Nucleotides and DNA templates

Unlabeled dideoxy- and deoxynucleotides were purchased from Pharmacia. The [ $\alpha$ - $^{32}$ P]dNTPs were from ICN and [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol) was obtained from Amersham International. Oligonucleotides SP1 (5'-GATCACAGTGAGTAC), SP1P (5'-GATCACAGTGAGTAG) and SP1C+6 (5'-TCTATTGTACTACTGTGATC) were prepared in a 380A synthesizer (Applied Biosystems) and subsequently purified by electrophoresis by 8 M urea-20% PAGE. Oligonucleotides SP1 and SP1P were 5'-end-labeled with [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase and further purified by PAGE. Labeled SP1 and SP1P (ss) were used as a substrate for the 3'→5' exonuclease activity of Ad DNA polymerase. Partially ds primer/template structures were created by hybridizing labeled SP1 or SP1P to the non-labeled SP1C+6 oligonucleotide in the presence of 0.2 M NaCl and 60 mM Tris-HCl, pH 7.5. The mixture was heated at 70°C and allowed to cool down slowly to room temperature.

### Purification of Ad DNA polymerase and the pTP-pol complex

*Ad DNA polymerase* Insect cells (Sf9) were grown in 25 T150 flasks in Grace medium supplemented with 10% fetal calf serum at 27°C to  $\sim 2 \times 10^7$  cells/flask and infected with recombinant baculovirus expressing Ad DNA polymerase for 1 h at 27°C. The viral inoculum was removed and the infected cells were further incubated for 48 h at 27°C. After 48 h, cells were dislodged from the flasks, collected by centrifugation at <200 g, washed once with ice-cold phosphate-buffered saline and resuspended in extraction buffer A containing 25 mM HEPES, pH 7.5, 5 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT), 0.35 mM phenylmethylsulfonyl fluoride (PMSF), 15 µg/ml sodium bisulfite, 2 µg/ml aprotinin, 1 µg/ml leupeptin. The cell suspension was incubated for 15 min on ice and disrupted by 25 strokes in a Dounce homogenizer (B pestle). To extract the nuclei, NaCl was added to a final concentration of 200 mM followed by a further incubation of 30 min on ice. Cell debris was removed by centrifugation at 15 000 g and finally the extract was cleared by centrifugation at 100 000 g for 40 min at 4°C. Glycerol was added to a final concentration of 20%. The extract was applied to two connected heparin cartridges (5 ml, each; BioRad Econopac) equilibrated with buffer B (25 mM HEPES, pH 7.5, 1 mM EDTA, 2 mM DTT, 0.5 mM sodium bisulfite, 0.35 mM PMSF and 20% glycerol) with 200 mM NaCl (B/200 mM NaCl). After washing with buffer B/200 mM NaCl the bound proteins were eluted with buffer B/600 mM NaCl. Peak fractions as determined by A<sub>280</sub> measurement were diluted with the same buffer to 200 mM NaCl and loaded on a 20 ml ssDNA cellulose column. The column was

washed extensively with buffer B/200 mM NaCl and bound proteins were eluted with buffer B/600 mM NaCl. Fractions (1 ml) were analyzed by SDS-PAGE followed by silver staining. Part of the peak fractions (200 µg) was further purified using a 5 ml glycerol gradient of 18–30% glycerol in buffer B/1 M NaCl. The gradients were centrifuged for 24 h at 50 000 r.p.m. in a SW50 rotor. Fractions (200 µl) were collected, screened for polymerase activity (using activated calf thymus DNA) and analyzed by SDS-PAGE followed by silver staining. The estimated purity was >95%. Furthermore, the fractions were screened for the presence of exogenous nucleases by monitoring the breakdown of Φ29 DNA, a 19 kb dsDNA molecule, in an alkaline agarose gel. Peak fractions containing the Ad DNA polymerase were negative in this assay.

*pTP-pol complex.* Insect cells were infected with baculoviruses expressing Ad pol and Ad pTP separately. Infections were as described above except that the incubation time for cells infected with pTP-expressing baculovirus was 72 instead of 48 h. Separate extracts were made as described above but to leach the nuclei of the pTP-expressing cells NaCl was added to 300 mM. The extracts were mixed, diluted to 150 mM NaCl and the mixture was applied to two connected heparin cartridges, which were equilibrated with buffer B/150 mM NaCl. After washing with buffer B/150 mM NaCl, the bound proteins were eluted with buffer B/600 mM NaCl. Peak fractions were determined by A<sub>280</sub> monitoring, diluted with buffer B to 150 mM NaCl and loaded on a heparin HiTrap cartridge for concentration. The column was washed extensively and bound proteins were eluted at 600 mM NaCl. Peak fractions were loaded on a gel filtration column (Superdex™ 200) equilibrated in buffer B with 600 mM NaCl. Fractions of 2 ml were collected and analyzed by SDS-PAGE followed by silver staining. Finally, the peak fractions of the Superdex column were loaded on a Mono S 5/5 FPLC column for further purification and concentration. The peak fractions were first diluted with buffer B/100 mM NaCl and then applied to Mono S equilibrated in the same buffer. After washing, the proteins were eluted with a linear gradient of 100–600 mM NaCl in buffer B. Fractions of 1 ml were collected and tested by SDS-PAGE followed by silver staining, the estimated purity being >95%.

