Molecular Architecture of Adenovirus DNA Polymerase and Location of the Protein-Primer.

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Molecular Architecture of Adenovirus DNA Polymerase and Location of the Protein-Primer.

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Adenovirus (Ad) DNA polymerase (pol) belongs to the distinct subclass of the pol α family of DNA polymerases that employs the precursor terminal protein (pTP) as primer. Ad pol forms a stable heterodimer with this primer and together they bind specifically to the core origin in order to start replication. After initiation of Ad replication, the resulting pTP-trinucleotide intermediate jumps back and pTP starts to dissociate. Compared to free Ad pol, the pTP-pol complex shows reduced polymerase and exonuclease activities, but the reason for this is not understood. Furthermore, the interaction domains between these proteins have not been defined and the contribution of each protein to origin binding is unclear. To address these questions, we used oligonucleotides with a translocation block and show here that pTP binds at the entrance of the primer binding groove of Ad pol, thereby explaining the decreased synthetic activities of the pTP-pol complex and providing insight into how pTP primes Ad replication. Employing an exonuclease-deficient mutant polymerase, we further show that the polymerase and exonuclease active sites of Ad pol are spatially distinct and that the exonuclease activity of Ad pol is located at the N-terminal part of the protein. In addition, by probing the distances between both active sites and the surface of Ad pol, we show that Ad pol binds a DNA region of 14-15 nucleotides. Based on these results, a model for binding of the pTP-pol complex at the origin of replication is proposed.

INTRODUCTION

Ad DNA replication requires three virally encoded proteins: Ad pol, pTP and the DNA binding protein (DBP). Replication initiates via a protein-priming mechanism involving Ad pol and the primer pTP which form the tight heterodimer pTP-pol. The precise interaction surface(s) between pTP and Ad pol have yet to be determined, although it has been suggested that the contacts extend over a large surface (24). Initiation of replication is catalyzed by Ad pol and can be stimulated by the two cellular transcription factors NFI and Oct-1, which function to recruit and position the pTP-pol complex to the origin of replication. The adenovirus (Ad) genome is a linear double-stranded DNA of about 36 kilobases with two origins located in the inverted terminal repeats (ITR). A terminal protein (TP) is covalently coupled to each 5'-end of the genome. The first 20 bp of the linear genome have been defined as the minimal replication origin. This minimal origin is highly conserved in all Ad serotypes and contains the pTP-pol binding site (nucleotides 9-18). Since both pTP and Ad pol bind to origin DNA with some specificity (31), the contribution of each protein is unclear. Next to the core origin an auxiliary region is present, containing binding sites for the transcription factors (for reviews see (9,13) and references therein).

After the formation of the pre-initiation complex at the origin of replication, initiation starts opposite the fourth base of the template strand with the covalent coupling of the initiating nucleotide, dCTP, to a serine residue in the primer pTP (20,21). When the third nucleotide is incorporated, the resulting trinucleotide intermediate (pTP-CAT) jumps back to basepair with template bases 1-3 (20). Concomitantly, pTP starts to dissociate from Ad pol (19). After dissociation, Ad pol replicates the Ad genome via a strand displacement mechanism that requires DBP and type I topoisomerase NFII (reviewed by de Jong et al,
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EXPERIMENTAL PROCEDURES

DNA templates, nucleotides and substrates

All oligonucleotides, unlabeled nucleotides, [α-32P]-dNTPs (3000 Ci/mmol), and [γ-32P]-dATP (5000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Streptavidin was purchased from Fishers. T30 (5'-AATCTAAAAGATATTATTGATGATG-3') represents the first 30 nucleotides of the template strand of the adenovirus 5 genome and D20 (5'-CATCTAAATATACCTT-3') is the complementary (displaced) strand of T30. Three oligonucleotides were used with an incorporated biotin molecule: Tbio5' (5'-bioATCCAAAAATAAGGTATATTGATGATG-3'), which is identical to T30 except for the 5' biotin being replaced with a biotin group, D7bio (5'-CATCATbioCAAATATACCT) which is identical to D20, only with an incorporated biotin group at position 7 and lacking the 3'-terminal nucleotide, and D7bio10 (5'-CATCATbioCAAATATACCTTTTGTGATG), which is identical to D7bio but with 10 extra nucleotides at the 3'-end. Labeling of the oligonucleotides was performed with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-32P]-dATP. The hybrid molecules D20/T30, D20/Tbio5', and D7bio/T30 were obtained by boiling oligonucleotides in 60 mM Tris-Cl, pH 7.5, 200 mM NaCl, followed by slow cooling to room temperature. All oligonucleotides used were purified by 10% polyacrylamide-1xTBE gel electrophoresis.

