Molecular architecture and function of adenovirus DNA polymerase

Moleculaire architectuur en functie van het adenovirus DNA polymerase

(met een samenvatting in het Nederlands)

Proefschrift

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Science should be made as simple as possible, but not simpler.
Albert Einstein (1879-1955)

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General Introduction

DNA polymerases

It is of fundamental importance for any living organism to assure the duplication of its genetic material. The central enzymes to perform this complicated task are the replicating DNA polymerases. The first polymerase described was the *E. coli* DNA polymerase I, identified in the Kornberg laboratory in 1956. It took another 15 years to isolate two other DNA polymerases in *E. coli*, DNA polymerase II and III and to realize that DNA polymerase III, and not DNA polymerase I, is the enzyme that replicates most of the DNA in *E. coli*. Later, polymerases were also identified in eukaryotes, archea, viruses and plasmids. All prokaryotic and eukaryotic DNA polymerases share the same synthetic activity: they add nucleotides to the 3'-OH of a growing DNA chain. In addition, a number of polymerases catalyse a second enzymatic activity, the 3'-5'exonuclease activity, which functions in proofreading. In recent years, the rapid unravelling of genome sequences of multiple organisms has led to the discovery of an impressive amount of novel DNA polymerases. These polymerases are not only involved in replicating the DNA but many are also involved in DNA repair and recombination. This chapter discusses the classification, structural aspects and properties of DNA polymerases. Characteristic of all polymerases whose structure is known is that they appear to share a common overall architecture and a general mechanism for the addition and removal of nucleotides. Therefore, the main discoveries leading to the understanding of the mechanisms of polymerization, exonuclease degradation, processivity and fidelity are described, based on the structure and biochemistry of selected enzymes. The structure of RB69 DNA polymerase, a member of the B family DNA polymerases, to which adenovirus DNA polymerase belongs will be discussed in more detail. Finally, the properties of adenovirus DNA polymerase and its role in adenovirus DNA replication are described.

Family A DNA polymerases

Based on amino acid sequence homology and structure analysis, the DNA polymerases can be classified into six families, A, B, C, D, X and Y (Table I) (6,10,44,79).

The family A DNA polymerases are named for their homology to *E. coli* DNA polymerase I, and are therefore also known as the pol I family. This is probably the best studied of all polymerase families and includes the first DNA polymerase of which the structure was determined, the Klenow Fragment (KF) of *E. coli* DNA polymerase I (80). Subsequent crystallization of the KF in complex with single-stranded (30) and double-stranded DNA (1) provided solid experimental evidence for a two-metal-ion mechanism that catalyses the 3'-5'exonuclease activity (53) as discussed later. In addition, the orientation of the DNA bound to the polymerase was proposed (1). The crystal structure of Taq DNA polymerase complexed with blunt-ended DNA confirmed this orientation and identified the location of the polymerase active site (27). With the crystallization of two other members of this family, T7 DNA polymerase (25) and the polymerase of *Bacillus stearothermophilus* (56) in the presence of primer-template DNA and nucleotides, the first structures of a replicative DNA polymerase locked in a polymerization mode were visualized. These structures provided a number of mechanistic insights into the chemistry and fidelity of DNA synthesis and supported a two-metal-ion mechanism of nucleotide addition (95), analogous to the mechanism used by the 3'-5'exonuclease of KF. Although the structures of the various members of the pol I family are homologous, their sequence identity is surprisingly low. Delarue et al. identified three regions of sequence homology (termed motif A, B and C) in DNA-dependent DNA polymerases of which two (motif A and C) are conserved between all known DNA and even RNA polymerases (19). These motifs contain residues critical for catalysis whose mutation severely reduces polymerase activity (83,84).

Family B DNA polymerases

The family B DNA polymerases are named for their homology with the *E. coli* DNA polymerase II (44) and are also called α-like DNA polymerases for their similarity with eukaryotic DNA polymerase α (113). The family can be divided into three subclasses: the cellular, bacterial/viral and protein-priming DNA polymerases (Table I). The cellular DNA

Table I. Classification of DNA polymerases

DNA polymerase family	Proposed main function
A (pol I)	
E. coli pol I	Bacterial repair/gap filling
Taq DNA polymerase	Bacterial replication
T7 DNA polymerase	Bacteriophage replication
Human pol γ	Mitochondrial DNA replication
Human pol θ	Repair of DNA crosslinks
B (α-like)	
Cellular	
Human pol α	Priming leading/lagging strand
Human pol δ	Replication human genome
Human pol ε	Replication human genome
Human pol ξ	Bypass synthesis
Bacterial/viral	
E. coli pol II	Repair
T4 DNA polymerase	Phage replication
RB69 DNA polymerase	Phage replication
Vaccinia DNA polymerase	Virus replication
Herpes simplex DNA polymerase	Virus replication
Protein-priming	
Adenovirus DNA polymerase	Virus replication
φ29 DNA polymerase	Phage replication
pGKL1	Plasmid replication
C	
E. coli pol III	Replication of *E. coli* genome
B. subtilis pol III	Replication of *B. subtilis* genome
D	
DNA pol D	Replication of euryarchaeotes
X	
Human DNA polymerase β	Base excision repair
Human pol λ	Meiosis-associated DNA repair
Human pol μ	Somatic hypermutation
Human pol σ	Sister-chromatid cohesion
Human terminal deoxynucleotidyl transferase (TdT)	Antigen receptor diversity
Y (*UmuC/DinB/Rev1p/Rad30* superfamily)	
E. coli pol IV (*DinB*)	Bypass synthesis/untargeted mutagenesis
E. coli pol V (*UmuDC*)	SOS lesion-targeted and untargeted mutagenesis
Human pol κ	Bypass synthesis
Human pol η (*XPV*)	Error-free translesion synthesis
Yeast pol η (*Rad30*)	Error-free translesion synthesis
Human pol ι	Error-prone translesion synthesis
Human Rev1	Abasic site synthesis

The nomenclature of the DNA polymerases is based on the guidelines of the Human Genome Organization Gene Nomenclature Committee and references therein (8,9,110). Note that only a selected number of polymerases for each family are shown.

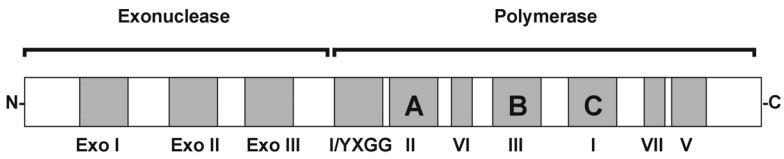

Figure 1. Sequence conservation in family B DNA polymerases: relative location of the conserved motifs. The relative location of all conserved motifs of the exonuclease and polymerase domain of family B DNA polymerases are indicated. Polymerase motif IV has been left out for clarity as it largely aligns with motif Exo II (2).

polymerases include human DNA polymerase α, the replicating polymerases δ and ε and the recently identified polymerase ξ. The bacterial/viral DNA polymerases contain several bacterial, bacteriophage and viral DNA polymerases. The distinct subclass of protein-priming DNA polymerases includes all adenovirus DNA polymerases, φ29 DNA polymerase and several plasmid encoded DNA polymerases. The unique feature of these polymerases is that they all use a protein instead of RNA as a primer (reviewed in (91)). Extensive alignment of the sequences of the polymerase domain of these polymerases revealed six regions of homology that were designated I to VI according to their extent of similarity, with region I being the most conserved and region VI the least similar (108,113). Recently two additional regions of homology were described; region VII, identified in herpes simplex virus and φ29 DNA polymerase (4,42) and the (I/Y)XGG motif, identified in φ29 DNA polymerase (102,103) and adenovirus DNA polymerase (7) (Chapter 3). Figure 1 shows the location of these regions within the primary sequence of a family B DNA polymerase. Regions I and II are homologous to motifs C and A, respectively, as described for all DNA and RNA polymerases. Furthermore, region III is homologous to motif B of the pol I family. Extensive biochemical studies, including drug resistance and mutational analysis in combination with available structural data have identified these regions as important for catalysis, nucleotide selection and substrate binding. In addition, three highly conserved motifs, called Exo I-III (Figure 1), have been identified in the amino-terminal domain of all polymerases that contain an exonuclease activity (2). Extensive mutational analysis has demonstrated that they are located at the exonuclease active site and play an important role in catalysis and substrate binding.

The first structure solved from this family of DNA polymerases was that of the RB69 DNA polymerase (107), soon followed by the structure of this enzyme locked in the editing and the polymerization mode (29,94). The structures of three other B family DNA polymerases, the hyperthermophilic archaeon *Thermococcus* sp. 9° N-7 (86), from *Thermococcus gorgonarius* (39) and from the archaebacterial *Desulfurococcus* strain *Tok* (116) have also been solved. Comparison with RB69 DNA polymerase shows a high degree of structural equivalence, suggesting that RB69 DNA polymerase is a good model for other polymerases belonging to this family despite the low sequence similarities. The structure of the replicating complex of RB69 DNA polymerase will be extensively discussed elsewhere in this chapter.

Family C DNA polymerases

The family C DNA polymerases are named for their homology to the product of the *polC* gene, the α subunit *E. coli* DNA polymerase III (Table I). *E. coli* DNA polymerase III is part of a multisubunit enzyme, the holoenzyme, that is the main replicator of the *E. coli* genome. This holoenzyme consists of ten different subunits including two α subunits that each replicate a different DNA strand, thereby coupling leading and lagging strand synthesis (reviewed in (55)). *E. coli* DNA polymerase III exhibits an extensive homology to *Bacillus subtilis* DNA polymerase III. Recently, it was shown that *Bacillus subtilis*, instead of one replicating polymerase, harbors a second one which is homologous to *E. coli* DNA polymerase III. (20). The structure of polymerases from this family has not yet been determined.

Family D DNA polymerases

A search for novel DNA polymerases in archea resulted in the characterization of a heterodimeric polymerase, pol II or pol D, from the hyperthermophilic archaeon *Pyrococcus furiosus* (43,104,105). Pol D is highly conserved among archea and since it does not belong to any of the known polymerase families it was proposed to be a member of a new family of DNA polymerase, the euryarchaeotic family D (10) (Table I).

Family X DNA polymerases

The members of the X family DNA polymerases include the eukaryotic DNA polymerase β, the terminal deoxynucleotidyl transferases (TdTs) and the recently identified polymerases μ (22), σ (109) and λ (33,76). DNA polymerase β is probably the best studied member of the family and is involved in DNA repair. The protein shows no sequence similarities to other polymerase families although it shares the conserved motifs A and C. The crystal structure of this polymerase is known (16,93). The structure of DNA polymerase β complexed with primer-template DNA and ddCTP (82) visualized for the first time the catalytic two-metal-ion mechanism for nucleotidyl transfer.

Family Y DNA polymerases

Another completely new family of prokaryotic and eukaryotic DNA polymerases has been identified over the last few years, called the *UmuC/DinB/Rev1/Rad30* superfamily of DNA polymerases (32,36). This family was named after early discovered prokaryotic members (34,71) and has been designated as the DNA polymerase Y family (79) in line with the orginal compilation (6,44). These polymerases are suggested to be involved in replacement of a stalled replication fork in order to bypass a lesion (translesion synthesis) (32,35,106,114) although the function of most members is less clear at present. A feature common to many of these polymerases is their tendency to copy undamaged DNA with remarkably poor fidelity. The crystal structures of two DinB homologs from *Sulfolobus solfataricus* have been solved recently and provide insight into the sources of low fidelity of these enzymes (69,117). Characteristic for all members of the family is the presence of five conserved sequence motifs (65). Despite the absence of sequence similarity to any other polymerase family, the conserved catalytic residues of motif A and C among the other polymerase families are structurally equivalent to those within motifs I and III and mutational analysis has demonstrated that these residues are absolutely required for polymerase activity (47).

Structure of RB69 DNA polymerase

The best studied polymerase belonging to the B family of DNA polymerases is RB69 DNA polymerase of which the structure of the editing and replicating complexes are known (29,94,107). The structure of the polymerase domain of RB69 DNA polymerase complexed with primer-template DNA is shown in figure 2A. It consists of three subdomains that resemble the shape of a right hand, with a palm, fingers and a thumb. The palm subdomain contains the two universally conserved aspartate residues from motifs A and C which, together with the dNTP, bind the two metal ions that catalyse the polymerase reaction. The fingers consist of two long antiparallel coiled-coil α-helices and the function of this subdomain includes important interactions with the template strand, the incoming nucleotide and the templating base. The thumb subdomain makes important interactions across the minor groove of the duplex DNA. The function of the thumb is to position the duplex DNA when it is either in the polymerizing or editing mode and plays a role in translocation and processivity.

Figure 2B shows the complete structure of this protein. In addition to the polymerase part, containing the palm, fingers and thumb (similarly oriented as in figure 2A), two subdomains are located at the amino-terminal part of the protein. These are the NH_2-domain and the exonuclease domain. The NH_2-terminal domain is non-conserved between polymerases and is dispensable for catalytic activity and subunit assembly of mouse pol α (74). Various functions have been attributed to this domain, including SV40 T-antigen binding in the case of human pol α (24) and origin DNA binding by the putative Zn finger located within this domain in adenovirus DNA polymerase (49, Chapter 4). The exonuclease domain harbours the catalytic residues required for exonuclease activity and binds single-stranded DNA with high affinity (Figure 2C).

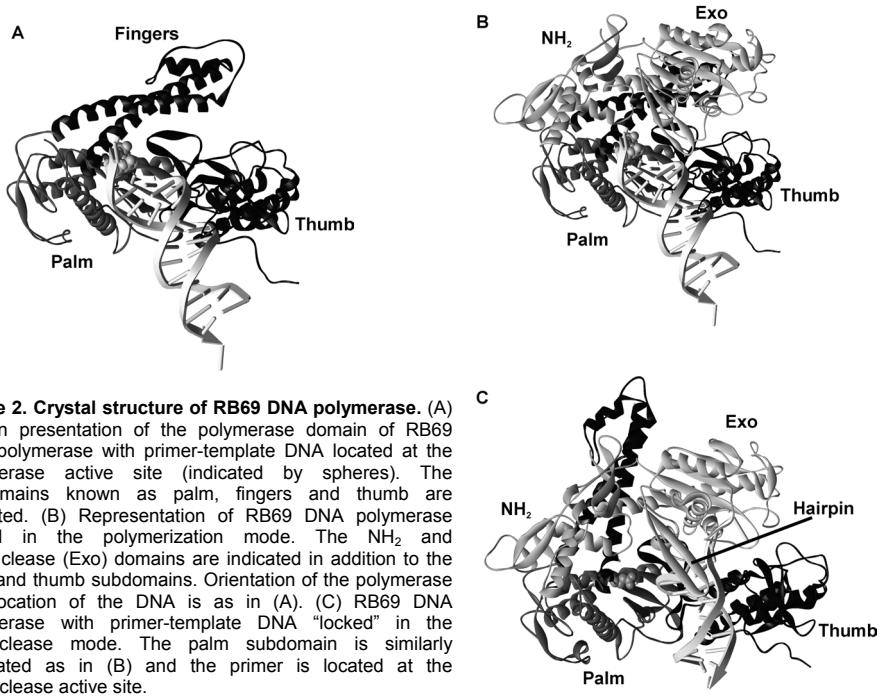

Figure 2. Crystal structure of RB69 DNA polymerase. (A) Ribbon presentation of the polymerase domain of RB69 DNA polymerase with primer-template DNA located at the polymerase active site (indicated by spheres). The subdomains known as palm, fingers and thumb are indicated. (B) Representation of RB69 DNA polymerase locked in the polymerization mode. The NH_2 and Exonuclease (Exo) domains are indicated in addition to the palm and thumb subdomains. Orientation of the polymerase and location of the DNA is as in (A). (C) RB69 DNA polymerase with primer-template DNA "locked" in the exonuclease mode. The palm subdomain is similarly orientated as in (B) and the primer is located at the exonuclease active site.

A remarkable feature of polymerases belonging to the B family is that their exonuclease domain is located opposite to the position in members of the A family.

Mechanism of polymerization

As mentioned before, the crystal structure of rat DNA polymerase β (82) visualized for the first time the molecular basis of the catalytic mechanism of polymerization. Central to this mechanism are two Mg^{2+} ions located at the polymerase active site. Therefore, this mechanism is referred to as the "two-metal-ion mechanism" of nucleotide addition (95,96), which was proposed by analogy to the nearly identical mechanism of the 3'-5'exonuclease mechanism of KF (1). These two metal ions (called A and B) are bound by two catalytic aspartates (Figure 3) that are invariantly conserved in polymerase motifs A and C (Figure 1). Mutational analysis of these residues in numerous polymerases has demonstrated that they are critical for catalysis. Metal ion A interacts with the 3'-hydroxyl of the primer strand and thereby lowers its affinity, facilitating the 3'-O^- attack on the α-phosphate of the incoming nucleotide triphosphate. Metal ion B assists the leaving of the pyrophosphate and both ions are proposed to stabilize the structure and charge of the pentacovalent transition state that occurs during the course of this reaction (Figure 3). This two-metal-ion catalyzed mechanism appears to be universally conserved among all DNA and RNA polymerase families as well as many other enzymes such as ribozymes (97).