### DNA polymerization (filling-in) assay

The standard incubation mixture (25 µl) contained 25 ng Ad DNA polymerase and 250 ng *Eco*RI-digested Φ29 DNA (1.6 pmol DNA ends) as a template in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 0.25 µM [ $\alpha$ - $^{32}$ P]dATP (2 µCi), 4% glycerol and 0.1 mg/ml bovine serum albumin (BSA). The filling-in reaction was allowed to proceed for 10 min at 37°C and stopped by adding EDTA to 10 mM. To determine the dAMP turnover during this assay, 2 µl of the reaction mixture were withdrawn at this point and analyzed by thin layer chromatography (Polygram Cel 300 PEI/UV254). The chromatogram was developed with 0.15 M lithium formate, pH 3.0, followed by autoradiography. Under these conditions, 5'-dAMP migrates while the DNA remains at the origin. To the remaining samples, SDS was added to 0.1% and the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volume was counted (Cerenkov radiation) and analyzed by agarose gel electrophoresis and autoradiography.

### 3'→5' Exonuclease assay

For the exonuclease assays, either ssDNA (SP1) or hybrid molecules between SP1 or SP1P and SP1C+6, made as described above, were used. In each case SP1 or SP1P was labeled at the 5'-end. Standard incubation mixtures of 12.5  $\mu$ l contained 0.12 ng ss or hybrid molecule (with matched or mismatched primer terminus) and 12.5 ng Ad DNA polymerase, in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 4% glycerol and 0.1 mg/ml BSA. After incubation for the indicated time and at the indicated temperature, the reactions were stopped by addition of EDTA to 10 mM. Samples were analyzed by 20% PAGE in 8 M urea and autoradiography.

### Polymerase/3'→5' exonuclease assay

The hybrid molecule between SP1 and SP1C+6 containing a 6 nt protruding 5'-end was used as a substrate for 3'→5' exonuclease activity and as a primer/template for DNA polymerization. Standard incubation mixtures of 12.5  $\mu$ l contained 0.12 ng hybrid molecule, 12.5 ng Ad DNA polymerase in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 4% glycerol, 0.1 mg/ml BSA and the indicated concentrations of the four dNTPs. After incubation for 10 min at 37°C, the reactions were stopped by addition of EDTA to 10 mM. Samples were analyzed by 20% PAGE in 8 M urea and autoradiography.

### Determination of the processivity during exonucleolytic degradation

To test the processivity of the 3'→5' exonuclease activity of Ad DNA polymerase, challenger M13mp18 ssDNA was added at different time points to the same incubation mixture as described above in the exonuclease assays. For these experiments the metal activator was omitted from the reaction mixture and was used to start the reaction. <sup>32</sup>P-labeled SP1 (0.12 ng) and Ad DNA polymerase were preincubated for 15 min at 4°C. The reaction was started by addition of MgCl<sub>2</sub> to 1 mM together with the challenger DNA (0.05  $\mu$ g), incubated for 10 min at 37°C and analyzed as described above. In the control experiment the challenger DNA was added in the preincubation step together with labeled SP1 DNA.

### Determination of the processivity during DNA synthesis

M13mp18 ssDNA primed with the 17mer universal primer was used. The primer/template was made by hybridization of 28 ng primer to 1  $\mu$ g M13mp18 ssDNA. The reaction mixture of 25  $\mu$ l contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 4% glycerol, 0.1 mg/ml BSA, 80  $\mu$ M of the four dNTPs, 2  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP, 1  $\mu$ g primed M13 DNA and the indicated amount of Ad DNA polymerase. After incubation for 10 min at 37°C, the reactions were stopped by addition of EDTA to 10 mM. NaOH was added to a concentration of 1 M and samples were subjected to electrophoresis in alkaline 0.7% agarose gels.

### Trapping experiment to detect transfer from the exonuclease to the polymerase site

The hybrid molecule between SP1P and SP1C+6 (mismatched primer terminus) was used to test if the polymerase dissociates from the template during proofreading. Standard incubation mixtures of 12.5  $\mu$ l contained 0.12 ng hybrid molecule, 12.5 ng

Ad DNA polymerase in a buffer containing 500 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA. Reactions were started by addition of MgCl<sub>2</sub> to a final concentration of 1 mM. Heparin (1 mg/ml) was added to trap the non-bound DNA polymerase at the indicated time point, either in a preincubation step or at the start of the reaction together with the MgCl<sub>2</sub>. When indicated, 500 nM of the four dNTPs or only dATP, dGTP and dTTP at 500 nM were added together with MgCl<sub>2</sub> and heparin. After incubation for 10 min at 37°C, the reactions were stopped by addition of EDTA to 10 mM. Samples were analyzed by 20% PAGE in 8 M urea and autoradiography.