Proteins and buffers

All Ad proteins used were from serotype 2. Wild type Ad DNA pol was expressed from a baculovirus expression system and purified to near homogeneity as previously described (4). The exonuclease-deficient mutant polymerase D422A was constructed by performing site-directed mutagenesis on full length Ad pol cDNA as described previously (4). The oligonucleotides for the PCR mutagenesis were: 5'-ATCACCGGCTTTG-3' for the active site and the entrance of the primer binding groove, and 5'-GAGCACGATCTCG-3', changes marked in bold. The presence of the desired mutation was confirmed by sequencing. Preparation of the recombinant baculoviruses, protein expression and purification to near homogeneity was performed as described (4). The pTP-pol complex was purified as described (20). The buffer used for dilution of the polymerases and the pTP-pol complex contained 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mg/ml bovine serum albumin (BSA) and 20% glycerol.

Determination of the distance between the exonuclease active site and the entrance of the primer binding groove

Exonuclease breakdown of 5'-labeled D7bio or D7bio10 was studied in the absence or presence of 7 nM streptavidin (pre-incubation for 5 min). The total reaction mixture (25 μl) contained 50 mM Tris-Cl, pH 7.5, 1 mM DTT, 4% glycerol, 1 mg/ml BSA, 10 mM MgCl₂, and 0.05 ng of 5'-labeled D7bio. The reaction was started by adding Ad pol or the pTP-pol complex respectively, to a final concentration of 28.5 nM. After incubation for the indicated times at 37°C, the reactions were stopped by addition of formamide loading buffer (98% formamide, 0.5 M EDTA, pH 8.0, 0.025% bromphenol blue, 0.025% Xylene Cyanol). Samples were analyzed on 8 M urea-20% polyacrylamide gel electrophoresis followed by autoradiography or analysis by phosphorimager. Exonuclease activity was detected as a decrease in size of the 5'-labeled D7bio primer.
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Determination of the distance between the polymerase active site and the entrance of the template binding groove

Partial duplex D20/Tbio5' in a primer-template structure with a 9-nt 3' overhang and a biotin at its 5'-terminus. The total reaction mixture (25 µl) contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 1 mg/ml BSA, 10 mM MgCl2, 1 mM dNTP's, 0.05 ng of 5'-labeled D7bio. After incubation in the absence or presence of 7 nM of streptavidin for 5 min., the reaction was started by adding Ad pol or the pTP-pol complex to a final concentration of 28.5 nM. After incubation at 37 °C for the indicated times, reactions were stopped by addition of formamide loading buffer. Samples were analyzed on 8 M urea-20% polyacrylamide gel electrophoresis followed by autoradiography or analysis by phosphorimager. Polymerization activity was detected as an increase in size of the 5'-labeled D20 primer.

Characterization of the exonuclease-deficient mutant polymerase D422A

The 3'-5' exonuclease assay and the DNA polymerase/exonuclease coupled assay used to characterize the exonuclease-deficient mutant polymerase D422A were performed as described (4) with the following changes: For the exonuclease assay, mutant or wild-type polymerase was used to reach a final concentration of 28.5 nM and degradation was studied in time as indicated in the figure legend; For the DNA polymerase/exonuclease coupled assay, mutant or wild-type polymerase to a final concentration of 28.5 nM were used in the presence of increasing amounts of dNTPs as indicated. Incubation was at 37 °C for 10 min.