Nucleotide binding

In addition to the palm domain with the highly conserved motifs A and C that position the metal ions, the fingers domain plays an equal important role in positioning of the incoming dNTP at the polymerase active site. Structural analysis of DNA polymerases demonstrated an important conformational change in the fingers domain upon binding a dNTP (25,56). When the

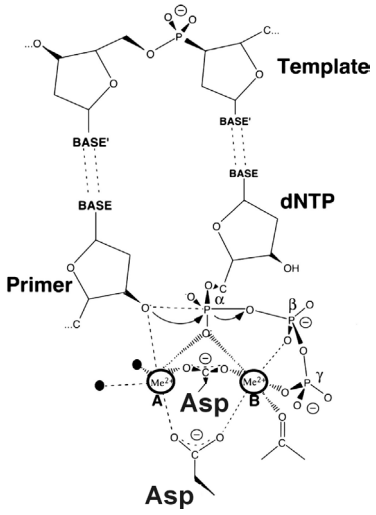

Figure 3. The two-ion-metal mechanism of polymerization. Two divalent metal ions, called A and B are positioned by aspartic-acid residues of the polymerase active site. The figure was adapted from (53). See text for details.

correct dNTP is bound by the fingers, they rotate by 60° towards the palm domain and close off the polymerase active site. Two of the eight highly conserved domains that can be found in family B DNA polymerases are located in the fingers domain (Figure 1). Rotation of the fingers brings the conserved residues of region III (motif B) and region VI in close contact with the dNTP at the polymerase active site leading to optimal positioning of substrates and residues required for catalysis. Mutational analysis of these motifs has confirmed that these regions are involved in dNTP binding (3,115).

Primer-template DNA binding

The primer-template DNA duplex lies in a groove between the palm and the thumb domains (Figure 2A, B). The single-stranded template strand is believed to enter the polymerase between the NH_2 and the exonuclease domain (29). The fingers sharply kink the template strand when it reaches the polymerase active site that allows optimal access of the templating base for the incoming dNTP. The template strand leaves the polymerase active site as duplex DNA and is stabilized by a number of interactions, including the residues of the recently identified (I/Y)XGG motif near the polymerase active site (7,103). The duplex portion of the primer strand is extensively contacted by the thumb domain that wraps around the DNA on the minor groove side, mainly by phosphate-backbone contacts. Both the primer and template strands are furthermore contacted next to the polymerase active site by the highly conserved motif VII, found in family B DNA polymerases (Figure 1). This motif stabilizes the DNA in the B-form by drawing the primer and template strand backbones together (29).

Transition of the primer strand to the exonuclease active site

When an error is sensed, the polymerase melts out three basepairs of primer-template DNA and the primer is located at the exonuclease active site in order to remove the mismatch. Comparison of the structure of RB69 DNA polymerase in the polymerase and the editing modes (Figure 2B and 2C) reveals that the duplex portion of the DNA rotates 40° around its helical axis (29,94). This allows the primer terminus to be located at the exonuclease active site, a distance of 40Å away from the polymerase active site. The motion of the DNA is guided by the movement of the tip of the thumb domain that remains bound to the DNA during the movement of the DNA. This movement allows the primer strand to enter the exonuclease active site that is otherwise occluded in the polymerization mode by a β-hairpin structure partitioning both active sites (indicated in figure 2C). This hairpin further stabilizes the partly melted primer-template DNA and prevents access of the duplex DNA into the exonuclease active site (29). Three nucleotides of single-stranded DNA are bound at the exonuclease active site in family B DNA polymerases. The mechanism of exonuclease activity will be discussed later in detail. The principles governing the switch from the polymerase to the editing mode are also applicable to polymerases belonging to the Pol I family.

Proofreading and fidelity

For replication of the genome of an organism it is of utmost importance to minimize the amount of mistakes made. Since DNA polymerases can choose for every catalytic cycle between four dNTPs and a number of analogues, it is perhaps

surprising that the fidelity, that is the ability to incorporate the correct substrate from a sea of potential imitators, is often very high. Replicative DNA polymerases have two general mechanisms to avoid making mistakes. These are base selection and proofreading. The combined action of these mechanisms ensures that only one mistake for every 10^8 newly incorporated bases is made. Accuracy is even further increased to only one mistake for every 10^9-10^{10} correct base pairings by the mismatch repair system, consisting of a number of proteins specialized in removing incorrectly inserted nucleotides in newly replicated DNA (reviewed in (45)). As this is a post-replicative repair system it is beyond the scope of this chapter.

Base selection (Insertion fidelity)

Base selection can be divided into three different steps at which the polymerase has the opportunity to remove or ignore a wrong dNTP. The first step is the binding of the dNTP to form a productive polymerizing complex. This favors the correct nucleotide over an incorrect nucleotide by a factor of 10^2-10^3 and depends primarily on basepair geometry (11,81). Second, when a nucleotide has bound, the fingers domain will close to allow optimal positioning of the dNTP for catalysis at the polymerase active site. The closure of the fingers is thought to be the main rate-limiting step before catalysis and is inhibited by an incorrect nucleotide (31,64). The discrimination against incorrect nucleotides here appears to depend on the shape of a correct Watson-Crick basepair (61) as there are no electrostatic of hydrogen-bonding interactions with the nascent primer-template basepair (25,29). Especially the conserved tyrosine of the finger motif B would sterically clash with the minor groove side of the DNA when a mismatch is incorporated as is supported by mutational analysis of this residue (92,115). This step raises the fidelity at the polymerization step up to 10^4-10^5 (46,112,118). Third, discrimination can result from a slower rate of phosphodiester bond formation with incorrect nucleotides as has been shown for KF (63).

Proofreading

When the polymerase has incorporated a wrong nucleotide, subsequent nucleotide addition (mismatch extension) is strongly inhibited possibly due to misorientation of the primer-terminus, allowing time to shuttle the mismatched primer to the exonuclease active site where proofreading can occur (23). The crystal structure of the replicating complexes of T7 DNA polymerase, *B. Stearothermophilus* and RB69 DNA polymerase provide clues for the destabilization of the DNA when an incorrect nucleotide has been incorporated (25,29,56). In these DNA polymerases, both ultimate and penultimate basepairs have hydrogen-bonding interactions with the polymerase. When a mismatch has been incorporated, one or both of the minor groove hydrogen-bond interactions will be moved out of position (40,41), breaking the interaction between protein and DNA and increasing the chances that the primer strand travels to the exonuclease domain in order to remove the incorrect base. This implies that these interactions contribute to binding affinity and the ability to sense base pair geometry (reviewed in (66)).

When the primer strand is located at the exonuclease active site, the incorrect base can be removed. The exonuclease active site basically consists of the three highly conserved sequence motifs, Exo I, II and III (Figure 1). Five residues of these motifs coordinate two metal ions. The molecular basis of proofreading was first visualized for KF complexed with ss- and dsDNA (1) and is known as the two-metal-ion mechanism. The mechanism is nearly identical to the polymerase mechanism that has been described in detail except that it is designed to remove a nucleotide (reviewed in (53). The presence of an 3'-5'-exonuclease activity could further increase the fidelity of a replicating DNA polymerase up to 10^8 (26) although itis far less important than base selection (66).

Processivity

One of the key enzymatic properties of replicating DNA polymerases is processivity; the capability to remain associated with the primer-template over a number of catalytic cycles. The numerous crystal structures of polymerases of different families gave some insights in how these polymerases can replicate thousands of nucleotides without falling off. The first clues came from the structure of the

KF complexed to DNA (1). Although the primer was located at the exonuclease active site, the thumb domain had rotated as compared to the apo-enzyme to encompass the duplex DNA. Such a conformational change after binding DNA allows the polymerase to bind the DNA more stabilly. In agreement with this notion, removal of 24 residues from the tip of the thumb of KF reduced the processivity of this enzyme (73). Despite this conformational change however, KF is not a very processive enzyme and further clues for structural features enhancing processivity needed to come from more processive DNA polymerases. The structure of T7 DNA polymerase gave another hint by which the polymerase may regulate processivity. Thioredoxin binds as a stable one-to-one complex to the thumb domain of the polymerase. This subunit is very flexible and might, in addition to the thumb domain, wrap over the duplex DNA thereby encircling it and ensuring high processivity (25).

Several polymerases, including the eukaryotic replicating polymerase δ (62), *E. coli* polymerase III (60) and RB69 DNA polymerase (94) only become processive in the presence of an additional protein, the sliding clamp. This is a trimeric protein that resembles the shape of a closed ring with a hole in the center that encircles duplex DNA (for reviews see (48,54)). The sliding clamp is tethered to the polymerase at the primer-template junction by means of a specific set of interactions with its cognate polymerase (94).

Adenovirus DNA replication

The first eukaryotic DNA replication system for which both initiation and elongation could be reconstituted in the test tube was that of the adenovirus. This virus contains a linear genome of approximately 36 kB with a protein covalently linked to the 5'-ends of the genome. Three viral proteins are required: these are the adenovirus DNA polymerase (Ad pol) that is responsable for replicating the genome, the precursor terminal protein (pTP) that primes the replication and the DNA binding protein (DBP) that is involved in several processes, including strand-displacement synthesis. In addition to these viral proteins, adenoviruses of serotypes 2 and 5 were found to make use of three additional cellular proteins. These are NFII, a topoisomerase I, required for replication of the entire genome, and the two transcription factors NFI and Oct-1 that stimulate initiation of replication up to 200-fold.

The mechanism of adenovirus replication

At pre-initiation complex formation, Ad pol is always found in complex with pTP. This pTP-pol complex needs to be positioned at the origins of replication found at each end of the viral genome (Figure 4A). Several mechanisms have evolved to assure that the pTP-pol complex is correctly loaded at the origins of replication. Two regions of these origins are required for efficient adenovirus replication for serotypes 2 and 5. The first twenty nucleotides were identified as the minimal origin of replication (100). Within this minimal origin, nucleotides 9-18 are invariant in all adenovirus serotypes identified to date and are known as the "core origin" (98, Figure 4A). Any mutation within this region alters replication activity (12,21).

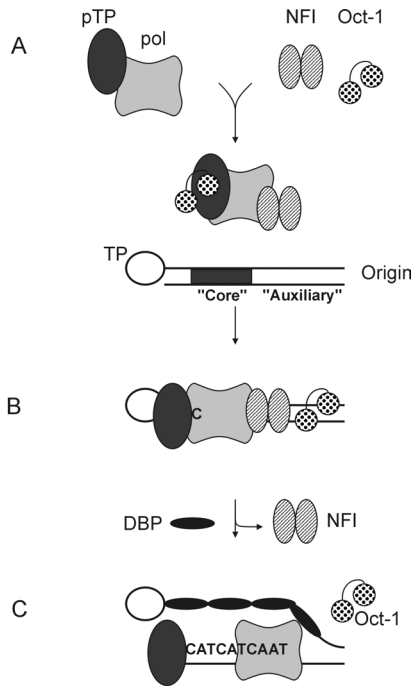

Figure 4. Schematic overview of adenovirus DNA replication. See text for details.

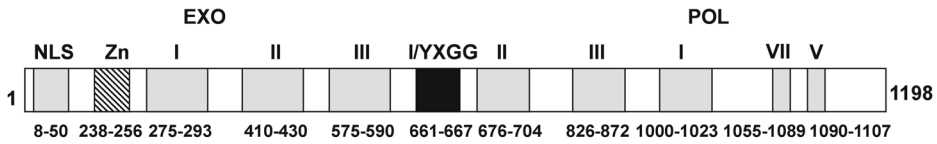

Figure 5. Sequence conservation in Ad pol. The relative location of the sequence motifs conserved in family B DNA polymerases and a putative Zn finger are indicated for Ad pol. A second putative Zn finger overlaps with motif VII and is therefore not indicated. NLS stands for nuclear localization sequence.

Footprinting analysis and EMSA furthermore showed that the pTP-pol complex specifically binds to this region (75,101). Next to the core origin, a second region called the "auxiliary origin" was identified, that contains sequences important for stimulation of adenovirus replication (111). This region contains the binding sites for the cellular transcription factors NFI and Oct-1 (Figure 4A). Both transcription factors stimulate replication by recruiting the pTP-pol complex to the origin through specific protein-protein and protein-DNA interactions. While Oct-1 binds to pTP (reviewed in (18, 17)), NFI specifically interacts with adenovirus DNA polymerase (5,13,75) as will be discussed later. DBP has also been shown to be involved in stabilization of the pTP-pol complex on the origin, albeit indirectly, by increasing the association rate and decreasing the dissociation rate of NFI (15,99). In addition, terminal protein (TP), the processed form of pTP, has been shown to be involved, although its role is less clear (85).

After pre-initiation complex formation on the origin, the pTP-pol complex initiates at an internal start site by the covalent coupling of a dCTP to serine 580 of pTP after which NFI dissociates from the origin (Figure 4B). A pTP-CAT trinucleotide intermediate is then formed that subsequently jumps back to basepair with template bases 1-3 (59). This process was termed the jumping-back mechanism and is discussed in detail in chapter 2. pTP now dissociates from Ad pol, Oct-1 dissociates from the origin when the polymerase passes and the genome is replicated in the presence of DBP (Figure 4C). The role of DBP in elongation is discussed in detail in chapter 2.

Adenovirus DNA polymerase

The main actor in adenovirus replication is Ad pol. Ad pol is a processive polymerase that contains, in addition to a polymerase activity, an 3'-5'exonuclease activity (28,57). The exonuclease activity is distributive although the removal of a mismatch and subsequent polymerization are coupled processively. Both polymerase and exonuclease activities are decreased in the presence of pTP for reasons that are unclear. Upon dissociation, the efficiency of Ad pol is increased (58), (Chapter 4).

Ad pol is a single polypeptide of 140 kDa and belongs to the B family DNA polymerases based on the presence of seven of the eight highly conserved motifs found within this family (108), (Figure 5). Linker-scanning and site-directed mutagenesis studies (13,49,52,70, 87,89,90) suggest a similar overall architecture to RB69 DNA polymerase, whose structure has been solved (29). A non-conserved putative Zn-finger has been identified at the NH_2-terminal domain of Ad pol (Figure 5). 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 (49).

Protein-protein interactions of Ad pol

Ad pol is known to interact with a number of proteins in the course of adenovirus DNA replication. Most importantly, Ad pol is complexed to the primer pTP for initiation. A number of mutational analysis studies have been unsuccessful in trying to identify particular sequences required for interaction with pTP or other proteins (13,14,50,51,87-89). In addition, a partial proteolysis study has been used that showed that Ad pol could be digested by Endolys C into four distinct domains. pTP was able to bind all of these, except for the domain covering the first 235 aa of Ad pol. Thus, a detailed characterization of the pTP-pol interaction surface is still lacking.

Another protein that directly interacts with Ad pol is NFI (5,13,75). NFI was originally identified from Hela extracts as a protein

capable of stimulating adenovirus replication (77) and it was shown that NFI was a site-specific DNA binding protein (78). A consensus NFI binding site was then identified in the auxiliary adenovirus origin (67,111), that proved essential for adenovirus replication *in vivo* (38). In addition to a role in adenovirus DNA replication, NFI proteins have been shown to regulate the transcription of a large variety of cellular genes (reviewed in (37)). The C-terminal part of the protein encodes a transactivation domain, involved in transcription regulation and the N-terminal part is responsable for DNA binding, dimerization and Ad pol binding (72). NFI simulates replication by stabilization of the pTP-pol complex on the origin through a direct interaction with Ad pol (75) although, as for the pTP, the interaction surface remains unclear.

For elongation, DBP is required and since no other single-stranded DNA binding protein could substitute for this protein, DBP is thought to also interact with Ad pol during elongation (68). Although DBP does increase the thermo-stability of Ad pol, a direct interaction has not been demonstrated to date.

Outline of this thesis

Central to this thesis is the role of adenovirus DNA polymerase (Ad pol) in adenovirus DNA replication. Ad pol is a member of the family B DNA polymerases but belongs to a distinct subclass of polymerases that use a protein as primer. As Ad pol catalyses both the initiation and elongation phases and needs to accomodate both DNA and protein as a primer, it is not surprising that a large number of protein-protein and protein-DNA interactions are involved in efficient replication. Indeed, Ad pol is known to interact with pTP, NFI and DNA, although our understanding of these interactions is limited. In this thesis, these interactions have been studied in greater detail.

After an introductory chapter on DNA dependent DNA polymerases and Ad replication, the jumping back mechanism that characterizes the change from initiation to elongation is extensively reviewed in chapter 2. In chapter 3, the highly conserved (I/Y)XGG motif of Ad pol is studied. In chapter 4, the interaction between Ad pol and DNA is further studied by the use of biotinylated oligo-nucleotides with a bulky streptavidin block. Chapter 5 examines the termination of Ad pol on the native TP-containing viral DNA. Finally, in chapter 6 the recruitment of the pTP-pol complex via a direct interaction between Ad pol and NFI is studied in detail.