## RESULTS

### Ad DNA polymerase possesses a 3'→5' exonuclease activity which is coupled to the polymerase activity

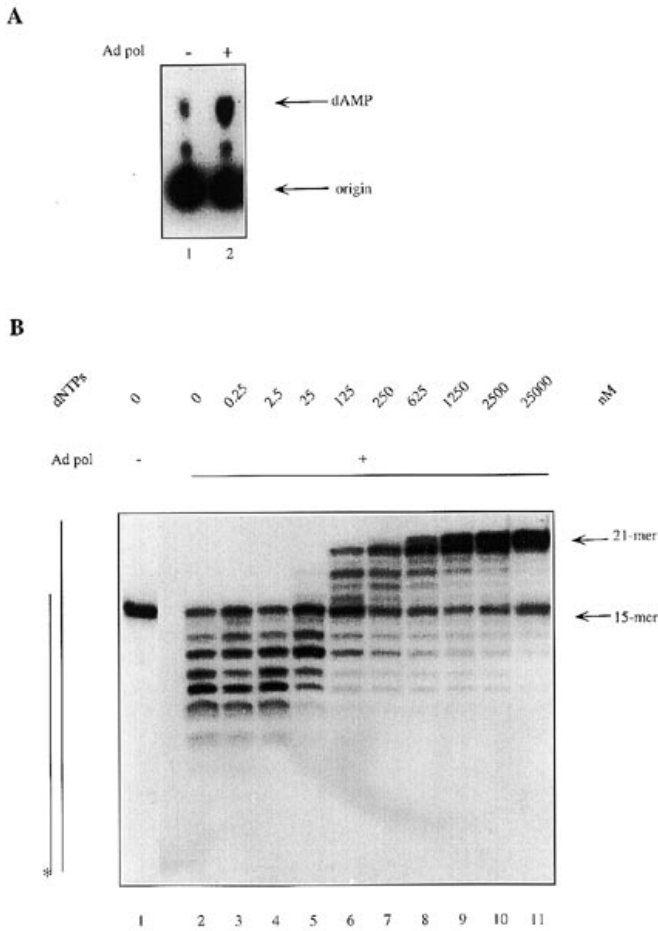
Ad5 DNA polymerase was expressed employing a recombinant baculovirus and purified to apparent homogeneity using a four step purification scheme as described in Materials and Methods. We used purified Ad DNA polymerase to fill-in recessive DNA 3'-ends in the presence of 0.2  $\mu$ M [ $\alpha$ -<sup>32</sup>P]dATP. During this incubation [ $\alpha$ -<sup>32</sup>P]dAMP was released as a secondary product, detected by thin layer chromatography (Figure 1A, lane 2). The control experiment where no protein was added shows the background level of [ $\alpha$ -<sup>32</sup>P]dAMP (Fig. 1A, lane 1). This result indicates the presence of a 3'→5' exonuclease activity that acts in association with the DNA polymerase activity. This exonuclease activity co-purified with the DNA polymerase activity throughout the purification and co-sedimented in a glycerol gradient run in a high salt (1 M) buffer, suggesting that both activities reside in the same polypeptide.

To determine coupling between the polymerization and exonuclease activities shown to be associated with Ad DNA polymerase, we monitored degradation and extension of a primer/template structure. The primer/template structure consisted of a 5'-end-labeled oligonucleotide fully base paired to a larger template oligonucleotide (SP1/SP1C+6; see Materials and Methods for sequence; Fig. 1B, lane 1). When Ad DNA polymerase was added to a primer/template in the absence of dNTPs or at low dNTP concentrations, the primer was degraded, giving rise to labeled products as low as 9 nt (Fig. 1B, lanes 2–5), although higher concentrations of Ad DNA polymerase produced degradation products with a minimum length of 3 nt (see Fig. 6). When increasing concentrations of dNTPs (125 nM and higher) were added, products longer than 15 nt were gradually formed (Fig. 1B, lanes 6–11), indicating that the exonucleolytic activity was competed away. This shows that there is a dynamic equilibrium between both activities and confirms that the exonuclease and polymerase activities are coupled and act coordinately.

### Preferential excision of a mismatched 3'-end

One of the basic criteria for an exonuclease to perform proofreading is the preferential excision of a mispaired primer terminus. Mismatch specificity was examined under non-polymerization conditions. Degradation patterns of a primer with a matched (C:G) (SP1/SP1C+6) or a mismatched (G:G) 3'-end (SP1P/SP1C+6) were compared at different incubation temperatures. Figure 2 shows that the exonuclease activity has a clear preference (4-fold) for excision of the mismatched primer/template, especially at the lower temperatures (10 and 25°C). At higher