RESULTS

The distance between the exonuclease active site and the entrance of the primer binding groove is 5 nucleotides

To measure the distance between the exonuclease active site and the entrance of the primer binding groove, we used a technique developed for T4 DNA polymerase based on a bulky biotin-streptavidin block located at a specific position within an oligonucleotide (8,12). The 20-mer D7bio contains a biotin at the seventh position from the 5'-end to which streptavidin strongly binds (Kd ≅ 10⁻¹⁵M) (35). D7bio can be degraded by the exonuclease activity of Ad pol, resulting in different product lengths. When streptavidin contacts the enzyme, it will block further entry of the oligonucleotide at the primer binding groove, due to steric hindrance (8,12). This approach allows us to determine the distance between the exonuclease active site and the entrance of the primer binding groove as schematically depicted in Fig. 1A.

When D7bio was degraded by Ad pol for the indicated times (Fig 1B), accumulation of a product of 9 nucleotides was observed starting after 5 min. incubation in the absence of streptavidin (lane 3-6). D7bio could thus be degraded up to 2 bases 3′ of the biotin group. Under these conditions, degradation of oligonucleotides without biotin group resulted in products of 3 nucleotides (data not shown and (18)). This indicates that the biotin group probably sterically impairs translocation before it reaches the exonuclease active site, preventing further degradation. When D7bio was pre-incubated with streptavidin and subsequently degraded, a drastic change in the degradation pattern was observed (Fig. 1B), showing accumulation of a 12 nucleotides long product (lanes 8-12). Streptavidin therefore has blocked entry and further degradation of D7bio once it has reached this length. This indicates that the distance between the enzyme surface at the primer binding groove and the exonuclease active site (indicated as the double-headed arrow in Fig. 1B) is 5 nucleotides. To ascertain entry of D7bio at the primer binding groove, degradation was also performed on the dsDNA primer-template D7bio/T30. The same accumulation product of 12 nucleotides was observed (data not shown).

pTP binds at the primer binding groove of Ad pol

Our previous study showed that the pTP-pol complex has a decreased rate of replication and exonuclease activities compared to free Ad pol (18). Furthermore, a difference in product lengths was found for the exonuclease activity of the pTP-pol complex (18). By using the experimental setup described for the previous
experiment, we probed the pTP-pol interaction. When the 20-mer D7bio was incubated with streptavidin and the pTP-pol complex, no exonucleolytic degradation was observed (data not shown). This suggests that either the pTP-pol complex cannot bind to the oligonucleotide in the presence of streptavidin, or the 3’-end of D7bio cannot reach the exonuclease active site because it is too short. To distinguish between these possibilities, a larger oligonucleotide (D7bio10) with 10 additional nucleotides at its 3’-end was designed keeping the internal biotin molecule at position 7. In the absence of streptavidin, degradation of this oligonucleotide to 8 nucleotides was observed (Fig. 1C, lane 5). Comparison of the degradation patterns in figures 1B and 1C shows that the exonucleolytic activity of the pTP-pol complex is slower than that of free Ad pol in agreement with our previous results (18). Furthermore, degradation of D7bio10 could now proceed up to one nucleotide from the biotin group rather than to 2 nucleotides (Fig. 1C), suggesting a more “open” exonuclease active site when pTP is complexed to Ad pol. When the experiment was performed in the presence of streptavidin, degradation led to the accumulation of products between 17 and 21 bp (Fig. 1C, lanes 9-10). This result shows that the pTP-pol complex is indeed able to bind to oligonucleotide D7bio10 in the presence of streptavidin and that pTP is located at the primer binding groove of Ad pol. The absence of an accumulated product of 12 bp further indicated that pTP was complexed to Ad pol throughout the experiment. The distance from the biotin to the exonuclease active site is therefore estimated at 10 to 14 nucleotides. Since the distance between the entrance of the primer binding groove and the exonuclease active site was 5 nucleotides (Fig. 1B), pTP may occupy a region between 5 and 9 nucleotides at the primer binding groove of Ad pol. The presence of several products of almost equal intensity might be explained by a flexible struc-
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Figure 2. Distance between polymerase active site and entrance of the template binding groove. A. Schematic representation of the experiment, legend as in Fig. 1A. B. Elongation of Ad pol was studied on primer/template D20/Tbio5’ in the absence (-, lanes 2-6) or presence (+, lanes 7-11) of streptavidin for the indicated times. Lane 1 is a control elongation reaction performed on primer/template D20/T30. Arrows indicate accumulated products. The double-headed arrow depicts the measured distance between the entrance of the template binding groove and the polymerase active site. C. Elongation of D20/Tbio5’ was studied on Ad pol and the pTP-pol complex in the absence or presence of streptavidin for 15 min. at 37°C.