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Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP

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Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP

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The adenovirus (Ad) genome is a linear double stranded (ds) molecule containing about 36 kbp. At each end of the genome an approximately 100 bp inverted terminal repeat (ITR) is found, the exact length depending on the serotype. To the 5'-end of each ITR, a 55 kDa terminal protein (TP) is covalently coupled. The Ad DNA replication system was one of the first replication systems that could be reconstituted *in vitro* (Challberg and Kelly 1979). The system requires three virally encoded proteins: precursor TP (pTP), DNA polymerase (pol) and the DNA binding protein (DBP). In addition, three stimulating human cellular proteins have been identified. These are the transcription factors NFI (Nagata et al. 1982) and Oct-1 (Pruijn et al. 1986) and the type I topoisomerase NFII (Nagata et al. 1983). Ad DNA replication uses a protein primer for replication initiation. The transition from initiation to elongation is marked by a jumping back mechanism (King and van der Vliet 1994), followed by elongation. In order to elongate DBP is required. In this review, we discuss the roles of DBP during initiation and elongation and we relate biochemical data on the jumping back mechanism used by Ad pol to the recently solved crystal structure of a pol α-like replication complex (Franklin et al. 2001). We comment on the conditions and possible functions of jumping back and propose a model to describe the jumping back mechanism.

Pre-initiation complex formation

In the process of characterizing the *in vitro* replication system, it was soon found that the combined action of two cellular transcription factors, NFI and Oct-1, could stimulate initiation of replication up to 200-fold depending on the pTP-pol concentration (de Jong and van der Vliet 1999) and references therein]. Conserved binding sites of NFI and Oct-1 were found in the ITRs of the Ad genome downstream of the core origin, in the so-called auxiliary region. NFI binds specifically to pol whereas Oct-1 binds to pTP. By using these direct interactions, NFI and Oct-1 recruit the pTP-pol complex to the core origin by increasing both binding affinity and specificity for this sequence. The binding of NFI to the replication origin was furthermore shown to be stimulated by DBP which increased the association rate and decreased the dissociation rate of the NFI-DNA complex (Cleat and Hay 1989; Stuiver and van der Vliet 1990). In addition, the genome-bound TP stabilizes core origin binding of the pTP-pol complex and induces changes in the origin structure (Pronk and van der Vliet 1993). Together these five proteins are involved in stabilizing the pre-initiation complex and correct positioning of pTP-pol.

The Ad DNA binding protein

The first viral replication protein to be discovered was the Ad DNA binding protein (DBP). In hindsight this was not surprising, since DBP is a very abundant protein with at least 10 million copies per infected cell. Moreover, its strong interaction with single stranded (ss) DNA makes it easy to detect. The DNA binding capacity and the genetically determined need for stoichiometric amounts immediately suggested a role in elongation. Indeed, mutant and electron microscopy (EM) studies as well as *in vitro* reconstitution confirmed its essential role in elongation, while

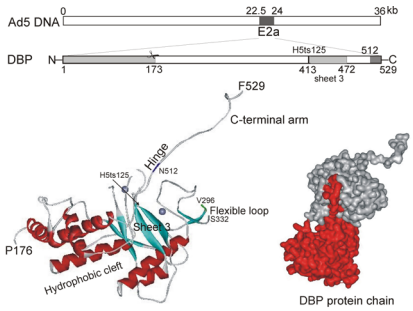

Figure 1. The Ad DNA binding protein DBP. The crystal structure of the conserved DNA binding region of Ad5 DBP is shown (Kanellopoulos et al. 1996; Tucker et al. 1994). The positions of the two Zn atoms (•) are indicated. Sheet 3 and the flexible loop are involved in DNA binding. The 17 amino acids C-terminal arm fits in the hydrophobic cleft of another molecule, giving rise to a protein chain. A space-filling example is given for two molecules. DNA is wound around the protein surface of the chain (not shown). The chain is flexible around the hinge region, enabling adaptation to different DNA sequences. Chain formation is essential for unwinding of DNA in the replication fork.

an enhancing function in initiation was established later. DNA replication turned out to be only one of the processes in which DBP is involved. Others include transcription control, mRNA stability, transformation, recombination, virus assembly and determination of the host range (Hay et al. 1995; van der Vliet 1995). It is also essential as a helper for adeno-associated virus replication (Ward et al. 1998), showing a high functional versatility. Here we restrict the discussion to DNA replication.

Structure of DBP

DBP is the product of the E2A gene. Its expression is controlled by two different promoters, active early and late in infection, respectively (Swaminathan and Thimmapaya 1995). Ad2/5 DBP consists of 529 amino acids (aa) with an apparent mobility of 72kDa in SDS- containing polyacrylamide gels. DBP can be separated into two domains by limited chymotrypsin treatment:, a highly phosphorylated N-terminal domain of 173 aa and a C-terminal domain of 356 aa. The latter is non-phosphorylated, contains two zinc atoms and is sufficient for all DNA replication functions *in vitro*. The crystal structure of this domain (see Fig. 1) shows a mainly globular core consisting of 7 alpha-helices, 3 beta sheets, and a remarkable protruding arm of 17 aa at the C-terminus (Tucker et al. 1994). This arm contains a hook that fits perfectly into a hydrophobic cleft of a neighboring molecule, thereby enabling multimerization. Indeed, a protein chain can be observed by EM and chain formation is abolished upon deletion of the C-terminal arm, confirming the X-ray diffraction results. Interestingly, a second crystal structure has been found in which only the angle between the C-terminal arm and the core is changed due to a different arrangement of residues 512-515, called the hinge region (Kanellopoulos et al. 1996). This suggests that the C-terminal arm has a high degree of rotational freedom, turning around the hinge region and in particular Asn512. Thus, the C-terminal arm can adopt different orientations leading to different conformations of the protein chain. The functional significance of this flexibility in DBP chains will be discussed below.

The structure of DNA is altered by DBP

DBP binds ssDNA as well as dsDNA and RNA without apparent sequence specificity but the modes of binding differ. Binding to ssDNA or RNA is stable and cooperative, with a cooperativity constant of 20-30 (van Amerongen et al. 1987). The length of the binding site varies between 10 and 15 bases. In contrast, dsDNA is bound in a non-cooperative fashion and binding is less stable (Stuiver et al. 1992). ssDNA is bound to the surface of the DBP chain, contacting the positively charged, three-stranded beta sheet 3, which is close to one of the zinc atoms. In this region also the mutant H5ts125 is located, which is temperature-sensitive for DNA binding (Fig. 1). Although the details of the DNA structure in the complex are not known, it is clear that DBP binding has a significant effect. In complex with DBP, ssDNA has an irregular, considerably extended structure with the bases unstacked as shown by spectroscopic and preliminary cocrystal data as well as model building (Kanellopoulos et al. 1995; Tucker et al. 1994; van Amerongen et al. 1987). This DBP-ssDNA structure increases the rigidity of ssDNA as indicated by a reduced intramolecular renaturation. Intermolecular renaturation is enhanced, presumably by removal of secondary structures or shielding the electrostatic repulsion between the two strands (Zijderveld et al. 1993). The dsDNA-DBP complex also forms a rigid

nucleoprotein structure in which tertiary structures are removed as measured by hydroxyl radical footprinting and several other techniques (Stuiver et al. 1992). Thus DBP changes the DNA conformation drastically, favoring DNA replication.

Role of DBP in chain elongation

Together with the DNA polymerase, DBP is essential for elongation. How does DBP achieve this? Four modes of action have been observed. First of all, DBP enhances the rate and processivity of the DNA polymerase and modifies the sensitivity to nucleotide analogues (Field et al. 1984; Lindenbaum et al. 1986; Mul and van der Vliet 1993). These effects could involve modification of the active site of the polymerase, possibly indirectly through modification of the DNA structure. The collaboration between pol and DBP is not understood in detail but it is specific, suggesting an interaction between the two proteins. Secondly, DBP can unwind short stretches of dsDNA or even longer stretches if short single-stranded protruding ends are present (Georgaki et al. 1992; Monaghan et al. 1994; Zijderveld and van der Vliet 1994). The strong and cooperative binding to ssDNA unwinds dsDNA independent of ATP hydrolysis, in agreement with the notion that DNA replication *in vitro* does not need a helicase. Thirdly, DBP removes secondary structures that could act as a roadblock for the advancing polymerase during displacement synthesis as well as during duplication of the displaced strand. Removal of secondary structures could also be instrumental in the very efficient recombination process in Ads and the renaturation of complementary strands originating from displacement (Zijderveld et al. 1993). Finally DBP protects ssDNA against nuclease attack, for instance in the vulnerable replication fork, a function common to many helix destabilizing proteins.

Multimerization is the driving force

How does the ability of DBP to multimerize come in? Deletion of the C-terminal arm resulted in a monomeric protein which bound with greatly reduced affinity to DNA, but still stimulated initiation. In contrast, the mutant did not support elongation on a ds template and was defective in unwinding (Dekker et al. 1997). This indicates that protein chain formation coupled to high affinity binding to ssDNA is the driving force for unwinding during DNA chain elongation.

Flexibility is the key to success

The different arrangement of the hinge region connecting the C-terminal arm to the core (Kanellopoulos et al. 1996) shows that the protein chain can adopt different conformations. What is the significance of this flexibility for the function of DBP? When proline residues were introduced in the hinge region to reduce flexibility, elongation was not possible and unwinding was severely impaired (van Breukelen et al. 2000). Still DBP could bind DNA efficiently and cooperatively. This suggests that flexibility of the protein chain is an essential prerequisite for DNA chain elongation. One explanation for the need of flexibility is that, when bound in the replication fork, the position of DBP could lead to slightly different orientations of the C-terminal arm. Assuming that conformational changes are required to accommodate the transition of DBP from binding to dsDNA to shifting to ssDNA, such a flexibility ensures that the C-terminal arm still can hook into its neighbour, giving rise to stable ssDNA-DBP complex in the displaced strand. If flexibility is lost, DBP will dissociate rapidly from the dsDNA in the fork, preventing unwinding and thus blocking elongation. Sequence differences in the fork could have an effect as well since some mutants in the hinge region are defective in binding polydA, which has an aberrant structure (van Breukelen et al. 2000). Thus, the ability of DBP to adopt more than one conformation by using a flexible C-terminal arm enables it to adapt to different DNA conformations thereby optimizing formation of the DBP-DNA chain and subsequent unwinding and elongation.

Another example of the need for flexibility has been observed. In the crystal, electron density for the region between aa 297 and 331 is not visible and this 34 aa long loop may well be flexible (Fig.1). This region contains several residues that contact ssDNA based on mutagenesis and crosslinking (Cleghon and Klessig 1992) and may fold after DNA binding, thereby stabilizing the complex. When the flexible loop was deleted, DBP could still enhance initiation but became defective in supporting elongation. Mixing experiments

showed that the flexible loop and the C-terminal arm have distinct functions in unwinding during replication. Apparently multimerization via the C-terminal arm is required for the formation of a protein filament that saturates the displaced strand whereas the flexible loop guarantees local destabilization of the fork by contributing to stability and high affinity, independent of multimerization (Dekker et al. 1998).

Role of DBP in initiation

The enhancing effect of DBP on initiation is strongly dependent on the pTP-pol concentration, being highest at low pTP-pol levels (Dekker et al. 1997). As with the transcription factors NFI and Oct-1, this suggests recruitment of pTP-pol to the origin and a specific interaction between DBP and the pTP-pol complex. In agreement with an interaction, DBP protects pol against thermal inactivation (Lindenbaum et al. 1986) and binding between the two proteins has been observed using immobilized DBP (B. van Breukelen, unpublished results). However, most other common assays to demonstrate such an interaction, such as pull-down or immune precipitation, have been unsuccessful suggesting that the interaction is weak or unstable. Besides aiding in recruitment, DBP might also help to destabilize the origin since initiation on a partially ss origin can not be stimulated by DBP anymore (B. van Breukelen, unpublished results). Mutants defective in unwinding can still stimulate initiation. Therefore, rather than ascribing the stimulation to simple unwinding of the origin, the effect could be more complex. DBP might stabilize pre-initiation complexes or prevent aberrant positioning of pTP-pol, effects less dependent on DNA binding and more on protein-protein interactions.

DBP has also a direct effect on the kinetics of the initiation reaction, which is independent of the pTP-pol concentration. It stimulates the formation of the pTP-CAT intermediate by lowering the Km for the reaction, indicating that it can influence the pol active center, either directly or by changing the template conformation (Mul and van der Vliet 1993). Finally, another way in which DBP enhances initiation is by enhancing the binding of NFI to the auxiliary origin (Cleat and Hay 1989; Stuiver and van der Vliet 1990). This is not due to a direct interaction between the two proteins but rather is based on a structural change leading to increased flexibility of the NFI binding site (Stuiver et al. 1992).

Table I.
Oligonucleotides used as templates directing *in vitro* Ad DNA replication.

Template	Sequence
Wildtype	3′-GTAGTAGTTATTATATGGAATAAAACCTAA-5′
G4A	3′-GTAATAGTTATTATATGGAATAAAACCTAA-5′
G7C	3′-GTAGTACTTATTATATGGAATAAAACCTAA-5′
Δ1	3′-·TAGTAGTTATTATATGGAATAAAACCTAA-5′
Δ2	3′-··AGTAGTTATTATATGGAATAAAACCTAA-5′
Δ3	3′-···GTAGTTATTATATGGAATAAAACCTAA-5′
Δ3G7C	3′-···GTACTTATTATATGGAATAAAACCTAA-5′
GAGA	3′-GAGAGAGATTATTATATGGAATAAAACCTAA-5′
position	····v····|····v····|····v····|

The templates referred to in the text are compared with the wildtype Ad5 template (top). Initiation takes place opposite template nucleotide G4, followed by synthesis of a CAT trinucleotide intermediate directed by template nucleotides 4-6. pTP-CAT pairs with template nucleotides 1-3 after jumping back. The core origin, which is essential for Ad DNA replication, is shaded. Mutations in the templates are underlined.

Ad pTP/pol uses a jumping back mechanism to start replication

Ad pol is a 140 kDa protein that belongs to the eukaryotic pol α family (Ito and Braithwaite 1991). Besides a DNA polymerase activity, it also contains a proofreading 3'-5' exonuclease activity. In the early stages of infection, the protein is found as a stable heterodimer with the 80 kDa pTP (Enomoto et al. 1981). The biochemical properties and characterization of pTP and pol are discussed in detail by Liu and Hay (this volume).

The Ad origin template* starts with the repetitive sequence 3'-GTAGTA-5' (see Table I for a summary of templates discussed). Ad pTP/pol initiates DNA replication opposite nucleotide 4 and synthesizes a pTP-CAT trinucleotide intermediate. This intermediate pairs with template nucleotides 1 to 3 after jumping back, after which elongation starts as illustrated in Figure 2

* Throughout this chapter, we will refer to the template strand as the strand that directs protein priming at the origin, in contrast to the displaced strand.

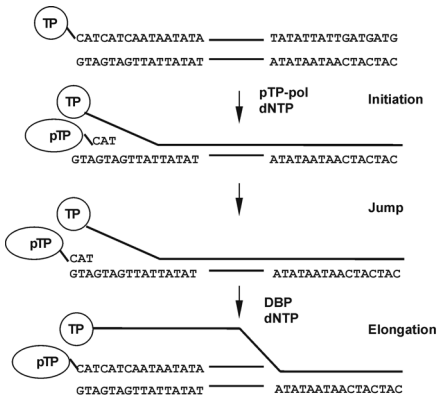

Figure 2. Ad replication initates opposite nucleotide 4, followed by jumping back. See text for details.

(King and van der Vliet 1994). A similar sliding-back mechanism was first identified by the group of M. Salas describing the replication of bacteriophages φ 29, PRD1, GA-1 and Cp-1 (Caldentey et al. 1993; Illana et al. 1996; Martin et al. 1996; Mendez et al. 1992). These phages use a protein-primed DNA replication system and contain nucleotide repeats at the genome termini. Since other protein-primed replicating phages contain sequence repeats as well, it seems safe to assume that an internal replication start followed by a sliding- or jumping-back mechanism is a common feature of all protein-primed DNA replication systems (Salas 1991; Salas et al. 1996).

This could extend to a group of RNA viruses including picornaviruses such as poliovirus, which contain a protein covalently attached to the 5' end of the viral genome (Kitamura et al. 1980). Interestingly, the poliovirus RNA polymerase initiates replication of the viral RNA genome by the coupling of two uridine nucleotides to its priming protein VPg directed by a hairpin structure containing a conserved 3'-ACAAA-5' sequence in the loop (Paul et al. 1998; Paul et al. 2000; Rieder et al. 2000). It has been proposed, that poliovirus might use a sliding back mechanism for VPg uridylylation, since mutation of the most 5' A produced the most severe replication defect, while mutation of the 3' preceding A resulted *in vitro* in the formation of a mono uridylylated VPg. This suggested that replication could start at the most 5' A, after which a putative sliding-back would be prohibited.

Which genomic features make jumping back possible?