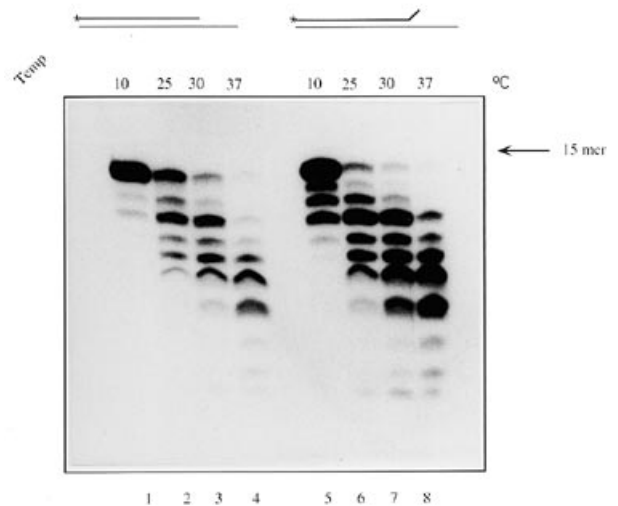


**Figure 1.** Ad DNA polymerase contains a 3'→5' exonuclease activity which is coupled to the polymerase activity. (A) Filling-in of recessive 3'-ends by Ad DNA polymerase using [ $\alpha$ - $^{32}$ P]dATP as described in Materials and Methods. dAMP was visualized by thin layer chromatography. (B) A partial duplex consisting of a 5'- $^{32}$ P-end-labeled 15mer and a non-radioactive 21mer (SP1/SP1C+6) was used as a substrate for 3'→5' exonuclease or as primer/template for DNA polymerization. Control lane 1 shows intact SP1/SP1C+6 prior to incubation with the polymerase. In the other lanes SP1/SP1C+6, Ad DNA polymerase and dNTPs, at the indicated concentrations, were incubated for 10 min at 37°C. The reaction products were analyzed on a 20% polyacrylamide-8 M urea gel. Arrows indicate the 15mer (non-elongated primer) and the 21mer (completely elongated primer) positions.

temperatures (30 and 37°C) the difference between the mismatched and matched primers/templates is less pronounced. This is in agreement with an editing function for which melting is an essential step (12). Taken together, the preference for a mismatch and the coordinated action of polymerase and exonuclease strongly indicate that Ad DNA polymerase is a proofreading enzyme.

### Distributive exonucleolytic degradation

Exonuclease assays on ssDNA (SP1) were carried out as described in Materials and Methods. The kinetics of degradation on ssDNA showed a gradual (stepwise) decrease in the length of the products (Fig. 3A). This might be explained by very slow degradation carried out by a processive enzyme or, more likely,

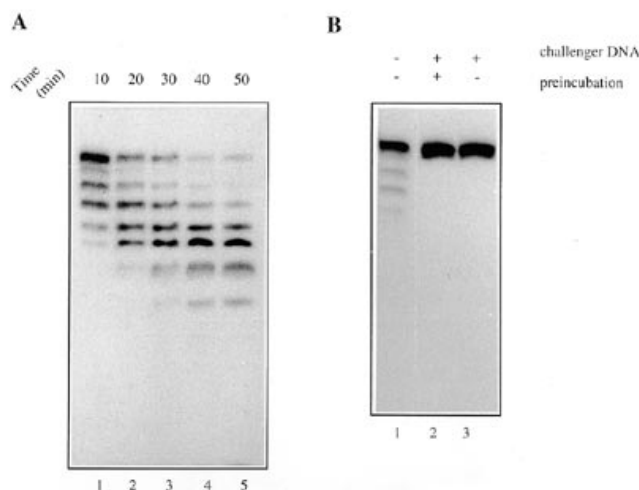


**Figure 2.** Preferential excision of a mismatched 3'-end by Ad DNA polymerase. Partial duplex molecules consisting of a 5'- $^{32}$ P-end-labeled 15mer with either a matched (SP1) or mismatched 3'-end (SP1P) and a non-labeled 21mer (SP1C+6) were used as a substrate for 3'→5' exonuclease activity. The exonuclease activity assay was carried out as described in Materials and Methods, using the primer/template and Ad DNA polymerase. The mixtures were incubated for 10 min at the indicated temperatures. Samples were analyzed on 20% polyacrylamide-8 M urea gels. An arrow indicates the position of the non-degraded primer.

by a distributive action where reassociation of the enzyme is the limiting factor. To further confirm this, similar exonuclease assays were performed in the absence or presence of challenger DNA, which was added to trap the dissociated DNA polymerase molecules. As shown in Figure 3B, addition of challenger DNA (0.05  $\mu$ g) either during the preincubation or at the beginning of the reaction (on addition of  $Mg^{2+}$ ) completely inhibited degradation. This indicates that Ad DNA polymerase dissociates rapidly from the oligonucleotide and thus acts in a distributive fashion.

### Processive DNA synthesis

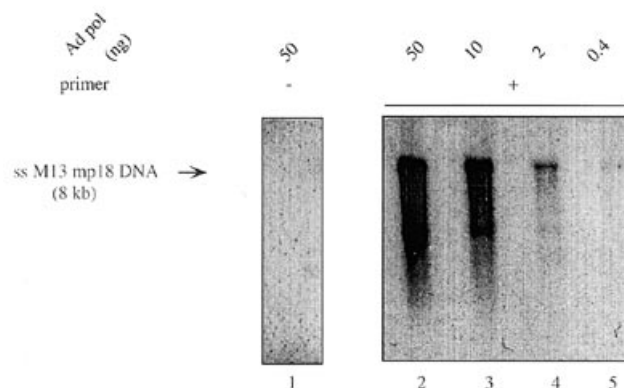
To assess the processivity of the polymerase activity we used M13mp18 ssDNA primed with the 17 nt universal primer as primer/template. Under the reaction conditions used, involving an excess of primer/template, an incubation time of ~10 min was needed to complete one round of full-length M13 DNA synthesis (result not shown). Whereas no products were obtained employing a template without a primer (Fig. 4, lane 1), full-length M13 replication products, but also some intermediates resulting from arrest of the polymerase at some sites of this template, were detected when primed M13 was used (Fig. 4, lane 2). DNA synthesis was studied using increasing dilutions of the enzyme. Under these conditions, non-processive synthesis would lead to a decrease in the size of the replication products as the polymerase was diluted. Upon dilution of the DNA polymerase up to 125-fold, a decrease in the amount of DNA synthesis was found, but still full-length M13 replication products were formed (Fig. 4, lane 5). This indicates that Ad DNA polymerase does not dissociate from the primer/template and polymerizes DNA on a ss template in a processive fashion in the absence of other proteins.