ture of pTP. When D7bio10 is degraded, streptavidin might approach pTP under various angles at the primer binding groove leading to a range of product lengths dependent of the geometry of the pTP surface. In addition, the flexibility of the biotin group could play a role.

The distance between the polymerase active site and entrance of the template binding groove is 5 nucleotides

Next, we wanted to determine the distance between the entrance of the template binding groove and the polymerase active site. For this, primer D20 was hybridized to the 30-mer Tbio5’, creating a primer-template with 9 nucleotides overhang at the 5’-end and a terminal biotin group. In the presence of streptavidin, the D20/Tbio5’ will be elongated by Ad pol until streptavidin blocks further entrance of the template strand at the template binding groove as indicated in the experimental scheme (Fig. 2A). When D20/Tbio5’ was incubated with all four dNTPs and Ad pol for the indicated times, D20 was elongated to a main product of 30 nucleotides (Fig. 2B, lanes 3-6). Since the template is 29 nucleotides long, this result shows that one base is added opposite the 5’-biotin molecule of Tbio5’. To test if this is due to the presence of the biotin group or due to a non-templated nucleotide addition, the same experiment was performed with primer/template D20/T30, which has a base instead of a biotin at its 5’-end. As shown in figure 2B, both a 30-mer and a 31-mer were found (lane 1). This indicates that Ad pol under these conditions could add a non-templated nucleotide to a blunt-ended DNA substrate, as has been described for several DNA polymerases (7).

When the primer/template D20/Tbio5’ was pre-incubated with streptavidin and subsequently elongated by Ad pol and dNTPs, a product of 25 nucleotides accumulated (lanes 10-11). Also some longer read-through products were formed possibly caused by the flexibility of the translocation block. Since the main product is 25 nucleotides long, our results suggest that the distance between the polymerase active site and the entrance of the template binding groove (indicated as a double-headed arrow in Fig. 2B) is 5 nucleotides.

pTP does not block the entrance of the template binding groove of Ad pol

The same experimental setup as described above (Fig. 2A) was used to determine if pTP could contact Ad pol at the entrance of the template binding groove in addition to the
entrance of the primer binding groove. As can be seen in figure 2C, both Ad pol and the pTP-pol complex are able to fully elongate primer/template D20/Tbio5', albeit with lower activity for the pTP-pol complex (compare lanes 2 and 6) in agreement with our previous results (18). In the presence of streptavidin, elongation stalled for both pTP-pol (lane 8) and free Ad pol (lane 4) at 25 nucleotides with some read-through product formed. Longer incubation for pTP-pol resulted in further accumulation of the 25 nucleotides product (data not shown). Therefore, these results suggest that, in contrast to the primer binding groove, pTP does not block at the entrance of the template binding groove and therefore any contacts in that region, if they exist, do not disturb passage of the template strand.

**Mutant polymerase D422A is exonuclease deficient**

To complete the measurements of the various DNA binding grooves within Ad pol, we wanted to determine the distance between the polymerase active site and the entrance of the primer binding groove. However, since Ad pol possesses a distributive 3'-5' exonuclease activity (Fig. 1B and (21)), discrimination between elongation and degradation of wild-type Ad pol is difficult. Therefore, the exonuclease-deficient mutant polymerase D422A was constructed by changing the catalytic aspartate residue present in the highly conserved Exo II motif (1) into an alanine residue (D422A). Mutant polymerase D422A was characterized by an 3'-5' exonuclease assay and a DNA polymerase/exonuclease coupled assay as shown in Fig. 3. In contrast to wild-type Ad pol (Fig. 3A, lanes 2-5), no exonucleolytic breakdown for mutant polymerase D422A on 5'-labeled oligonucleotide D20 was observed (Fig. 3A, lanes 6-9), confirming the exonuclease-deficient phenotype. The polymerase-exonuclease coupled assay showed that at low nucleotide concentrations (e.g. 1 mM), the polymerase activity of D422A was wild-type like (data not shown). Both enzymes could also elongate primer D20 up to 31 nucleotides, indicating that a non-templated nucleotide was added, as was shown previously in Fig. 2B.