Jumping back has been observed both on natural templates and on single stranded oligonucleotide templates, indicating that the process does not depend on the presence of the TP-moiety or the double stranded structure of the genome terminus (King and van der Vliet 1994). Unwinding of the origin must precede the basepairing of incoming nucleotides, because the template strand terminus bound by the polymerase will be single stranded during replication initiation of genomic DNA. Indeed, removal of 5' terminal nucleotides of synthetic ds templates facilitates *in vitro* DNA replication by exposing a single stranded template region (Kenny and Hurwitz 1988).

Almost all Ads sequenced to date contain di-, tri- or tetra-nucleotide repeats at their genome termini and we suspect that the exceptions to this rule are due to the difficulty of sequencing the genome termini. Such repeats are necessary to allow optimal basepairing of internal replication intermediates after jumping back, but small mismatches and deletions of terminal template nucleotides did not block DNA replication (Graham et al. 1989; King and van der Vliet 1994).

When replication was performed on a GAGA template (see Table I), G5 was used as the start site and a pTP-tetranucleotide intermediate was formed (King et al. 1997b). Ad pTP/pol apparently possesses some flexibility in both the choice of the start site and the size of the jumping intermediate. A strong preference for coupling C to pTP was observed, since even on the G4A template (Table I), a pTP-C product was formed, probably caused by initiation on G7 or G1 (King and van der Vliet 1994).

Template strand movement is essential during jumping back

During replication, the polymerase stays bound to its DNA template, since it can elongate primers using an M13 template to completion and is able to combine its exonuclease and polymerase activities in a processive manner (King et al. 1997a). Flexibility in the binding to the template must allow the template to translocate along the catalytic site in order for

jumping back to occur. While the template strand has to be retracted relative to the polymerase active center, the pTP-CAT primer still has to present its 3'-OH to the catalytic site to accept the incoming nucleotide. The movement of pTP-CAT relative to the pol catalytic site is therefore restricted. The essential motion during jumping back is the movement of the template strand relative to the pTP-CAT/pol complex.

This template strand movement causes a paradox, since pTP/pol has been shown to bind the core origin (bp 9-18), a conserved sequence feature which is essential to DNA replication of both ss and ds templates (Temperley and Hay 1992). When the interaction between pTP/pol and the core origin would remain intact, it would be hard to envisage a movement of the template strand of more than 10 Å relative to the polymerase. The paradox might be solved by dissociation of the pTP/pol complex during the jump, changing its DNA binding affinity for the core origin and allowing the polymerase to proceed. An alternative explanation could be a flexibility of the pTP/DNA interaction that allows pTP to slide along the template during the early steps of replication. The interaction with specific downstream sequences like the core origin is not needed for elongation of a DNA primer by pol, so core origin binding is important primarily during the initiation phase of DNA replication. When ds templates were mutated in their core nucleotides and assayed for their ability to support both initiation and elongation, mutations had similar effects in both phases (Temperley et al. 1991). No elongation specific defects were observed, suggesting that the interaction might be more important before the start of replication, than after initiation.

Why jump back during genome replication?

Jumping back has two major advantages: first, it enables the polymerase to correct mistakes during the early phase of replication and second, it allows an internal start site of the replication of a linear genome.

When the proofreading exonuclease activity of φ29 and PRD1 was assayed using ss oligonucleotides, terminal protein severely inhibited exonucleolytic breakdown by its native DNA polymerase (Caldentey et al. 1993; Esteban et al. 1993). This was verified for Ad DNA polymerase. When bound to pTP, the

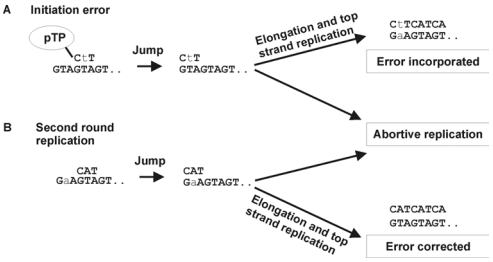

Figure 3. Jumping back mechanism corrects initiation errors. pTP is schematically presented for only one initiation event and pol is omitted for clarity. A) When an error is made during initiation, it can either result in abortive replication after the jump since it will not correctly basepair with the template or it will be incorporated into the Ad genome leading to a mutated genome when the new top strand is replicated. B) When in the second round the bottom strand of the mutated genome is replicated, a correct initiation intermediate is formed that jumps back. This will result in either abortive replication if basepairing is ineffective or it will result in elongation, thereby correcting the error after replication of the top strand.

polymerization efficiency of Ad pol is low, whereas the exonuclease activity is inhibited (King et al. 1997b; King et al. 1997a). By starting replication internally, mistakes in the synthesis of the first nucleotides which are not corrected by the impaired exonuclease activity, will be corrected in the next round of replication, because these nucleotides will not serve as a template (see Figure 3). Mismatched intermediates could in principle fail to basepair to the genome terminus and thereby lead to abortive replication, although this was not observed when mutating the third nucleotide, inhibiting the intermediate to basepair at position 3 (King and van der Vliet 1994). Finally, small deletions of the genome termini can be repaired using the jumping back mechanism (Graham et al. 1989; King and van der Vliet 1994), providing a safe-guard for the integrity of the replication start site.

Apart from being an error-correction mechanism, jumping and sliding back could also be a requirement of starting DNA replication of a linear genome internally. Jumping back will then prevent shortening of this genome at every replication cycle. Protein priming in general may depend on the ability to initiate replication internally, since not only Ads, but also φ29, PRD1 and Cp-1 start replication internally. When DNA or RNA

primers are used to initiate DNA replication, DNA polymerases are presented a matched primer/template hybrid to elongate. One could explain the need for an internal start by assuming that the protein primer has to interact with the preceding unpaired template nucleotide(s) to stably present its serine OH group to the incoming dNTP.

Interacting with a preceding template nucleotide might have yet another advantage. DNA polymerases do not select Watson-Crick basepairs by hydrogen bonding to (Doublie et al. 1998) and/or interacting hydrophobically with the base moieties of the nascent basepair (Franklin et al. 2001). Rather, this new basepair is held in position by numerous interactions of the polymerase with the phosphates of the incoming dNTP, the sugar of the dNTP and the phosphate backbone of the pairing template nucleotide. Moreover, the preceding primer-template basepair provides a stacking surface to the nascent basepair, further confining the dimensions of the active site to harbor only Watson-Crick basepairs. During protein-primed initiation, the preceding basepair is absent, but pTP has to present its serine to the active site like a DNA primer. Interestingly, the priming serine of many Ad pTPs is preceded by an asparagine or histidine that could partially substitute for the lost stacking interactions. In this way, an interaction with 3' terminal template nucleotides could help to restrict the size of the catalytic site to Watson-Crick basepair dimensions, adding to the fidelity of the initiation reaction.

How to use a protein primer?

When pTP presents its serine to the polymerase active site during the initiation reaction, it will come close to the template strand. In RB69, the phosphate of a DNA primer terminus is held in place by a water-mediated contact with Y619 and direct contacts with K726 and with Y708 of the highly conserved KKRY motif (Franklin et al. 2001). RB69 residues K705 and R707 contact the template strand, while the conserved K706 and Y708 bind the primer. This draws the two strands together and forces the DNA to adopt a B-DNA structure. In almost all protein-primed DNA polymerases however, residues corresponding to K705 and R707 are absent (Blasco et al. 1995), suggesting that the use of a protein primer prohibits a compact primer-template hybrid during initiation. Mutation of Y1081 (Y708 from RB69) in Ad pol resulted in a strong reduction of pTP interaction, initation activity and DNA binding (Liu et al. 2000). When in φ29 this tyrosine was mutated to a serine, DNA polymerization was much more affected than initiation activity, in agreement with the notion that this region is involved in primer/template binding in both DNA- and protein-primed polymerases (Blasco et al. 1995).

The backbone contacts are normally supplemented by stacking on top of the penultimate basepair and hydrogen bonding to its template pairing nucleotide. The primer 3'-OH group should be liganded by one of the two metals in order to lower its affinity for the leaving hydrogen, facilitating its attack on the dNTP α-phosphate. In this framework of potentially 'lost' interactions, the pTP Ser580-OH still has to be positioned correctly. We consider it highly likely, that interactions with the template strand nucleotides will contribute to proper positioning of pTP Ser580 in the polymerase active site (see Figure 1). In agreement with this, initiation by bacteriophage PRD1 and Cp-1 polymerase is severely inhibited by mutations preceding the initiation start site (Caldentey et al. 1993; Martin et al. 1996).

As we discussed before, in DNA primed polymerization the preceding basepair may contribute to the size exclusion of non Watson-Crick basepairs by the active site structure. Moreover, preceding template (and primer) bases are proofread by polymerase contacts with universal minor groove hydrogen bond acceptors (Franklin et al. 2001). Both types of interactions will be different during protein priming. It is interesting to note, that the exonuclease activity of pol is inhibited in the presence of pTP (King et al. 1997a). This could prevent exonucleolytic degradation of the primer initiation product caused by non-Watson Crick basepairing of template nucleotides downstream of the active site. Exonuclease activity could be inhibited because the protein primer Ser580 region is too large to enter the exonuclease active site. Alternatively, pTP could sterically block entry to this active site by binding the exonuclease domain. Finally, pTP might restrict the movement of the thumb needed to transfer the primer strand from the polymerase to the exonuclease active site by

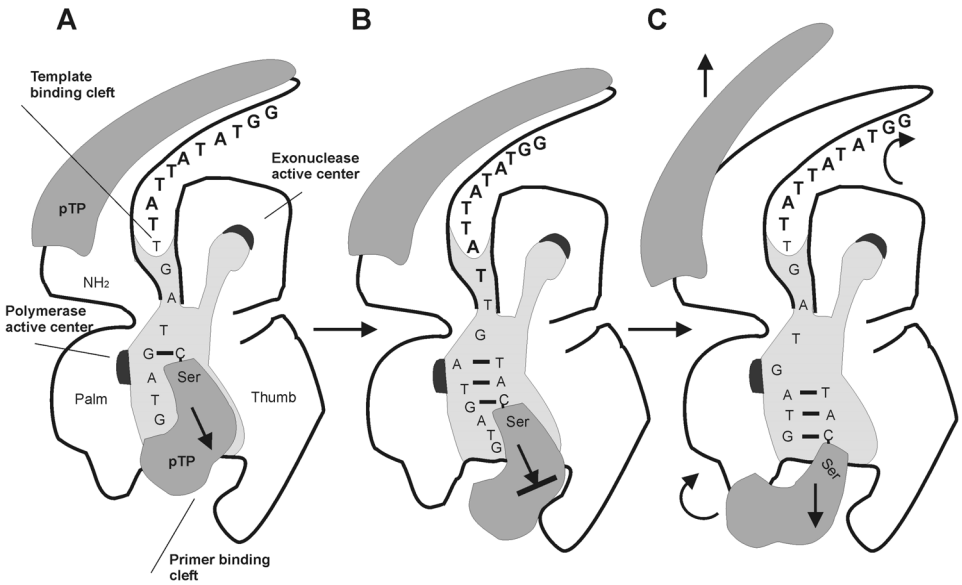

Figure 4. A schematic model for the jumping back mechanism. The Ad polymerase is presented as a cloverleaf following the structural data of the ternary complex of RB69 DNA polymerase (Franklin et al. 2001). The lower part of the cloverleaf shows the polymerase domain with the palm containing the polymerase active site, and the thumb. The fingers are omitted for clarity. The exonuclease domain containing its active site and a putative N-terminal domain (indicated as NH_2) are indicated at the upper part of the cloverleaf. pTP is displayed as two gray modules. One of the pTP modules binds the primer binding cleft, locating the priming serine hydroxyl group (Ser) at the pol active site. The other module together with the N-terminal pol domain contacts the core origin and lies close to the template binding cleft. The sequence of the core origin is marked in bold. Arrows mark the direction of DNA- or protein-movement. (A) After pre-initiation complex formation, pTP-C is formed. The primer will move the distance of one nucleotide out of the pol active site. (B) When pTP-CAT is formed, the region of pTP fixed to pol forms a roadblock and pTP-CAT cannot leave the pol active site. (C) Repositioning of pTP releases the roadblock and results in dissociation of the pTP region fixed to pol. The template strand has jumped back the distance of three bases and pTP-CAT has re-annealed with template strand residues 1-3. The resulting hybrid can move out of the pol active site for subsequent elongation.

binding in the dsDNA binding channel of the DNA polymerase, where the unpaired template strand is located.

The mechanism of jumping back

A model of the mechanism of jumping back is illustrated in figure 4. Ad polymerase is drawn schematically analogous to the structural data of RB69, a member of the eukaryotic DNA pol α-like family to which Ad pol belongs (Franklin et al. 2001). RB69 DNA polymerase was the first eukaryotic DNA polymerase of which the crystal structures of the apo-enzyme, as well as the editing- and replicating complexes were solved. Three other structures of the pol α family DNA polymerases display a high degree of structural similarity (Hopfner et al. 1999; Rodriguez et al. 2000; Zhao et al. 1999), which makes it likely that Ad pol adopts a similar structure. All polymerases of which the structure is known at present show a common architecture that resembles the shape of a right hand, with the palm, thumb and finger domains (Steitz 1999).

In figure 4, the palm domain containing the polymerase active site and the thumb domain are shown. The finger domain lies to the back of the figure and is omitted for clarity. The

exonuclease domain with its active site, and the N-terminal domain are indicated as well. Although not conserved, the presence of a separate N-terminal domain in Ad pol will be discussed below. Initiation starts opposite the fourth nucleotide of the template strand of the origin of replication. Based on the ternary structure of the replicating complex of RB69 DNA polymerase, it was recently suggested that the template enters at a template binding cleft located between the N-terminal and exonuclease domains, as indicated in figure 4. Several biochemical studies for e.g. T4 DNA polymerase (Munn and Alberts 1991) and phage 29 DNA polymerase (de Vega et al. 1999) have shown that these polymerases cover a region of 10 basepairs, with a distance of four bases from the entrance of the template binding cleft to the polymerase active site. Therefore, during pre-initiation complex formation, the polymerase likely spans at least 7 template bases. In addition, footprinting analysis of pTP, Ad pol and the pTP/pol complex on origin DNA demonstrated specific binding of the pTP-pol complex to the core origin basepairs 9-18 (Mul and van der Vliet 1992; Temperley and Hay 1992). Since the left border of the core origin would be located just before the entrance of the template binding cleft during initiation, it is possible that the N-terminus of Ad pol folds into a domain that binds to the core origin sequence as indicated in figure. 4. Indeed, there are about 250 aa 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 (Joung and Engler 1992).

The protein primer for DNA replication, pTP, is indicated in figure 4 at the entrance of the primer binding cleft presenting the priming 3' hydroxyl group of serine 580 to the polymerase active site. Part of the pTP is also shown at the entrance of the template binding cleft, contacting the core origin.

After pre-initiation complex formation, replication initiates with the covalent coupling of dCTP to pTP (Figure 4A). After incorporation of the first and second dNMP residues, the protein primer and the template strand have to move one nucleotide out of the polymerase active site in order to make room for incorporation of the next nucleotide. However, after the incorporation of the third nucleotide (Figure 4B), the template strand jumps back to basepair with pTP-CAT on template residues 1-3 (Figure 4C). The sequence of these events is at present unclear. We propose that the pTP region located at the primer binding cleft becomes strained by the movement of the primer-template after each nucleotide incorporation. At the formation of pTP-CAT, further elongation is blocked by the presence of pTP, which is fixed to the polymerase. This block allows the polymerase to melt three bases, after which the strain on pTP could be released by the repositioning of pTP which leads to dissociation. The template jumps back either actively or passively and pTP starts to dissociate from the polymerase occupying the now available space in the primer binding cleft. Guided by DBP, the polymerase now further copies its genome with high fidelity and processivity.

pTP might trigger the jump by acting as a lever

Instead of incorporating a fourth nucleotide, the template jumps back. To achieve this, an event must take place within the pTP-pol complex that determines if and when the jump has to occur. During DNA primed replication, the newly formed duplex product binds in a groove formed by the palm and thumb domains, making contacts with the protein over one full turn of the DNA helix (Franklin et al. 2001). After incorporation of a correctly basepaired nucleotide, the newly synthesized duplex DNA moves one basepair out of the polymerase active site. This movement is not only a vertical translocation but a helical rotation as well. In protein-primed initiation, pTP likely moves in a similar fashion upon each nucleotide incorporated although pTP remains bound to the polymerase (King et al. 1997b). While pTP interacts over a large surface of the polymerase during initiation (Parker et al. 1998), a region must be present at the primer binding cleft to provide the hydroxyl group of serine 580 to the polymerase active site as discussed previously. pTP-CAT movement is thus restricted since it interacts stably with the polymerase. We therefore presume that there is a flexibile region of pTP that binds in the primer binding cleft.

After formation of pTP-CAT, not only the template strand jumps back, but concomitantly the pTP-pol complex starts to dissociate (King et al. 1997b; King and van der Vliet 1994). pTP might therefore trigger the jump and dissociation by acting as a lever. From each primer-template movement (both vertical and helical) strain will result in the pTP region that binds in the primer binding cleft while the rest of pTP remains fixed to the polymerase. When pTP-CAT is formed, the strain on pTP is maximal and is released by jumping back of the template strand and simultaneous dissociation of pTP, permitting further primer-template elongation. Alternatively, the jumping back mechanism could be an intrinsic property of the DNA polymerase that can only be observed when unwinding of the primer-template hybrid is energetically inexpensive. Since a self-imposed block would probably affect the processivity when encountering repeats that cannot be jumped, we favor the first explanation.