**Figure 3.** The 3'→5' exonuclease activity degrades ssDNA distributively. A 5'-<sup>32</sup>P-end-labeled ss oligonucleotide (15mer) was used as substrate for the exonucleolytic activity of Ad DNA polymerase. (A) Ad DNA polymerase was incubated with labeled SP1 for the indicated times as described in Materials and Methods. (B) The same amounts of Ad DNA polymerase and the labeled oligonucleotide as in (A) were used. This mixture was preincubated on ice in the absence of Mg<sup>2+</sup>. In lanes 2 and 3 challenger M13mp18 ssDNA was added either during preincubation or at the start of the reaction. The exonucleolytic reaction was initiated by addition of Mg<sup>2+</sup>. After incubation for 10 min at 37°C, the reactions were stopped and analyzed on a 20% polyacrylamide-8 M urea gel.

### Processive proofreading

During replication both exonuclease and polymerase activities must act in concert to ensure a high overall elongation rate. Since the polymerase and exonuclease active sites are believed to be located distantly in most DNA polymerases, based on studies with several DNA polymerase enzymes such as the Klenow fragment of *Escherichia coli* DNA polymerase I (2), this coordinated action requires a switch of the primer from one active site to the other. This switching can occur via either an inter- or intramolecular pathway. The processivity of Ad DNA polymerase during synthesis could suggest an intramolecular pathway because of a tight association of the polymerase with the template under these conditions. On the other hand, the distributivity in the exonuclease action may suggest dissociation of the polymerase after exonucleolysis. To elucidate whether Ad DNA polymerase can remove a misincorporated nucleotide and resume synthesis without undergoing dissociation from the template, we used single hit conditions as described by Reddy (13). This implies the use of heparin to trap DNA polymerase molecules that dissociate from the primer/template. The primer/template used here had a mismatched 3'-end (SP1P/SP1C+6). Using multiple hit conditions (untrapped), addition of Mg<sup>2+</sup> to the primer/template preincubated with Ad DNA polymerase resulted in normal degradation of the primer (Fig. 5, lane 1). To investigate the effectiveness of the trap we preincubated the primer/template with heparin together with polymerase and initiated the reaction by addition of Mg<sup>2+</sup>. This procedure indeed prevented binding of the polymerase to the primer/template, since no exonucleolytic degradation of the primer was observed (Fig. 5, lane 2). When the primer/template was preincubated only with Ad DNA polymerase and the reaction was initiated by simultaneous addition of Mg<sup>2+</sup> and heparin (Fig. 5,

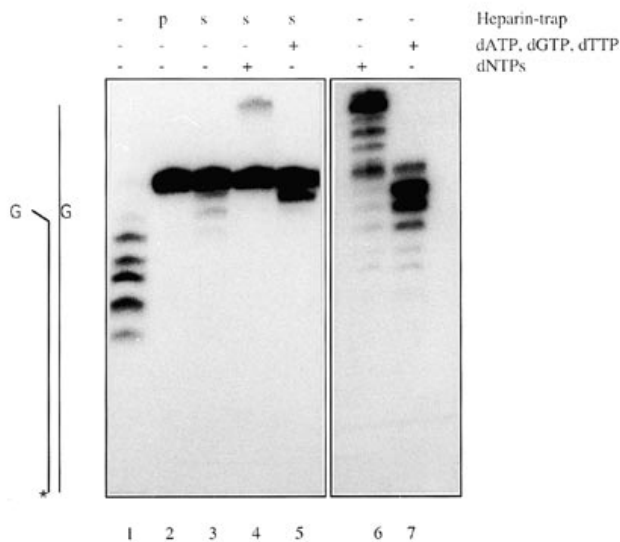


**Figure 4.** Processive DNA synthesis by Ad DNA polymerase. M13mp18 ssDNA primed with 17mer universal primer was used as primer/template for DNA polymerization. Decreasing Ad DNA polymerase concentrations, as indicated, were used. In lane 1 non-primed M13 ssDNA was used. Products were analyzed on an alkaline 0.7% agarose gel.