**The distance between the polymerase active site and the entrance of the primer binding groove is 9-10 nucleotides**

With the exonuclease-deficient mutant polymerase D422A, the distance between the polymerase active site and the entrance of the primer binding groove could be measured. The 20-mer D7bio was partially degraded at the 3'-end as described in Experimental procedures and hybridized to T30, generating a primer/template mix with various primer lengths. When these primer/templates are elongated in the presence of streptavidin, only primers that are long enough to reach the polymerase active site (measured from the 3' end) are elongated (Fig. 3B). The polymerase activity of mutant polymerase D422A was slightly affected (Fig. 3B) but as expected, no degradation was observed, explaining at least in part the lower elongation activity. At higher nucleotide concentrations (e.g. 1 mM), the polymerase activity of D422A was wild-type like (data not shown). Both enzymes could also elongate primer D20 up to 31 nucleotides, indicating that a non-templated nucleotide was added, as was shown previously in Fig. 2B.
Figure 4. Distance between the polymerase active site and the entrance of the primer binding groove. A. Schematic representation of the experiment, legend as in Fig. 1A. B. Partially degraded D20 was hybridized to template T30. The resulting primer/template mix (lane 1) was elongated at 30°C for the indicated times in the absence (-, lanes 2-4) or presence (+, lanes 5-6) of streptavidin by the exonuclease-deficient mutant polymerase D422A. Arrows indicate the length of the elongation products.

streptavidin-biotin block) can support elongation. This assay therefore allows us to determine the distance (in nucleotides) between the polymerase active site and the entrance of the primer binding groove (Fig. 4A). Figure 4B shows the result of the elongation of the partially degraded D7bio/T30 mix (lane 1). In the absence of streptavidin, all primers longer than 14 nucleotides could be elongated by mutant polymerase D422A after 60 min. (Fig 4B, lane 4), up to 31 nucleotides. Quantitation of the products of 13 and 14 nucleotides showed a decreased intensity after 60 min. (lane 4) indicating that small amounts of these products were also elongated. Longer incubation did not change this pattern (data not shown). When the mixture was pre-incubated with streptavidin and elongated, a change in the elongation pattern was observed (lanes 5-6). Only primers longer than 17 nucleotides could be efficiently elongated even after 60 min. Since the 17-mer product also showed some decreased intensity, we estimate a distance of 17 nucleotides from the entrance of the primer binding groove to the polymerase active site. Combining the data for exonucleolytic degradation and elongation, we propose that there is a difference of 4-5 nucleotides in the length of the primer in contacting the polymerase active site (9-10 nucleotides) or the exonuclease active site (5 nucleotides). Therefore these results support the notion that the exonuclease active site and the polymerase active site in Ad pol are spatially distinct. Furthermore, since we already showed a distance of 5 nucleotides between the polymerase active site and the entrance of the template binding groove (Fig. 2B), Ad pol can bind a DNA region of approximately 14-15 nucleotides in length.