Early studies on the pTP-pol complex demonstrated a clear difference in the kinetics of initiation and elongation. During initiation, the Km for nucleotides is lower than during elongation (Mul and van der Vliet 1993). Moreover, initiation is resistant to ddNTPs and aphidicolin, whereas elongation is partially sensitive (Lichy et al. 1981). These results suggest that a major change within the pTP-pol complex takes place upon the transition from initiation to elongation. Further experimental evidence for such a change comes from studies on the dissociation of the pTP-pol complex. When an oligonucleotide template was used, which cannot jump back because of lack of nucleotides 1-3 (oligonucleotide Δ3G7C, Table I), about 60% of pTP dissociated from pol after the formation of pTP-CAT, indicating a change within the pTP-pol complex (King et al. 1997b). In addition, this result suggested that jumping back and dissociation are separate processes.

We consider it likely that the changes observed after pTP-CAT formation, are a consequence of reorientation of pTP as described above. This would not only enable the polymerase to processively elongate the entire genome without inhibition of bound pTP (King et al. 1997b) but would also prevent another jump after the synthesis of pTP-CATCAT. We further propose that the size of the pTP-intermediate is a determining factor for triggering dissociation and jumping back. Interestingly, when a GAGAGAGA repeat (Table I) was used as template, initiation of replication started at G5. Instead of making a dinucleotide and jumping back twice, a pTP-tetranucleotide was synthesized (King et al. 1997b). This result is in agreement with the idea that a pTP-intermediate of a certain length is required to release the pTP lever.

How does the template strand move?

During the jump, three Watson-Crick basepairs are disrupted and the template strand moves. Members of the pol α family of DNA polymerases have an intrinsic capacity to destabilize the dsDNA necessary for their editing function (Shamoo and Steitz 1999) and this may play a role during jumping back. The tertiary structure of the editing and replicating complexes of RB69 DNA polymerase show, that when a mismatch is incorporated or when a primer-template remains too long at the polymerase active site without replicating, three basepairs are unwound and the primer strand is transferred to the exonuclease active site (Franklin et al. 2001; Shamoo and Steitz 1999). Although exonuclease activity is strongly decreased when pTP is complexed with the polymerase (King et al. 1997a), the capacity to melt three bases might still be intact. A fourth nucleotide cannot be incorporated into pTP-CAT and elongation is blocked. This may give the polymerase the opportunity to melt three bases similar to an exonucleolytic event. Instead of locating the primer strand at the exonuclease active site, the template strand jumps three bases and reanneals with the primer at template residues 1-3. The template strand is contacted by the polymerase by multiple hydrogen bonds and charge-charge interactions (Franklin et al. 2001). At the polymerase active site, the I/YXGG motif in Ad DNA polymerase has recently been demonstrated to stabilize the template strand (Brenkman et al. 2001), in agreement with the ternary structure of the replicating RB69 DNA polymerase (Franklin et al. 2001). Mutation of both glycines to alanines (GG666/7AA) inhibited the transition from initiation to elongation, illustrating that template stabilization is important for jumping back (Brenkman et al. 2001).

When the template strand unwinds, it could move either simultaneously or just after pTP dissociation. To dissociate from the polymerase, pTP might be sterically hindered by the 3'-template overhang located at the primer-template binding groove. It is therefore possible that the strain released from pTP after formation of pTP-CAT, actively or passively pushes the template back the required distance. This could mean that the 3'-template overhang is an additional determining factor for the initiation start site and the jump of the template. Dissociation of pTP then allows it to leave the primer binding groove, explaining why the polymerase does not stall again after nucleotide 6.

Experimental evidence for this mechanism

Replication of template Δ3G7C (Table I) resulted in dissociation after pTP-CAT formation in the absence of jumping back (described above, (King et al. 1997b)). Although this experiment suggests that dissociation of pTP and jumping back are independent processes, this template has no 3'-overhang and initiation is restricted to residue G1. When pTP-CAT is formed, reorientation of pTP within the pTP-pol complex could therefore be facilitated since more space is available at the primer-template binding cleft, due to the lack of a 3'-template overhang. Dissociation of pTP then starts, without the need to jump back. In agreement with this, when Ad origin templates were used with deletions of one or two terminal 3'-template residues, jumping back proceeds and recovers the terminal deletions (King and van der Vliet 1994). However, the major product of the Δ2 template (Table I) results from readthrough without jumping back, suggesting that there is enough space for pTP to reorientate without movement of the template strand. When a template was mutated at position G4 to A (3'-GTA**A**TAG, Table I), pTP-C formation started on G7, providing a long 3'-template overhang of six nucleotides. Remarkably, no further elongation was permitted in the presence of dATP, dCTP and dTTP, indicating a block in pTP movement possibly due to limited space available at the primer-template binding cleft. It is furthermore interesting to note that initiation of Ad DNA replication can not start at an internal origin but needs to be linearized unless head to tail origins are provided (Graham et al. 1989).

In conclusion, besides the recruitment of the pTP-pol complex by the cellular replication proteins to the core origin, the preference for binding termini with a distinct length may provide an additional prerequisite to direct the exact initation starting position.

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The "(I/Y)XGG" motif of adenovirus DNA polymerase affects template DNA binding and the transition from initiation to elongation.

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The "(I/Y)XGG" motif of adenovirus DNA polymerase affects template DNA binding and the transition from initiation to elongation.

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Adenovirus DNA polymerase (Ad pol) is a eukaryotic-type DNA polymerase involved in the catalysis of protein-primed initiation as well as DNA polymerization. The functional significance of the "(I/Y)XGG" motif, highly conserved among eukaryotic-type DNA polymerases, was analyzed in Ad pol by site-directed mutagenesis of four conserved amino acids. All mutant polymerases could bind primer-template efficiently but were impaired in binding duplex DNA. Three mutant polymerases required higher nucleotide concentrations for effective polymerization, and showed higher exonuclease activity on dsDNA. These observations suggest a local destabilization of DNA substrate at the polymerase active site. In agreement with this, the mutant polymerases showed reduced initiation activity and increased Km_{app} for the initiating nucleotide, dCMP. Interestingly, one mutant polymerase, while capable of elongating on primer-template DNA, failed to elongate after protein-priming. Further investigation of this mutant polymerase showed that polymerization activity decreased after each polymerization step and ceased completely after formation of the precursor terminal protein-trinucleotide (pTP-CAT) initiation intermediate. Our results suggest that residues in the conserved motif "(I/Y)XGG" in Ad pol are involved in binding the template strand in the polymerase active site and play an important role in the transition from initiation to elongation.

INTRODUCTION

Adenoviruses contain a linear double-stranded genome of approximately 36 kb with two origins of replication located in the inverted terminal repeats. At each 5' end of the adenovirus genome a terminal protein (TP) is covalently linked. Replication initiates via a protein-priming mechanism (1) involving the Ad pol and precursor terminal protein (pTP). Ad pol and pTP form a tight heterodimer of which the pTP acts as a primer and is covalently linked to the initiating nucleotide dCMP. 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 on the origin of replication ((2) and references therein). Ad pol initiates replication opposite the fourth base of the template strand and synthesizes a pTP-CAT intermediate. For elongation to occur, this intermediate jumps back to be paired with template residues 1-3, whereafter pTP dissociates from Ad pol and elongation starts (3;4). Elongation occurs via a strand displacement mechanism which requires the viral DNA binding protein (DBP) (reviewed in (5;6)). Late in infection, pTP is cleaved by a virus encoded protease into TP and the precursor part (reviewed in (5;6)). The actual role of pTP processing is at present unclear. Initiation and elongation are performed by the same polymerase, but the two processes differ in sensitivity to inhibitors (7;8). This suggests that a conformational change occurs upon transition from initiation to elongation, most likely after the formation of pTP-CAT. In agreement with this notion, kinetic studies

revealed that the Km for dCTP is lower for initiation than for elongation (9). In addition to its synthetic activities, Ad pol also possesses a distributive 3'-5' exonuclease activity, shown to be involved in proofreading (10).

Many DNA polymerases have been characterized and were generally found to have a polymerase and a 3'-5' exonuclease activity. Sequence comparisons of DNA polymerases from bacterial, viral and cellular origin led to a classification into four groups A, B (also known as α-like), C and D, based on amino acid similarities with *Escherichia coli* Pol I, II and III, and human DNA pol β (11-13). Based on their extent of similarity, six highly conserved motifs (I-VI), which were proposed to lie in the polymerase active site, were identified in human pol α (14). DNA polymerases containing these six motifs (see Fig. 1) were designated α-like DNA polymerases (12). Further alignments showed that motifs I-III are conserved in all groups of DNA polymerases (12), while a seventh conserved motif was identified in the α-like DNA polymerases (15;16). Besides conserved motifs located in the C-terminal part of DNA polymerases, three sequence motifs (Exo I-III) were shown to form an evolutionary conserved 3'-5'exonuclease site (17-19). Extensive biochemical analysis of a number of prokaryotic and eukaryotic DNA polymerases, such as the Klenow fragment of *E. coli* Pol I (12), T4 (reviewed in (20)), HSV (21), ϕ29 (reviewed in (22)) and pol α (23) has shown that residues located in conserved motifs of these different DNA polymerases play similar roles in dNTP or DNA binding, or in catalysis of polymerase or 3'-5' exonuclease activity. Analysis of a number of DNA polymerases suggests that the polymerase active site is structurally and functionally conserved for both prokaryotic and eukaryotic DNA polymerases (24-28). They are all proposed to utilize an identical two-metal ion-catalyzed polymerase mechanism but differ extensively in many of their structural features (29). The crystal structure of phage RB69 DNA polymerase (24) can serve as prototype of the pol α family of DNA polymerases, since the recently solved crystal structures of *Thermococcus gorgonarius* DNA polymerase (30) and *Thermococcus* sp. 9^{o} N-7 (31) are topologically similar to this DNA polymerase.

Ad pol is an α-like DNA polymerase belonging to the subclass of protein-priming DNA polymerases. Site-directed mutagenesis studies have identified motif I as a motif important for initiation and elongation activity of Ad pol (32). Furthermore, two putative zinc finger domains were identified (33) and linker mutagenesis studies have shown that multiple regions, including motif IV and V, in Ad pol are essential for Ad DNA replication (34-36). Recently, a set of 22 alanine substitutions of conserved residues in the C-terminal part of Ad pol suggests an arrangement of conserved motifs in Ad pol similar to RB69 DNA polymerase (37).

An additional motif, "YXGG/A", located N-terminal of motif II (12;18) of the polymerase active site, was shown to be highly conserved among α-like DNA polymerases (38). Mutational analysis of this motif in ϕ29 DNA polymerase, that starts replication by protein-priming, led to the proposal that it is involved in the binding stability of the DNA template at the polymerization active site (38). Additionally, it was shown to be important for the formation of a stable complex between TP and DNA polymerase, resulting in transition defects from TP-priming to DNA-priming during replication of ϕ29 TP-DNA (39). A multiple alignment of the YXGG/A motif in eukaryotic-type DNA-dependent DNA polymerases has been shown previously in Truniger *et al.* (38). In eukaryotic-type DNA polymerases the motif has the consensus sequence "YXGG/A" but for the subclass of protein-primed DNA polymerases, the motif could be restricted to the consensus YXGG. For these DNA polymerases, including Ad pol, the highly conserved tyrosine residue is often an isoleucine. This led us to define the motif as "(I/Y)XGG".

Here, we report the detailed characterization of the "(I/Y)XGG" motif in Ad pol, which has been subjected to site-directed mutational analysis. We propose that the motif is involved in the stabilization of the template strand at the polymerase active site. During pTP-primed initiation this indirectly affects the binding of the initiating nucleotide, as well as the transition of the initiation intermediate pTP-CAT from initiation to elongation thereby leading to abortive replication. Based on the crystal structure of RB69, modeled with primer-template and dNTP, we propose a hydrophobic

interaction between the conserved isoleucine and the ribose moiety of the nucleotide preceding the template base.

EXPERIMENTAL PROCEDURES

DNA templates and substrates

All oligonucleotides, unlabeled nucleotides, [α-^{32}P] dNTPs (3000 Ci/mmol) and [γ-^{32}P] ATP (5000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. T30 (5'-AATCCAAAATAAGGTATATTATTGATGATG) represents the first 30 nucleotides of the bottom strand of the adenovirus 5 genome, and T20 the first 20. D20 (5'-CATCATCAATAATATACCTT) is the complementary strand of T20. Labeling of D20 was performed with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-^{32}P] ATP. D20 was used for the 3'-5'exonuclease assay on ssDNA. For the polymerase/exonuclease coupled assay, gel retardation and 3'-5'exonuclease assay on dsDNA, 5'-labeled D20 was hybridized to T30 or to T20. The hybrid molecules D20/T30 and T20/D20 were obtained by boiling oligonucleotides in 60 mM Tris-HCl, pH 7.5, 200 mM NaCl, followed by slow cooling to room temperature. D20/T30 and D20/T20 were purified by 10% polyacrylamide-1xTBE gel electrophoresis. Ad 5 TP-DNA was isolated as described (40).

Site-directed mutagenesis

A full length Ad pol cDNA encoding amino acids 1 to1199 (provided by Henk G. Stunnenberg (41)) was cloned in the EcoRI and Sph I sites of the pFastBac donor plasmid. Site-directed mutagenesis was performed using the Quickchange method from Stratagene. The oligonucleotides for the PCR mutagenesis were for R_{661}A: 5'-ATGCTGGCG**GC**CACGTA-ATCG and 5'-CGATTACGTG**GC**CGCCAGCAT; for I_{664}S 5'-TCTTCCACCGCGG**GA**GCTGGCG and 5'- CGCCAGC-**TC**CCGCGGTGGAAGA; for I_{664}Y 5'-TCTTCCACCGCGG-**TA**GCTGGCG and 5'-CGCCAGC**TA**CCGCGGTGGAAGA; for $GG_{666/7}$AA 5'-GTAGCATCTT**G**CA**G**CGCGGATGCTC and 5'-GAGCATCCGCG**C**TG**C**AAGATGCTAC, with changes marked bold.

The presence of the desired mutations was confirmed by complete sequencing of each mutant gene. The recombinant plasmids were transformed into DH10Bac competent cells, which contain the bacmid with a mini-*att*Tn7 target site and a helper plasmid. The mini-Tn7 element on the pFastBac plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids were identified by disruption of the lacZα gene. Bacmid DNA was isolated by means of a high molecular weight mini-preparation. This DNA was then used to transfect insect cells with lipofectin (Life Technologies) according to the manufacturer manual. After 72 h of transfection, the recombinant baculoviruses were harvested and amplified for several rounds.

Expression and purification of Ad DNA polymerase mutants

Insect cells (Sf-9) were grown as monolayers on 167.5 cm^2 plates in SF900 II medium (Life Technologies) at 27 °C. Plates were infected with recombinant baculovirus expressing the wild-type or mutant Ad pol when approximately 80 % confluence was reached. After 56 h of infection, cells were harvested and washed with ice-cold PBS. Cells were resuspended in a hypotonic lysis buffer containing 25 mM HEPES pH 7.5, 10 % glycerol, 5 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 2 µg/ml aprotinine and 1 µg/ml leupeptine and placed on ice. After 10 min, cells were disrupted by 20 strokes of a Dounce homogenizer (B-Pestle) and NaCl was added to a final concentration of 200 mM. The lysate was cleared by ultracentrifugation at 25000 rpm in a SW28 rotor for 30 min at 4 °C.

For purification to near homogeneity, the lysate was loaded on a SP-Sepharose column, equilibrated with buffer A (25 mM HEPES, pH 7.5, 20 % glycerol, 1 mM EDTA, 1 mM PMSF, 1 mM DTT) containing 200 mM NaCl. The column was washed extensively with buffer A/200 mM NaCl and eluted with buffer A/450 mM NaCl. Fractions were collected and analyzed on a 7.5 % polyacrylamide/SDS gel followed by silver staining. Peak fractions were collected, dialyzed against buffer A/100 mM NaCl and loaded on a single-stranded DNA-cellulose column (Sigma Chemical Co.). After washing with buffer A/150 mM NaCl, protein was eluted at 600 mM NaCl. Peak fractions were dialyzed against buffer B (25 mM HEPES, pH 8.0, 20 % glycerol, 1 mM EDTA, 1 mM PMSF, 1 mM DTT) containing 100 mM NaCl and loaded onto a 1ml Mono Q HR 5/5 column (Amersham Pharmacia Biotech). After washing with buffer B/100 mM NaCl, protein was eluted in a gradient of buffer B/100-500 mM NaCl. Peak fractions were collected and the purity of the protein was estimated to be > 95 % by gel electrophoresis and Coomassie staining.

Proteins and buffers

pTP was a gift from Panagiotis N. Kanellopoulos. ΔN-DBP was purified as described (42). The buffer used for dilution of the replication proteins contained 25 mM HEPES, pH 7.5, 20 % glycerol, 120 mM NaCl and 1 mg/ml bovine serum albumin (BSA).