lane 3), the distributive character of the exonuclease reaction was again obvious. Only 1 or 2 nt of the primer were removed, indicating that Ad DNA polymerase dissociates rapidly from the primer/template. In the presence of all four dNTPs (500 nM) and in the absence of heparin, Ad DNA polymerase was able to extend the primer, giving rise to the full-length (21 nt) product as well as smaller products (Fig. 5, lane 6). The initial event here is removal of the mismatch followed by subsequent DNA synthesis. However, due to the low dNTP concentration (500 nM) the polymerase/exonuclease equilibrium allows some exonuclease activity, leading to partial degradation of the newly synthesized product, thus explaining the shorter products. When in a similar experiment the primer/template was preincubated with Ad DNA polymerase and the reaction was initiated by addition of Mg<sup>2+</sup>, dNTPs and heparin (single hit conditions), full-length products were also formed (Fig. 5, lane 4). This indicates that Ad DNA polymerase was not trapped after removal of the mismatch and can extend the primer without undergoing dissociation of the template. To confirm that the mismatch was removed before extension of the primer and that the extended product was not the result of mismatch elongation, the same reaction was repeated with three rather than four dNTPs (Fig. 5, lane 5). These conditions do not allow correction of the mismatch, for which dCTP is needed. In lane 5 no extension of the primer occurred, showing that the product observed in lane 4 is only formed after correction of the mismatch. Figure 5, lane 7 is the control showing the products of the same experiment as described for lane 5 in the absence of heparin. Together, these results show that Ad DNA polymerase is able to translocate the primer from the exonuclease to the polymerase active site of the molecule without dissociation and thus is performing processive proofreading.

### Limited degradation in the presence of pTP

In infected cells Ad DNA polymerase is complexed to the precursor terminal protein (pTP). This complex dissociates during replication (King *et al.*, submitted). However, early in replication Ad DNA polymerase is still bound to pTP. To investigate whether pTP can influence the exonuclease activity of Ad DNA polymerase we compared equimolar amounts of DNA polymerase free or in complex with pTP. In this experiment we



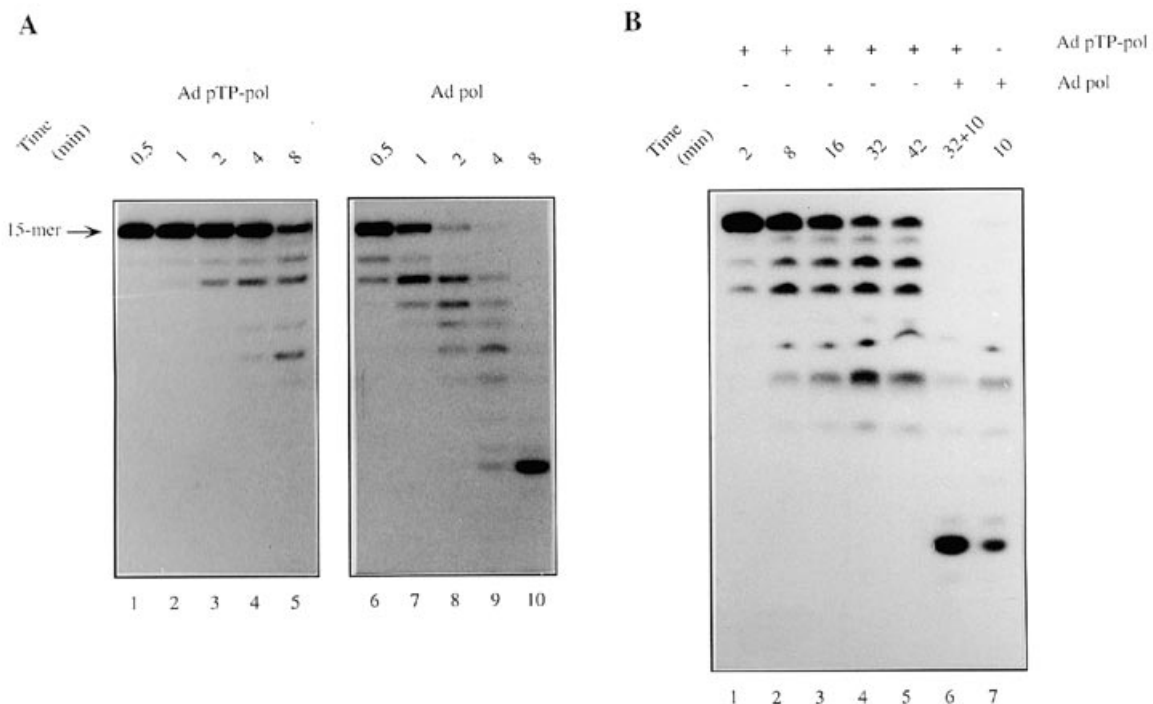
**Figure 5.** Processive proofreading by Ad DNA polymerase. Exonuclease and synthesis assays were performed as described in Materials and Methods using the 15/21 primer/template construct with a mismatched 3'-end (SP1/SP1C+6). The primer/template and Ad DNA polymerase were mixed and preincubated on ice. Reactions were started by addition of Mg<sup>2+</sup> and analyzed as described above. In lanes 2–5 the reaction was allowed to proceed for 10 min in the presence of heparin. Heparin was added either during preincubation (p) or at the start of the reaction (s), as indicated above the lanes. All four or only three (lacking dCTP) dNTPs (500 nM) were added at the start of the reaction when indicated (+).