DISCUSSION

Spatial relationship between polymerase and exonuclease active sites

We observe that the distance between the enzyme surface and the exonuclease active site is approximately 5 nucleotides. This is very similar to the distances observed for T4 DNA pol (4-5 nucleotides, (12)) and for φ29 DNA pol (5 nucleotides, (10)). The distance between the entrance of the primer binding groove and the polymerase active site (9-10 nucleotides) was shown to be 2-4 nucleotides longer than what was measured for T4 DNA pol (7 nucleotides) and φ29 DNA pol (6 nucleotides). This difference may, in the case of φ29 DNA pol, simply reflect its smaller size (66 kDa versus 140 kDa for Ad pol). Ad pol, T4 DNA pol and φ29 DNA pol all belong to the pol-α like DNA polymerases. Recently, the structure of the replicating and editing complexes of RB69 DNA polymerase have been resolved (11,29) that can be used as a model for DNA polymerases belonging to this family. The structure of RB69 DNA polymerase shows that it contains a polymerase domain that, like other DNA polymerases, resembles the shape of a right hand consisting of a palm, fingers and a thumb. In addition, an exonuclease domain is present, with its catalytic site located away from the polymerase active site (29). When the primer-template is bound, it is stabilized by numerous interactions between residues in the thumb domain and the minor groove of the DNA (11). Based on the structure of this replicating complex, it can be estimated that the distance between the polymerase active site and the entrance of the primer binding groove is approximately 10 nucleotides (11). The switch from the polymerase to the exonuclease active site is accompanied by a conformational change
as observed for RB69 DNA polymerase (11). The thumb domain confers a “closed” conformation when the polymerase is in the polymerizing mode but is in a more “open” conformation when the polymerase is in the editing mode, having fewer contacts with the DNA (29). The distance between the exonuclease active site and the entrance of the primer binding groove, measured in the editing mode, is estimated at approximately 6 nucleotides (29). These distances are close to what was measured in this study for Ad pol (9-10 nucleotides and 5 nucleotides respectively). The similar spatial relationship for the exonuclease and polymerase active sites for RB69 DNA polymerase and for Ad pol and the fact that they all belong to the same family of α-like DNA polymerases, supports the proposal that they all have a similar structural organization. This conclusion is further supported by mutational analysis of a set of conserved residues in the C-terminal part of Ad pol that suggest an arrangement of conserved motifs in Ad pol similar to RB69 DNA polymerase (22). Moreover, we confirmed that the exonuclease activity resides in the N-terminal part of Ad pol, since mutant polymerase D422A lost its exonuclease activity while the polymerase activity remained almost wild-type like (Fig. 3), similar to what was found for other characterized α-like DNA polymerases.

pTP interacts at the primer binding groove of Ad pol

Here we present data suggesting that pTP binds at the entrance of the primer binding groove of Ad pol (Fig. 1C). This finding is in agreement with the proposed role of pTP to present its priming serine residue at the polymerase active site. Mutations in Ad pol, including amino acid Y1080, E1057 and Y673, resulted in a strong reduction of pTP interaction, initiation activity and DNA binding (22). Accordingly, extensive mutational analysis of the protein-priming φ29 DNA polymerase (reviewed in (2)) has indicated that several amino acids proposed to interact with the DNA primer-template cause defects in TP-interaction (3,32), suggesting that both primers are bound by the enzyme in a similar fashion (11). Furthermore, a partial proteolysis study on φ29 DNA polymerase revealed that the protection and digestion pattern of TP was similar to that obtained with DNA, suggesting that both primers, DNA and TP, fit in the same double-stranded DNA-binding channel and protect the same regions of φ29 DNA polymerase (33). All these data are in agreement with the location of pTP at the entrance of the primer binding groove.

When the pTP-pol complex was probed with primer template and a terminal biotin, it was demonstrated that pTP did not block the entrance of the template binding groove (Fig. 2C). This result indicates that pTP does not bind this side of the polymerase, although it cannot be excluded that pTP dissociates first before the primer-template was elongated. Two observations however argue against this option. First, the rate of polymerization is much lower for the pTP-pol complex (Figs. 1,2), suggesting that pTP remains bound to Ad pol when it is in the polymerase mode and secondly, it was shown that dissociation of pTP is not a prerequisite for DNA-primed polymerization (20).