DNA polymerase/exonuclease coupled assay

Partial duplex D20/T30 containing a stretch of 10 nucleotides protruding from the 5'end was used as primer-template to study DNA-dependent DNA polymerization and 3'-5' exonuclease activity. The reaction mixture (12.5 µl) contained 50 mM Tris-HCl, pH 7.5, 4 % glycerol, 1 mM DTT, 1 mg/ml BSA, 1 mM $MgCl_2$, 0.05 ng 5'-labeled D20/T30, 12.5 ng wild-type or mutant DNA polymerase and the indicated amounts of dNTPs. Reactions were stopped after 10 min at 37 °C by addition of sequencing loading buffer (10 mM EDTA, 98 % formamide and 0.025 % bromophenol blue). Samples were analyzed on 8 M urea-20 % polyacrylamide gel electrophoresis followed by autoradiography. Polymerization or 3'-5' exonuclease activity were detected as an increase or decrease in size, respectively, of the 5'-labeled D20 primer.

3'-5' exonuclease assays

Exonucleolytic breakdown of ss and dsDNA was tested using 5'-labeled D20 and 5'-labeled D20/T30, respectively. The reaction mixture (25 µl) contained 50 mM Tris-HCl, pH 7.5, 4 % glycerol, 1 mM DTT, 1 mg/ml BSA, 50 mM NaCl, 1 mM $MgCl_2$, 0.05 ng ss or dsDNA and the reaction was started by addition of 25 ng of wild-type polymerase or mutant polymerases. Incubation was at 37 °C, allowing conditions to be linear both in time and enzyme concentration. Reactions were stopped by addition of sequencing loading buffer. After analysis by 8 M urea-20 % polyacrylamide gel electrophoresis, the 3'-5' exonuclease activity is measured as a decrease in size of the DNA by densitometry. From these data the catalytic efficiency of the mutants (indicated in Table II) was calculated relative to wild-type Ad pol.

DNA binding assays

Gel retardation was performed using 5'-labeled D20/T30 and

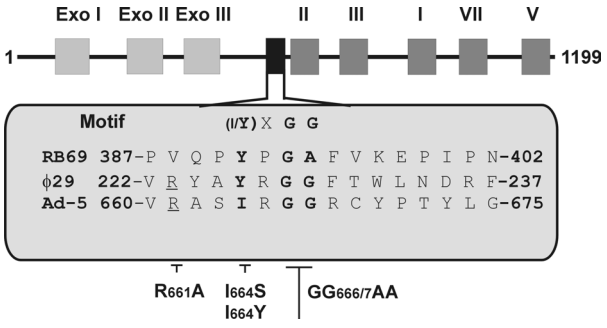

Figure1. Sequence conservation in Ad pol; relative location and alignment of the "(I/Y)XGG" motif. The relative location of the motifs conserved in eukaryotic-type DNA polymerases are indicated for Ad pol. Motif VI is lacking in Ad pol (14) and motif IV largely aligns with Exo II (19). In the lower panel, the motif "(I/Y)XGG" as defined by (38) is aligned for the DNA polymerases RB69, ϕ29 and Ad pol (indicated as Ad-5), with the conserved amino acids in bold. An arginine, specifically conserved among protein-primed and cellular α-like DNA polymerases has been underscored. Mutant polymerases are shown below the panel and are designated by the original amino acid (in single letter notation), its position in Ad pol and the replacing amino acid.

5'-labeled D20/T20. The binding reaction (20 µl) contained 25 mM HEPES, pH 7.5, 4 % Ficoll, 1 mM EDTA, 55 mM NaCl, 4 mM DTT, 0.1 mg/ml BSA, 1 mM $MgCl_2$, 0.05 ng of either 5'-labeled D20/T30 or 5'-labeled D20/T20 and the indicated amounts of Ad pol or the corresponding mutants. After incubation for 5 min at 4 °C, samples were loaded and separated on a 10 % polyacrylamide-1x TBE gel at 4 °C. Gels were dried, autoradiographed, and quantified using a Phosphor Imager.

Initiation and partial elongation of DNA replication

Initiation of replication was performed in a standard incubation mixture of 25 µl in the presence of 25 mM HEPES, pH 7.5, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 µg BSA, 50 nM [α-^{32}P] dCTP, and the indicated amounts of Ad pol and pTP. As template either 0.6 µg of origin-containing T30 or 60 ng of TP-DNA were used. When TP-DNA was used, 250 ng ΔN-DBP was added per reaction. Initiation coupled to partial/truncated elongation was performed under similar conditions as initiation in the presence of the indicated concentrations of dCTP, dATP and dTTP. No dGTP was added in the reaction mixture. Reactions were performed at 37 °C for 45 min and were stopped by adding EDTA to a final concentration of 80 mM. The samples were precipitated with 20 % trichloroacetic acid (TCA) on ice. Precipitates were washed with 5 % TCA, dissolved in sample buffer and analyzed on a 7.5 % polyacrylamide/SDS gel and autoradiographed. Replication products were quantified by densitometric analysis following exposure on a Phosphor Imager.

The Km_{app} for pTP deoxynucleotidylation was determined by performing initiation assays with [α-^{32}P] dCTP with wild-type and mutant polymerases using increasing concentrations of unlabeled dCTP (1-1000µM). The Km_{app} was calculated from three experiments.

Glycerol gradient sedimentation

The standard incubation mixture (200 µl) for glycerol gradient analysis contained 2 µg Ad pol, 1.2 µg pTP, 25 mM HEPES, pH 7.5, 1 mM DTT, 1 mM $MgCl_2$, and NaCl to a final concentration of 55 mM. After incubation for 30 min on ice, the mixture was layered on top of a 4.8 ml linear 10 %-30 % (v/v) glycerol gradient containing 25 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.5 M NaCl and 100 µg BSA as an internal control. Gradients were centrifuged for 24 h at 50,000 rpm in a SW50 rotor at 4 °C. A control gradient with 1.2 µg pTP was run under similar conditions. Fractions were collected from the bottom of the tube and analyzed on a 7.5 % polyacrylamide/SDS gel. BSA was visualized by silver staining and pTP, Ad pol and the pTP-pol complex by immunoblotting using an anti-pol and anti-pTP-pol antiserum (43) Quantitation of the relative amounts of pTP and pol present in each fraction was carried out by densitometry.

RESULTS

In order to understand the role of the "(I/Y)XGG" motif in Ad pol, individual residues of this region were mutated (Fig. 1) as described under Experimental Procedures. The isoleucine was changed into tyrosine ($I_{664}Y$) as present in RB69, ϕ29 and most other cellular, bacterial and viral DNA polymerases (39) and also into serine ($I_{664}S$) to study the effect of another non-conservative change. The two glycines are invariantly conserved among protein-primed DNA polymerases but the second glycine is often an alanine among bacterial, viral and many cellular DNA polymerases (39). Both glycines were changed into alanines, giving mutant polymerase $GG_{666/7}AA$. A positive charge preceding the "(I/Y)XGG" motif (R_{661}) appears to be specifically conserved among protein-primed and cellular DNA polymerases (38) and was changed into alanine ($R_{661}A$). Construction of baculoviruses, and expression and purification

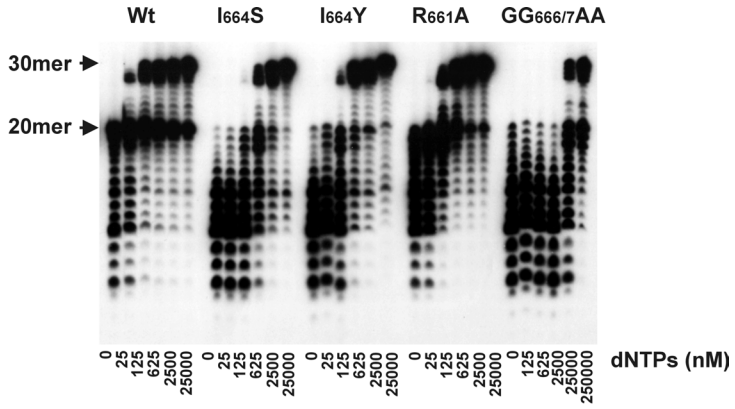

Figure 2. DNA polymerase-exonuclease coupled assay. The assay was carried out using 5'-labeled D20/T30 primer-template, the indicated concentration of each dNTP, and 25 ng of wild-type or mutant Ad pol. Arrows indicate the position of the 20mer (non-elongated primer) and the 30mer (elongated primer).

Table I.
Enzymatic activities of Ad wild-type and mutant polymerases

Activity assay	Wild-type	$I_{664}S$	$I_{664}Y$	$R_{661}A$	$GG_{666/7}AA$
3'-5'Exonuclease ssDNA (%)[a]	100	62	74	68	61
3'-5'Exonuclease dsDNA (%)[b]	100	189	299	105	254
Pol/Exo (nM dNTPs)[c]	125	625	625	125	25.10^3

The assays were performed as described in Experimental Procedures.
[a] 3'-5'Exonucleolytic activity on ssDNA. Numbers indicate the percentage of the wild- type enzyme activity.
[b] 3'-5'Exonucleolytic activity on dsDNA. Numbers indicate the percentage of wild-type enzyme activity.
[c] In the polymerase/exonuclease coupled assay, the dNTP concentration (in nM) required to complete full polymerization is indicated

of the recombinant proteins was performed as described under Experimental Procedures. During purification all mutant polymerases behaved essentially as wild-type Ad pol.

Mutations at the "(I/Y)XGG" motif alter the wild-type polymerase/exonuclease balance.
A polymerase/exonuclease coupled assay was performed to study the coordination of both degradative and polymerization activities with the mutant polymerases. The functional coupling between synthesis and degradation on primer-template D20/T30 was assayed as a function of the dNTP concentration. As shown in Fig. 2, 3'-5'exonucleolytic digestion of the primer strand occurred in the absence of nucleotides. By addition of increasing amounts of dNTPs, the equilibrium was shifted towards synthesis, exonucleolysis being competed by DNA polymerization. In the presence of 25 nM dNTPs, the wild-type Ad pol was able to extend D20/T30 until 27-28 nucleotides, and from 125 nM dNTPs full extension of the primer-template was accomplished (D30/T30). Whereas mutant polymerase $R_{661}A$ allowed full polymerization at approximately similar nucleotide concentrations as the wild-type enzyme (125 nM, Fig. 2), mutant polymerases $I_{664}S$ and $I_{664}Y$ required a 5-fold higher amount (Fig. 2 and Table I). On the other hand, mutant polymerase $GG_{666/7}AA$ required a 200-fold higher dNTP concentration compared to the wild-type enzyme for full polymerization (Fig. 2 and Table I). Furthermore, an increased exonuclease activity was observed for mutant polymerases $I_{664}S$, $I_{664}Y$ and $GG_{666/7}AA$ upon comparison of their degradation activities (Fig. 2, lanes 0 nM) with that of the wild-type polymerase, as can be seen by the higher intensity of the faster moving bands. The higher amount of dNTPs, required to fully elongate the primer-template for these three mutant polymerases, might be explained, at least partially, by their higher exonuclease activity. The 3'-5' exonuclease activity was therefore determined on both ss and dsDNA and the results are quantified in Table I. Indeed, the exonuclease activity on primer-template DNA was increased for mutant polymerases $I_{664}S$,

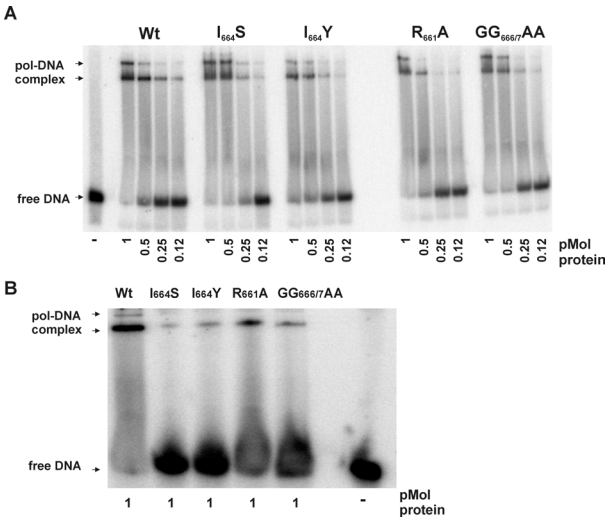

Figure 3. DNA binding on duplex DNA is affected for all mutant polymerases. The gel retardation assay was performed using 5'-labeled dsDNA, in the presence of the indicated amounts of wild-type or mutant Ad pol. Bands corresponding to free DNA and to polymerase-DNA complexes are indicated with an arrow. (A) Ad pol binding to primer-template DNA D20/T30. (B) Ad pol binding to duplex DNA D20/T20.

Table II.
Protein-primed activities of Ad wild-type and mutant polymerases

Polymerase	Initiation activity (%)[a]		pTP-pol[b] interaction	Km_{app}[c] (μM dCTP)
	TP-DNA	ssDNA		
Wild-type	100	100 (100)	+	8.3 ± 1.0
$I_{664}S$	16	14 (57)	+	22.6 ± 1.3
$I_{664}Y$	28	25 (63)	+	28.5 ± 0.8
$R_{661}A$	23	17 (54)	+	12.3 ± 2.7
$GG_{666/7}AA$	7	11 (36)	+	76.5 ± 17.6

The assays were performed as described in Experimental Procedures.
[a]Data taken from initiation reactions on T30 and TP-DNA (the natural viral template) , under standard conditions as described in Experimental Procedures. Numbers in parentheses indicate the % of activity in the presence of 10 μM unlabeled dCTP. Wild-type Ad pol initiation activity was set at 100%.
[b]pTP-pol interaction, as determined by glycerol gradient centrifugation.+, wild-type (100% ± 20%) pTP-Ad pol interaction.
[c]Numbers show Km_{app} values of pTP-deoxycytidylation with standard deviations (n=3 for each polymerase).

$I_{664}Y$ and $GG_{666/7}AA$ (Table I). Degradation of ssDNA by all mutant polymerases was slightly lower than that by the wild-type polymerase and proceeded in a distributive manner. An increased exonuclease activity on primer-template DNA might be the result of a lower DNA binding stability of the mutant polymerases in the polymerase active site.

DNA binding of the mutant DNA polymerases is affected on duplex DNA.
To examine the dsDNA binding capability of the different mutant DNA polymerases, gel retardation assays were performed with the primer-template molecule D20/T30 and duplex DNA molecule D20/T20. The formation of Ad pol/DNA complexes with the primer-template D20/T30 was similar for the wild-type and the mutant DNA polymerases (Fig. 3A). However, all mutant polymerases were affected in binding duplex DNA (Fig. 3B), although mutant polymerase $R_{661}A$ to a lesser extent than mutant polymerases $I_{664}S$, $I_{664}Y$ and $GG_{666/7}AA$. As can be seen in Fig. 3A and 3B, a second migrating band is visible for wild-type polymerase and on primer-template DNA for all mutant polymerases. A likely explanation for the presence of two migrating complexes is the existence of two forms of binding DNA by Ad pol (Ad pol monomeric and dimeric forms under conditions of low ionic strength (44)). These results are in agreement with the increased exonuclease activity found for three mutants on dsDNA (Table I) and indicate a decreased stability of the DNA in the polymerase active site.

Initiation activity is affected in all DNA polymerase mutants in the "(I/Y)XGG" motif.

Since Ad pol uses pTP as primer in a template-dependent fashion during initiation, the formation of pTP-C was studied using T30 as template. As shown in Table II, the initiation activities of all mutant polymerases were severely affected with values of 14 %, 25 %, 17 % and 11 % compared to wild-type Ad pol for mutant polymerases $I_{664}S$, $I_{664}Y$, $R_{661}A$ and $GG_{666/7}AA$, respectively.

When the mutant polymerases were tested for initiation using TP-DNA (the adenovirus genome with covalently linked TP to each 5' DNA end), similar differences could be observed (Table II). Initiation performed without template or with an oligonucleotide of unrelated sequence did not result in any activity (data not shown) in agreement with previous data showing origin dependency (3;45;46). When initiation was performed with a 200-fold higher dCTP concentration, the initiation activity of the mutant polymerases showed an increase from 11-25 % to 36-63 % of the wild-type level (Table II), suggesting a reduced affinity for the initiating nucleotide, dCMP. Therefore, the apparent Km (Km_{app}) for incorporation of dCTP was determined (Table II). Wild-type Ad pol showed a Km_{app} of 8.3 μM for the initiating nucleotide dCTP. Mutant polymerase $R_{661}A$ showed a Km_{app} value which was only slightly increased compared to wild-type Ad pol. On the other hand, the Km values of mutant polymerases $I_{664}S$ and $I_{664}Y$ were both 3-fold higher, while the Km_{app} of mutant polymerase $GG_{666/7}AA$ was shown to be 9-times higher than that of the wild-type polymerase (Table II).