used highly purified pre-formed pTP-pol complex to ensure the presence of the pTP-pol complex. A primer/template with a matched 3'-end (SP1/SP1C+6) was used to monitor the breakdown kinetics. Uncomplexed Ad DNA polymerase degraded the 15mer primer about four times faster than the pTP-pol complex (Fig. 6A). Furthermore, not only was a quantitative difference observed, but also a qualitative one. Ad DNA polymerase alone was able to degrade the primer to a minimum length of 3 nt (Fig. 6B, lane 7), while the polymerase in complex with pTP could only degrade the primer to a 8 nt product (Fig. 6B, lane 5), even after an extensive incubation. When the primer/template was incubated with the pTP-pol complex for 32 min and free Ad polymerase was added for an additional 10 min, the primer was degraded to a 3 nt product (Fig. 6B, lane 6), the same degradation pattern found after incubation with Ad DNA polymerase for 10 min (Fig. 6B, lane 7). This shows that degradation of the primer was still possible, indicating that pTP prevents maximal degradation of the primer, possibly due to steric hindrance. Since pTP is only associated with Ad DNA polymerase in an early step of replication, this suggests an impaired proofreading capacity at this stage of replication.

**DISCUSSION**

**Proofreading**

In this paper we have characterized Ad DNA polymerase-associated exonuclease, an intrinsic property, and shown that it enables Ad DNA polymerase to act as a proofreading enzyme. Proofreading was first proposed by Brutlag and Kornberg (12), who showed that the 3'→5' exonuclease of *E.coli* DNA polymerase I removed



**Figure 6.** Limited degradation in the presence of pTP limited. (A) Kinetics of exonucleolytic degradation of SP1/SP1C+6 was monitored. Degradation of the primer using pTP-pol complex (37.5 ng) and Ad DNA polymerase (25 ng) alone were compared at equimolar amounts of polymerase. (B) The exonuclease assay was carried out as above using the pTP-pol complex or Ad DNA polymerase. Incubation times were as indicated. In lane 5 SP1/SP1C+6 was incubated with pTP-pol for 32 min and then Ad DNA polymerase was added and incubation continued for 10 min.



a 3'-terminal mismatch before the DNA was elongated. Since this finding, this protein has served as a model for studying proofreading. A detailed analysis of the Klenow fragment structure contributed considerably to a better understanding of the editing function. Crystallographic data revealed a physical separation of the polymerase and exonuclease active sites (14). Furthermore, they showed that the exonuclease site must function solely as a ss exonuclease, because there is no space for dsDNA. To explain editing by the Klenow fragment of DNA polymerase I, a 'melt and slide' model was proposed by Joyce *et al.* (15) describing a rapid equilibrium between DNA bound to the polymerase site as a duplex and 3'-terminus bound to the exonuclease site as ssDNA. To reach the exonuclease site at least four bases must be unwound (15). Since this is easier for a mismatched 3'-terminus, this will drive the equilibrium to binding in the exonuclease site, resulting in removal of the mismatch. This model might be general for most prokaryotic and eukaryotic DNA polymerases, including Ad DNA polymerase, since the overall organization of the exonuclease domain of all these DNA polymerases is thought to be very similar to that of the Klenow fragment (16), now also confirmed for T4 DNA polymerase (N388 fragment) (17). Supporting this idea, we found that such an equilibrium between synthesis and degradation also exists for Ad DNA polymerase, as depicted in Figure 1B, where the equilibrium was shown to be influenced by the dNTP concentration. In addition, the 3'→5' exonucleolytic activity of Ad DNA polymerase shows a clear preference for degradation of a mispaired 3'-terminus compared with a correctly paired 3'-terminus. This preference is slightly diminished at increasing temperature, which is in agreement with the notion that melting is a crucial step in the editing function (12). Figure 5, lane 5 stresses that the exonuclease activity of Ad DNA polymerase contributes to the fidelity of DNA synthesis, since DNA synthesis was shown to stall if error editing was not allowed.

### Processivity during proofreading

Besides the fidelity of DNA synthesis, the rate of synthesis is also very important in DNA replication. An acceptable rate can be achieved if few association and dissociation events occur during DNA polymerase action. Maintenance of the enzyme-DNA association during polymerization, i.e. processivity, is often achieved by complexation with accessory proteins, such as the  $\beta$  subunit of *E.coli* DNA polymerase III or PCNA, both acting as a clamp (18,19). For Ad DNA replication the only other protein required during elongation besides the polymerase is the SSB-type Ad DBP. In this paper we show that Ad DNA polymerase alone can perform processive synthesis on a ss template. Processivity was not influenced by the addition of Ad DBP (not shown). Unlike in DNA synthesis mode, a polymerase should be distributive during exonucleolytic degradation in order to prevent excision of correctly paired bases. In agreement with this, Ad DNA polymerase degrades ssDNA in a strictly distributive manner. When a misincorporation is made, exonucleolytic correction will take place. For this to occur the primer is translocated from the polymerase to the exonuclease active site. Depending on the intrinsic processivity of each DNA polymerase, this can occur through intramolecular shuttling of the primer terminus or through an intermolecular pathway involving dissociation of the polymerase from the template. Immediately after exonuclease action, the corrected primer should be moved back

to the polymerase active site, again via intra- or intermolecular shuttling. Our data show a clear intramolecular transfer in the shift from exonuclease to polymerase site after mispair hydrolysis, in agreement with the processive polymerization displayed by Ad DNA polymerase. Comparable studies on the processive T4 DNA polymerase also showed an intramolecular shuttling between the exonuclease and polymerase sites (13), while the moderately processive Klenow fragment edits errors predominantly via an intermolecular pathway (20).