In the presence of pTP, Ad pol is able to perform both exonuclease activity and polymerase activity (this study, (18,19)). For this, Ad pol needs to accommodate both DNA and pTP at the primer binding groove. Both exonuclease and polymerase activities are decreased in the presence of pTP (this study, (18,19)). This is not caused by an altered DNA binding affinity (31). Rather, we assume that catalysis or the translocation of DNA after each catalytic event is hampered. This could be caused by competition for DNA and pTP binding at the primer binding groove of Ad pol. At least three nucleotides need to be incorporated before pTP starts to dissociate (19), suggesting that some flexibility in the priming part of pTP exists. The crystal structure of the pTP-pol complex or of any other protein-priming polymerase is required to determine the exact space constraints of both proteins.

Origin binding of the pTP-pol complex

Based on the results discussed above, a model for origin binding of the pTP-pol complex preceding replication initiation can be proposed (Fig. 5). pTP is located in the model as binding to the entrance of the primer binding groove, with its priming part located at the polymerase active site close to the fourth nucleotide of the template strand. Since pTP is a DNA binding protein (31), it could be located near or even in
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**Figure 5. Model for origin binding of the pTP-pol complex.** Model for origin binding of the pTP-pol complex preceding replication initiation. The Ad origin DNA (thick lines) containing the core origin (nucleotides 9-18) are bound by Ad pol. pTP is complexed to Ad pol with its priming part depicted at the primer binding cleft (arrow) close to the polymerase active site (P) and the templating nucleotide for initiation (nucleotide 4 of the template strand). The exonuclease active site (E) has been indicated for clarity. The NH2-domain represents the putative N-terminal domain of Ad pol that could bind to the core origin. See text for more details.

The distance from the polymerase active site to the entrance of the template binding groove is 5 nucleotides (Fig. 2) and 9-10 nucleotides to the entrance of the primer binding groove (Fig 4), indicating that Ad pol covers 14-15 nucleotides of DNA when it is in the polymerase mode. Although these data are approximate due to the flexibility of the biotin group, they correspond well to what has been observed in RB69 DNA pol. Here, a minimum of 12 nucleotides are covered, although the exact number is difficult to estimate since the template strand of the replicating complex was unstructured at its 5' end (11). The template strand enters RB69 DNA polymerase in a groove formed between the NH2 and exonuclease domains (11). A DNase I footprint of Ad pol on the Ad 2 origin showed protection of the first 20 nucleotides (31). The pTP-pol complex showed increased specificity on origin-containing DNA and protected bases 9-18 (31) but the contribution of each protein is unclear (23). Since the distance between the polymerase active site and entrance of the template binding groove is 5 nucleotides, our results indicate that at pre-initiation complex formation, nucleotides 1-9 are covered by Ad pol. These data then do not explain the DNase I footprinting results that show that nucleotides 9-18 are covered. In order to explain this discrepancy, we propose that the N-terminus of Ad pol folds into a domain that binds to the core origin sequence (Fig. 5). Although this N-terminal domain is not conserved between DNA polymerases, it may play a role in origin recognition in the case of Ad pol. Indeed, there are about 250 amino acids located N-terminally that contain a putative Zn finger motif. Point and linker-insertion mutants in this motif retained DNA elongation, but had severely reduced initiation activity and lost the ability to bind the Ad core origin DNA, essential for Ad DNA replication (16). Furthermore, one of the four fragments of Ad pol obtained after partial digestion with endolys C contained these 250 aa, suggesting that it could fold as an independent domain (24). Alternatively, pTP could contribute to binding of the core origin. However, this is unlikely as no blocking of the entrance of the template binding cleft by pTP was observed which would be expected when pTP is binding the core origin. Interestingly, the N-terminal domain of Ad pol was the only domain that could not bind to pTP as was demonstrated in the partial proteolysis study (24), further indicating that it is unlikely that pTP binds at this side of the polymerase.

In summary, our results and those reported previously support the proposal that Ad pol has a similar molecular architecture as RB69 DNA polymerase. Furthermore the location of pTP was directly probed, binding at the primer binding groove of Ad pol, providing an explanation for the observed decrease in polymerase and exonuclease activity in the presence of pTP and allowing insight in the use of a protein to prime replication. These results have led to a model for pTP-pol binding on the origin of replication. Since no structural information exists on any protein-priming polymerase nor any priming protein, these results are an important contribution to the understanding of Ad DNA replication and protein-primed replication in general.
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