The initiation impairment observed could be explained by an affected template strand binding of the mutated residues of the "(I/Y)XGG" motif. This could lead to an incorrect positioning of the templating nucleotide, explaining the increased Km_{app} shown for the initiating nucleotide. Another explanation for an increased Km_{app} for the initiating nucleotide, could be a defective pTP/pol interaction. To discriminate between these possibilities, glycerol gradient analysis was performed as described in Experimental Procedures to study the interaction of pTP and the Ad pol mutants. Interaction with pTP was found to be stable, whereby all mutant polymerases behaved essentially as the wild-type Ad pol (Table II). It is therefore likely that the increased Km_{app} observed for the mutant polymerases is a result of destabilization of the template DNA strand, indirectly affecting dNTP binding. These results support the role of the "(I/Y)XGG" motif as a motif involved in binding template DNA.

Mutant polymerase $GG_{666/7}AA$ is elongation defective after protein-primed initiation

Initiation on adenovirus DNA starts opposite the fourth nucleotide in the origin of replication with the formation of pTP-C, which extends to position 6, forming a pTP-CAT intermediate (3). The transition from initiation to elongation in adenovirus DNA replication is characterized by a jumping-back mechanism which recovers the terminal three nucleotides, resulting in DNA primer-template elongation and dissociation of pTP (3;4). To study elongation after protein-priming, truncated replication reactions were performed in the presence of 50 nM dCTP and additionally 40 μM dATP and 40 μM dTTP but without dGTP. Under the conditions chosen (low dCTP), only part of the pTP-CAT intermediate is elongated until position 26 (the first C-residue in the template), thus providing an internal control for elongation efficiency. The pTP-26 product will migrate as a band of 90 kDa in SDS-polyacrylamide gels and is clearly distinguishable from the pTP-CAT intermediate (Fig. 4A). Wild-type Ad pol shows both the pTP-CAT intermediate and pTP-26 formation with a 5-fold higher intensity for pTP-26 than for pTP-CAT. Taking into account the 5 C-residues in pTP-26, this indicates that equal amounts of pTP-CAT and pTP-26 were synthesized under these conditions resulting in an elongation to initiation ratio of 1. Whereas the absolute levels of DNA synthesis were lower for mutant polymerases $I_{664}Y$ and $R_{661}A$, the elongation to initiation ratios were similar to those of wild-type Ad pol. On the other hand, for mutant polymerase $I_{664}S$, a pTP-26 product was visible only after long exposure giving an elongation to initiation ratio of 0.01. For mutant polymerase $GG_{666/7}AA$, no pTP-26 formation could be detected. When elongation was performed with increasing concentrations of dCTP, elongation activity could be partially restored for mutant polymerase $I_{664}S$ increasing

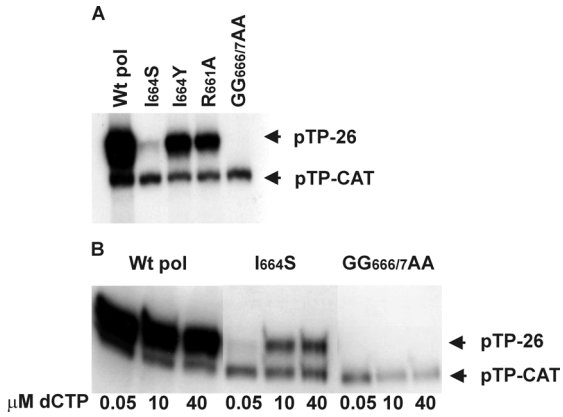

Figure 4. Protein-primed initiation and partial elongation on single stranded origin containing DNA. Activity was assayed using 600 ng of T30 (the template DNA strand of the Ad 5 origin), 50 nM [α-^{32}P]dCTP, 90 ng Ad pTP and 100 ng of either wild-type or mutant Ad pol. (A) Initiation followed by partial elongation. In this assay, 40 μM each of dATP and dTTP were added to allow elongation up to 26 nucleotides. The positions of the pTP-CAT intermediate and the pTP-26 product are indicated. (B) Initiation followed by partial elongation as a function of the dNTP concentration. Partial elongation was allowed in the presence of 50nM [α-^{32}P]dCTP, 40 μM each of dATP and dTTP and increasing amounts of unlabeled dCTP.

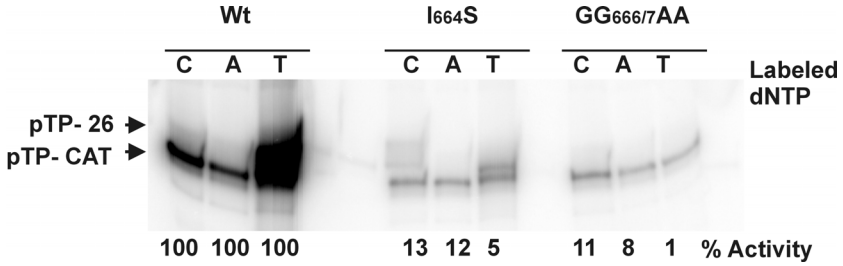

Figure 5. Mutant polymerase $GG_{666/7}AA$ is capable of pTP-CAT formation. Initiation was performed using 600 ng of T30, 90 ng Ad pTP and 100 ng of either wild-type or mutant Ad pol. Each reaction contained either 50 nM [α-^{32}P] dCTP marked as C, 200 μM dCTP and 50 nM [α-^{32}P] dATP marked as A, or 200 μM dCTP, 200 μM dATP and 50 nM [α-^{32}P] dTTP marked as T. The percentage (%) of initiation activity is based on the incorporation of each radiolabeled nucleotide relative to the wild-type polymerase and corrected for the elongation activity of mutant polymerases $I_{664}S$ and $GG_{666/7}AA$.

the elongation to initiation ratio to 0.2 (10 μM dCTP), while mutant polymerase $GG_{666/7}AA$ remained elongation-defective (Fig. 4B).

As mutant polymerase $GG_{666/7}AA$ showed no detectable elongation activity following initiation, we wondered whether initiation of mutant polymerase $GG_{666/7}AA$ resulted in the formation of pTP-CA and pTP-CAT intermediates. Therefore, initiation was assayed in the presence of labeled dATP or dTTP as shown in Fig. 5. To ascertain that the labeled nucleotide incorporated occupied the second or third position in pTP-CAT, respectively, 200 μM non-labeled dCTP (in the case of [α^{32}-P] dATP) or 200 μM of each dCTP and dATP (in the case of [α^{32}-P] dTTP) were included in the reaction. When any of the three [α^{32}-P] labeled dNTPs were supplied separately as the only nucleotide, only dCMP could be directly linked to pTP ((3) and data not shown). Mutant polymerase $GG_{666/7}AA$ could incorporate three nucleotides to form the pTP-CAT intermediate. This result shows that mutant polymerase $GG_{666/7}AA$ is capable of dNTP incorporation using both the OH-group of the serine (the priming amino acid of pTP) and the 3'OH of nucleotides for polymerization in agreement with the DNA-primed results (Fig. 2). However, DNA synthesis by mutant polymerase $GG_{666/7}AA$ stalls after pTP-CAT intermediate formation. Additionally, we observe a decrease in activity during each polymerization step from pTP-C to pTP-CAT resulting in abortive replication for mutant polymerase $GG_{666/7}AA$. These results suggest a defective translocation of the pTP-pol complex along the template DNA during the transition from initiation to elongation.

DISCUSSION

The "(I/Y)XGG" motif (defined as " YXGG/A" in ϕ29 DNA polymerase), highly conserved among eukaryotic-type DNA polymerases and located at the N-terminal site of the polymerase domain (38), was mutated in Ad pol to determine the function of these conserved residues in both DNA- and protein-primed reactions. Four mutant polymerases were obtained: $R_{661}A$, $I_{664}S$, $I_{664}Y$ and $GG_{666/7}AA$.

The "(I/Y)XGG" motif in Ad pol stabilizes the template strand in the polymerase active site

Mutational analysis in the "(I/Y)XGG" motif of ϕ29 DNA polymerase, revealed an important role of this motif in the binding stability of the template strand at the polymerase active site (38). Different mutations in the same residue of this motif resulted in a pol/exo balance shifted either towards polymerization or towards exonucleolysis, depending on the stabilization of the template strand at a particular active site. During DNA-primed replication three of the four mutant Ad pols described here showed a pol/exo balance shifted towards exonucleolysis (low pol/exo balance). Mutant polymerases $I_{664}S$, $I_{664}Y$ and $GG_{666/7}AA$ were shown to have a 2-3-fold increased exonuclease activity on dsDNA, while their activity on ssDNA was wild-type like. The increased exonuclease activity of these three mutant polymerases explains, at least partially, the higher dNTP concentrations required for effective polymerization on the same primer-template and indicates that DNA binding in the polymerase active site is affected. Indeed, when binding of duplex DNA was tested, the mutant polymerases were clearly affected in dsDNA binding in comparison to the wild-type Ad pol, indicating that a local destabilization of the template DNA at the polymerization active site exists in these mutant polymerases. However, no defective primer-template binding could be seen for any of the mutant polymerases. A possible explanation for this difference is that the mutant polymerases destabilize primer-template DNA only locally. Duplex DNA at the polymerase active site is held in position by numerous interactions of the fingers, palm and thumb as has been shown in the crystal structure of *Taq* polymerase complexed with duplex DNA (25). However, in the presence of a 5'-template overhang, additional DNA contacts with the fingers are possible as shown in the crystal structure of bacteriophage T7 complexed with a primer-template (47). These additional contacts could further stabilize the primer-template at the polymerase active site, therefore affecting the DNA binding detectably only on duplex DNA. In the case of the ϕ29 DNA polymerase mutants, defective retardation of dsDNA indicated a defective stabilization of the template strand at the polymerization active site and resulted in a low pol/exo balance (38). For mutant polymerase $GG_{666/7}AA$ the local destabilization of the primer-template at the polymerization active site might have an additional indirect effect on the positioning of the template strand. This could result in a template base which is incorrectly positioned for base-pairing with the incoming nucleotide and thereby affecting the affinity of this mutant polymerase for nucleotides during polymerization (200-fold higher nucleotide requirement).

The possible role of the "(I/Y)XGG" motif as a DNA binding motif is in agreement with the proposed localization of its residues (39) in the crystal structure of RB69 DNA polymerase modeled with DNA. The structure of RB69 DNA polymerase, one of the three DNA polymerases belonging to the α-like DNA polymerases (family B) of which the crystal structure is known (24);(30;31) can serve as a prototype for eukaryotic-type DNA polymerases. The RB69 polymerase active site modeled with a primer-template and a dNTP (crystallographic data from PBD ID: 1WAH, (39)), shows that the tyrosine of the "(I/Y)XGG" motif (Y_{391}, corresponding to I_{664} in Ad pol) interacts directly with the phosphate between the two nucleotides preceding the one acting as the template. Also G_{393} and A_{394} are positioned close to the template strand. The motif is located more than 12Å away from the dNTP binding site indicating that a direct role of the "(I/Y)XGG" motif in dNTP binding highly unlikely.

Decreased template binding affects pTP-primed initiation

All four mutants described here were affected in pTP-primed initiation. The affected initiation activity and increase in the Km_{app} for the

initiating nucleotide are likely a consequence of the local destabilization of the template strand at the polymerization active site, resulting indirectly in the incorrect positioning of the template strand for base-pairing with the incoming nucleotide. This is in agreement with the results of the DNA binding experiments (Fig. 3) and with the position of the "(I/Y)XGG" motif in the RB69 structure, modeled with a primer-template and incoming dNTP (crystallographic data from PBD ID: 1WAH, (39)). A decrease in initiation activity for the "(I/Y)XGG" mutants of ϕ29 DNA polymerase was also observed but was explained by an impaired interaction between TP and ϕ29 pol (39). Since in ϕ29 DNA polymerase protein-primed initiation is templated by the second nucleotide of the TP-DNA, the "(I/Y)XGG" motif could be located at the position of the TP during this reaction. Such an interaction defect is less likely for Ad pol during initiation since it occurs opposite the fourth nucleotide, a situation where the motif is proposed to be in direct contact with the template strand (crystallographic data from PBD ID: 1WAH, (39)) and located rather distant from the protein-primer. Indeed, we did not observe interaction differences between the wild-type and mutant polymerases and pTP as determined by glycerol gradient centrifugation. However, minor differences in interaction with pTP may not be detected in this assay. The increase in the Km_{app} for dCTP did not completely explain the decrease of the initiation activity in the case of mutant polymerase $R_{661}A$. One possible explanation is that the interaction between pTP and Ad pol for mutant polymerase $R_{661}A$ was not fully functional, as has been shown to be the case for some ϕ29 DNA polymerase mutants (48).

The "(I/Y)XGG" motif is involved in the transition from initiation to elongation

Whereas the phenotypes of mutant DNA polymerases $I_{664}S$ and $I_{664}Y$ were mostly comparable, they clearly differed in their ability to elongate after protein-priming. The elongation to initiation ratio of mutant polymerase $I_{664}Y$ was wild-type like, while mutant polymerase $I_{664}S$ showed a reduced elongation activity and was unable to restore the wild-type elongation to initiation ratio even at high dNTP concentrations. Mutant polymerase $GG_{666/7}AA$ had no detectable elongation activity even at high dNTP concentrations. However, both mutant polymerases, $GG_{666/7}AA$ and $I_{664}S$, had been shown to be able to elongate a primer-template (Fig. 2) and were capable of pTP-CAT formation (Fig. 5). These results show that both mutant polymerases can use protein and DNA as a primer and therefore the elongation defect of mutant polymerase $GG_{666/7}AA$ must lie in the transition from initiation to elongation. A decrease in the activity of mutant polymerases $I_{664}S$ and $GG_{666/7}AA$ during each additional polymerization step following initiation (C-CA-CAT) was observed, indicating a defective translocation of the pTP-pol complex along the template DNA. In the case of mutant polymerase $GG_{666/7}AA$ this defective translocation led to abortive replication after the formation of the initiation intermediate pTP-CAT. The role of the "(I/Y)XGG" motif in stabilization of the template strand at the polymerase active site probably leads during initiation of protein-primed replication to a defect in the translocation of the template strand before, during and after the jumping-back mechanism. A transition defect from initiation to elongation in TP-DNA has been described for three ϕ29 mutant polymerases studied at the "(I/Y)XGG" motif (39). One of these mutated residues was G_{228} corresponding to G_{666} in Ad pol, which was changed into Ala. Their interaction defect with TP and/or DNA was proposed to cause a premature dissociation of TP, DNA polymerase and DNA, resulting in the transition defect of the mutant polymerases. These mutant polymerases were not able to repolymerize short DNA fragments. Although a pTP-pol interaction defect was not found for the Ad pol mutants described here, they might not be able to efficiently bind the pTP-CAT intermediate. This would result in abortive replication. Glycine residues are often found in loops, as is G_{393}, the first glycine of the "(I/Y)XGG" motif of RB69 polymerase. Thus, the glycine pair in Ad pol most likely functions as a structural element. The conservation of the glycine pair among many α-like DNA polymerases suggests that it may play a critical role in creating the optimal environment for accommodation of the template strand. Recently, a study of Ad pol was carried out with crude lysates of 22 site-directed mutations to identify conserved residues involved in Ad pol

function (37). In this study, mutant polymerases $G_{667}D$ and $GG_{666/7}AA$ from the "(I/Y)XGG" motif were tested for initiation activity, DNA binding, pTP-pol interaction and polymerization activity on calf thymus DNA. Both mutant polymerases were mainly affected in initiation and dsDNA binding activity and were shown to interact with pTP, in agreement with our observations. In contrast, the initiation activity of mutant polymerase $GG_{666/7}AA$ was found to be much higher (50-75%) than in our study (11%). A likely explanation for this apparent discrepancy is that their assay conditions included nuclear extract that could contain cellular factors that might stimulate initiation. In addition, the presence of Mn^{2+} instead of Mg^{2+} used in the present study, strongly reduces the specific nucleotide selection (3) which may lead to abberant initiation products.

A hydrophobic contact which positions the template strand

In DNA polymerases of different Ad serotypes, the tyrosine in the "(I/Y)XGG" motif is an isoleucine. In contrast to tyrosine, isoleucine cannot hydrogen bond with the phosphate between the two nucleotides preceding the one acting as a template as described for ϕ29 pol (39). When the motif is aligned with the amino acid sequences of eukaryotic DNA-dependent DNA polymerases, the tyrosine is a phenylalanine in several bacterial and viral polymerases and an isoleucine in several TP-primed DNA polymerases, like Ad pol (38). This suggests that a hydrophobic residue at this position is important and might interact with the template DNA in the polymerase active site. Indeed, our results show an affected elongation for mutant polymerases $I_{664}S$, whereas mutant polymerase $I_{664}Y$ has a wild-type like elongation to initiation ratio (Fig. 4A). Moreover, changing the tyrosine of the "(I/Y)XGG" motif of ϕ29 pol into serine ($Y_{226}S$) to keep the hydroxyl group resulted in a drastic phenotype with null polymerization and no dsDNA binding (38). Substitution into phenylalanine ($Y_{226}F$) however, retained polymerization and dsDNA binding (38). We therefore propose that the isoleucine in the "(I/Y)XGG" motif of Ad pol interacts directly with the ribose moiety of the 3' nucleotide preceding the templating nucleotide. This interaction might be important in the case of a tyrosine as well. Such a model is consistent with our results, but also explains why the "(I/Y)XGG" motif in ϕ29 pol controls the pol/exo balance immediately after the formation of the initiation product TP-dAMP (39). The TP-primed initiation of ϕ29 pol starts opposite the second nucleotide of the template and uses a sliding-back mechanism to recover the terminal nucleotide (49). If the hydrogen bond between Y_{226} (the equivalent of I_{664} in Ad pol) and the phosphate between the two nucleotides preceding the template base would be important, the pol/exo balance could only start at the formation of TP-$(dAMP)_3$. However, the pol/exo balance (which is due to the role of the motif for stably binding DNA at the polymerase active site (38)), starts at TP-$(dAMP)_2$ (39) when the hydrogen bond does not exist. This result is readily explained when the template is positioned correctly by a hydrophobic interaction with the nucleotide next to the template base. A similar hydrophobic interaction has been shown to be involved in discriminating between deoxy-versus ribonucleotides in T7 DNA polymerase (47). Y_{526} together with E_{480} wedge the ribose moiety of the incoming nucleotide, thereby forming a hydrophobic pocket at the C2' position of the ribose that could exclude ribonucleotides from the polymerase active site (47).