### Diminished exonuclease activity of the pTP-pol complex

We show here a clearly diminished rate of degradation of a primer/template by the pTP-pol complex compared with uncomplexed Ad DNA polymerase. Moreover, degradation is less extensive, leading to larger degradation products formed by the pTP-pol complex. The pTP-pol complex is only present in the early steps of replication, since there is dissociation of the complex after replication of several nucleotides (King *et al.*, submitted). Therefore, this result is compatible with a diminished proofreading capacity during these early steps. Editing on short replication products could be difficult in any case, since, at least for the Klenow fragment of DNA polymerase I, the minimal distance needed to transfer the primer from the polymerase to the exonuclease site is 4 nt (15). Based on these data, problems of editing could be expected when the growing chain is  $\leq 4$  nt long, as it occurs at the early stages of TP-primed replication. Moreover, the fact that a protein (TP) is attached to the first nucleotide could prevent fitting the first nucleotides in the exonuclease active site. This, together with the reduced exonuclease activity of Ad DNA polymerase when in complex with pTP, could predict a problem in proofreading during initiation of DNA replication. This may explain the need for a jumping- or sliding-back mechanism to recover and correct the DNA ends during protein-primed DNA replication (6,9).

### ACKNOWLEDGEMENTS

We are grateful to Dr R.T.Hay (St Andrews) and Dr E. Winnacker (Munich) for gifts of baculovirus expressing Ad DNA polymerase and Ad pTP. We thank J.M.Lázaro for help with purification of Ad DNA polymerase. This work was supported in part by an EMBO short-term fellowship to A.J.K., by the Netherlands Foundation for Chemical Research (SON) with financial support from the Netherlands Organization for Scientific Research (NWO) to P.C.van der V., by research grant 5R01 GM27242-17 from the National Institutes of Health to M.S., by grant no. PB93-0173 from Dirección General de Investigación Científica y Técnica to M.S., by grant CHRX-CT 93-0248 from the European Economic Community to P.C. van der V. and M.S. and by an Institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular 'Severo Ochoa'.

### REFERENCES

- 1 Kunkel,T.A. (1988) *Cell*, **53**, 837-840.
- 2 Ollis,D.L., Brick,P., Hamlin,R., Xuong,N.G. and Steitz,T.A. (1985) *Nature*, **313**, 762-766.
- 3 King,A. and van der Vliet,P.C. (1994) *EMBO J.*, **13**, 5786-5792.
- 4 van der Vliet,P.C. (1995) *Curr. Topics Microbiol. Immunol.*, **199**, 1-30.
- 5 Hay,R.T. (1996) *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 699-720.

- 6 Méndez,J., Blanco,L., Esteban,J.A., Bernad,A. and Salas,M. (1992)*Proc. Natl. Acad. Sci. USA*, **89**, 9579–9583.
- 7 Caldentey,J., Blanco,L., Bamford,D.H. and Salas,M. (1993)*Nucleic Acids Res.*, **21**, 3725–3730.
- 8 Martín,A.C., Blanco,L., García,P., Salas,M. and Méndez,J. (1996)*J. Mol. Biol.*, **260**, 369–377.
- 9 Esteban,J.A., Bernad,A., Salas,M. and Blanco,L. (1992)*Biochemistry*, **31**, 350–359.
- 10 Field,J., Gronostajski,R.M. and Hurwitz,J. (1984)*J. Biol. Chem.*, **259**, 9487–9495.
- 11 Lindenbaum,J.O., Field,J. and Hurwitz,J. (1986)*J. Biol. Chem.*, **261**, 10218–19227.
- 12 Brutlag,D. and Kornberg,A. (1972)*J. Biol. Chem.*, **264**, 241–248.
- 13 Reddy,M.K., Weitzel,S.E. and von Hippel,P.H. (1992)*J. Biol. Chem.*, **267**, 14157–14166.
- 14 Joyce,C.M. and Steitz,T.A. (1987)*Trends Biochem. Sci.*, **12**, 288–293.
- 15 Joyce,C.M. and Steitz,T.A. (1994)*Annu. Rev. Biochem.*, **63**, 777–822.
- 16 Bernad,A., Blanco,L., Lázaro,J.M., Martín,G. and Salas,M. (1989)*Cell*, **59**, 219–228.
- 17 Wang,J., Yu,P., Lin,T.C., Konigsberg,W.H. and Steitz,T.A. (1996)*Biochemistry*, **35**, 8110–8119.
- 18 Maki,S. and Kornberg,A. (1988)*J. Biol. Chem.*, **263**, 6561–6569.
- 19 Prelich,G., Tan,C.K., Kostura,M., Mathews,M.B., So,A.G., Downey,K.M. and Stillman,B. (1987)*Nature*, **326**, 517–520.
- 20 Joyce,C.M. (1989)*J. Biol. Chem.*, **264**, 10858–10866.