Summarizing, our results support the conclusion drawn from the mutational analysis of ϕ29 DNA polymerase that the "(I/Y)XGG" motif is involved in the coordination of synthesis and degradation due to its importance for the binding stability of the template DNA at the polymerization active site. Its role in DNA binding makes it additionally important for the transition steps from initiation to elongation. In addition, we propose that I_{664} directly interacts with the ribose moiety of the nucleotide next to the template nucleotide, which holds the template into position for correct initiation, jumping-back and subsequent elongation.

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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,

in press). Late in infection, pTP is processed by a virus-encoded protease into the mature TP (30).

Ad pol is a 140 kDa protein that belongs to the Pol α family of DNA-dependent DNA polymerases based on amino acid sequence comparison (15,34,36). Within this family it is part of the subclass of protein-priming DNA polymerases (15). Mutational analysis of the polymerase domain has shown that, like other Pol α-like DNA polymerases, Ad pol is functionally conserved with the polymerase activity located at the C-terminus (4,5,6,17,22,26,27). Biochemical analysis of Ad pol has shown that it replicates DNA in a processive manner but that it has a distributive 3'-5' exonuclease activity on ssDNA, although removal of a mismatched nucleotide and subsequent switching to polymerization proceeds processively (18). Both polymerase and exonuclease activities are decreased when pTP is complexed with Ad pol and dissociation likely increases processivity (18,19). The lack of structural data for the pTP-pol complex or of any other protein-priming DNA polymerase has hampered the detailed characterization of Ad pol and its binding to pTP and DNA.

Here, we have further examined the interaction of the polymerase with pTP and DNA while it is in the polymerase or the exonuclease mode. By using a biotin-streptavidin translocation block developed by the Benkovic group (8,12), we demonstrate that the exonuclease and polymerase active sites are spatially distinct and when bound to DNA, Ad pol covers a region of 14 to 15 nucleotides. Moreover, an exonuclease-deficient mutant was constructed by mutating a conserved residue located at the proposed exonuclease domain of Ad pol. Combined with mutational studies (22,26,27), these results suggest a similar molecular architecture for Ad pol to RB69 DNA polymerase, a model enzyme for the family B polymerases (11). Furthermore, we demonstrate that pTP binds at the primer binding groove of Ad pol. The decreased exonuclease and polymerase activity in the presence of pTP is therefore most likely the result of competition between pTP and the DNA, located at the primer binding groove. Based on these results, a model is proposed for binding of the pTP-pol complex to the origin.

EXPERIMENTAL PROCEDURES

DNA templates, nucleotides and substrates

All oligonucleotides, unlabeled nucleotides, [α-^{32}P] dNTPs (3000 Ci/mmol), and [γ-^{32}P]ATP (5000Ci/mmol) were purchased from Amersham Pharmacia Biotech. Streptavidin was purchased from USB. T30 (5'-AATCCAAAATAAGGTATATTATTGATGATG-3') represents the first 30 nucleotides of the template strand of the adenovirus 5 genome and D20 (5'-CATCATCAATAATATACCTT-3') is the complementary (displaced) strand of T30. Three oligonucleotides were used with an incorporated biotin molecule: Tbio5'(5'-bioATCCAAAATAAGGTATATTATTGATGATG-3'), which is identical to T30 except for the 5'dATP being replaced with a biotin group, D7bio (5'-CATCATbioCAATAATATACCT) which is identical to D20, only with an incorporated biotin group at position 7 and lacking the 3'-terminal nucleotide, and D7bio10 (5'-CATCATbioCAATAATATACCTTATTTTGGAT), 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-^{32}P]ATP. The hybrid molecules D20/T30, D20/Tbio5', and D7bio/T30 were obtained by boiling oligonucleotides in 60 mM Tris-HCl, 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**C**CGAGATCGTGCTC-3' and 5'-GAGCACGATCTCG**G**CAAAGCCGGTGAT-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

Exonucleolytic 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-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 1 mg/ml BSA, 10 mM $MgCl_2$ 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. Exonucleolytic activity was detected as a decrease in size of the 5'-labeled D7bio primer.

Determination of the distance between the polymerase active site and the entrance of the template binding groove
Partial duplex D20/Tbio5' is a primer-template structure with a 9 nucleotide template 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 $MgCl_2$, 1 mM dNTP's, 0.05 ng of 5'labeled-D20/Tbio5'. 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 were used to a final concentration of 28.5nM 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 28.5nM were used in the presence of increasing amounts of dNTPs as indicated. Incubation was at 37 °C for 10 min.

Determination of the distance between the polymerase active site and the entrance of the primer binding groove
5'-labeled D7bio was partially degraded by 1µg Ad pol in a reaction volume of 25 µl in the presence of 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 1 mg/ml BSA and 10 mM $MgCl_2$ in the absence of nucleotides. After incubation at 37 °C for 2 min., the degraded products were boiled in order to inactivate Ad pol and hybridized to T30 in presence of 60 mM Tris-HCl, pH 7.5 and 200 mM NaCl, followed by slow cooling to room temperature. The resulting primer/templates with various primer lengths were used in the polymerization assay and were incubated for 5 min., with or without 7 nM streptavidin. 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 $MgCl_2$, 1 mM dNTP's and 0.05 ng primer/template mix. The reaction was started by the addition of mutant polymerase D422A to a final concentration of 28.5 nM and incubated at 30 °C for the indicated times. Reactions were stopped at the indicated times by the addition of formamide loading buffer. Samples were analyzed on 8 M urea-20% polyacrylamide gel electrophoresis followed by autoradiography or analysis by phosphorimager.

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^{-15}M) (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

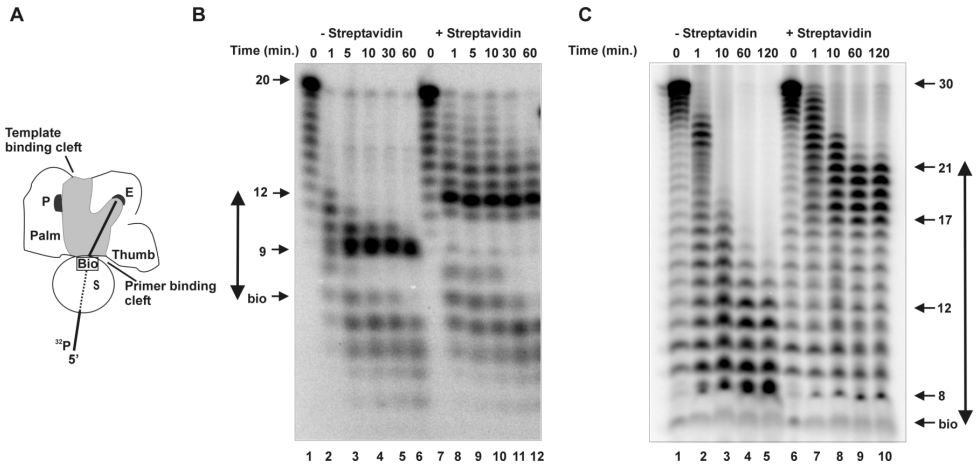

Figure 1. Distance between exonuclease active site and entrance of the primer binding groove. A. Schematic representation of Ad pol based on the crystal structure of the replicating RB69 DNA polymerase complex (11). The lower part of the polymerase shows the palm domain containing the polymerase active center (P) and the thumb. The upper part of the polymerase shows the exonuclease domain with its active center (E). The N-terminal domain and the fingers have been left out for clarity. The grey area indicates the grooves where DNA can bind. The entrances of the primer and template binding grooves are depicted. 5'-labeled D7bio is schematically presented as a solid line, or dashed when it is covered by streptavidin (S). The biotin group (Bio) is depicted as a box. B. For Ad pol, exonucleolytic degradation was studied on 5'-labeled 20-mer D7bio, that contains a biotin group at position 7. Degradation was studied for the indicated times in the absence (-, lanes 1-6) or presence (+, lanes 7-12) of streptavidin. Arrows indicate accumulated degradation products and the position of the biotin group. The measured distance is presented as a double-headed arrow. C. For the pTP-pol complex, exonucleolytic degradation of the 5'-labeled 30-mer D7bio10 was studied in the absence (-, lanes 1-5) or presence (+, lanes 6-10) of streptavidin for the indicated times. Arrows indicate accumulated degradation products and the position of the biotin group. The measured distance is presented as a double-headed arrow.

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-

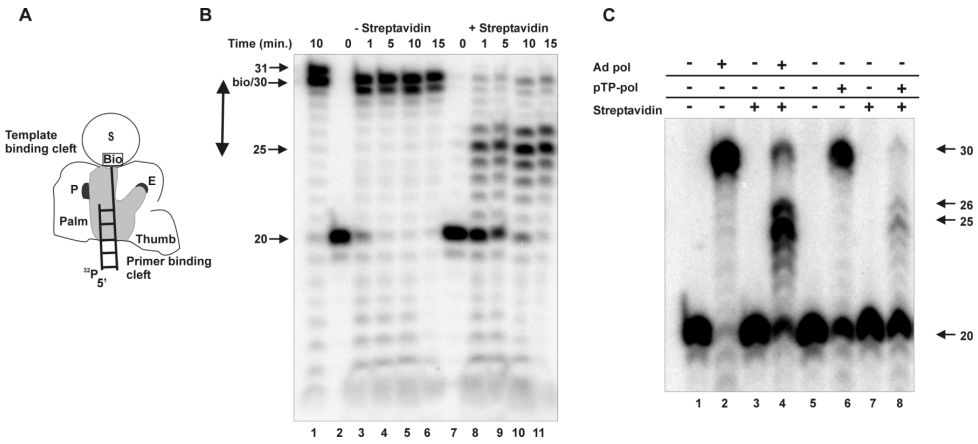

Figure 2. Distance between polymerase active site and entrance of the template binding groove. A. Schematic representation of the experiment, legend as in Fig. 1A. B. Elongation of Ad pol was studied on primer/template D20/Tbio5' in the absence (-, lanes 2-6) or presence (+, lanes 7-11) of streptavidin 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;25)), 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, wild-type Ad pol could both polymerize and degrade the primer-template (Fig. 3B). The polymerase activity of mutant polymerase D422A was only mildly affected (Fig. 3B) but as expected, no degradation was observed, explaining at least in part the lower elongation activity. At higher nucleotide

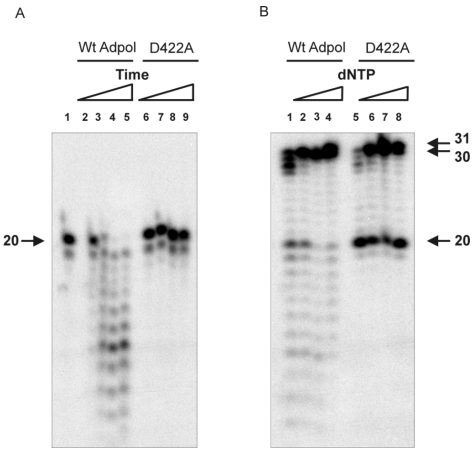

Figure 3. Characterization of the exonuclease deficient mutant polymerase D422A. Mutant polymerase D422A was tested for exonuclease activity on 5'-labeled D20 and for polymerase activity on primer/template D20/T30 as decribed in Experimental procedures. A. Exonucleolytic degradation was studied on D20 for wild-type (Wt) Ad pol and mutant polymerase D422A. The incubation times were as follows: 1 min. (lanes 2,6), 5 min. (lanes 3,7), 10 min. (lanes 4,8) and 15 min. (lanes 5,9). B. To study elongation of Wt Ad pol and mutant polymerase D422A, a DNA polymerase/exonuclease coupled assay on D20/T30 was performed in the presence of increasing concentrations of dNTPs: 25nM (lanes 1,5), 125nM (lanes 2,6), 625nM (lanes3,7) and 2500nM (lanes 4,8). Incubation was at 37 °C for 10 min.

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

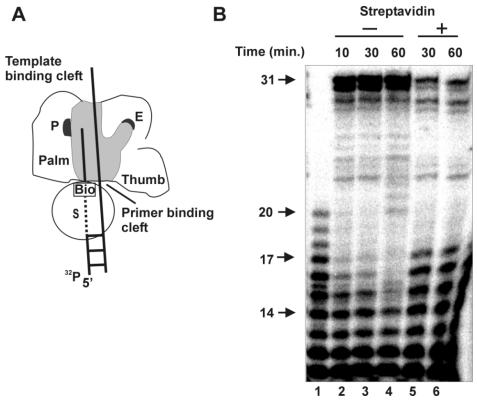

Figure 4. Distance between the polymerase active site and the entrance of the primer binding groove. A. Schematic representation of the experiment, legend as in Fig. 1A. B. Partially degraded D20 was hybridized to template T30. The resulting primer/template mix (lane 1) was elongated at 30°C for the 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 that 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 9-10 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 dicussed 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

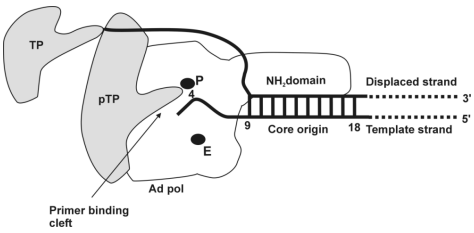

Figure 5. Model for origin binding of the pTP-pol complex. Model for origin binding of the pTP-pol complex preceding replication initiation. The Ad origin DNA (thick lines) containing the core origin (nucleotides 9-18) are bound by Ad pol. pTP is complexed to Ad pol with its priming part depicted at 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 NH_2-domain represents the putative N-terminal domain of Ad pol that could bind to the core origin. See text for more details.

contact with the displaced parental TP-containing DNA strand. Furthermore, the location of pTP at the entrance of the primer binding groove of Ad pol, positions it close to the parental TP. Parental TP has been shown to be involved in stabilizing the binding of the pTP-pol complex at the origin, possibly via a direct interaction with pTP (25) (R.N. de Jong, manuscript in preparation). A direct interaction between parental TP and pTP (p2 in ϕ29) has been described for ϕ29 (14,28).

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 NH_2 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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Termination of Adenovirus DNA Replication implicates a Role for the Terminal Protein

Manuscript in preparation

Termination of Adenovirus DNA Replication implicates a role for the Terminal Protein

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Adenoviruses contain linear genomes of approximately 36 kB with a terminal protein (TP) covalently attached to each 5'-end. DNA replication initiation and elongation on this genome have been well characterized, but the mechanism by which adenovirus (Ad) DNA replication terminates is unknown at present. Here we describe the development of an assay to study the role of TP during termination. We compared replication on templates containing the natural TP with templates containing streptavidin at the same terminal position. In case of streptavidin, Ad pol arrested synthesis 5 nucleotides before reaching the terminal template base suggesting that the protein acts as a steric barrier. With TP-DNA, in contrast, Ad pol replicated to the very end of the template. These results suggest that TP is not simply a barrier for Ad DNA polymerase but that there is a specific interaction between Ad pol and TP, required to complete replication of the Ad genome.

INTRODUCTION

DNA replication forks of many prokaryotic and some eukaryotic replicons are terminated at specific sequences, called replication termini (reviewed in (2)). These replication termini (Ter) are bound by a replication terminator protein (RTP) that arrests replication either in a polar or bipolar fashion (2,15). Replication termination has been studied in detail in for example *E. coli*. The *E. coli* chromosome has 10 ter sites that are located diametrically opposite the replication origin. These sites are clustered in two groups of five that have opposite polarity. The polarity is such that a replication fork coming from one side can pass through the ter sites but is trapped when it comes from the other side. A monomeric Tus protein, the RTP in *E. coli*, binds at each ter site in the genome (9). The crystal structure of the Tus-ter complex has been solved at 2.7 Å (10). Mutational analysis has demonstrated that the mechanism of termination of DNA replication involves contrahelicase activity of the Tus protein with the *E. coli* DnaB helicase. Recently, a direct specific interaction between a loop in the Tus protein and DnaB was demonstrated (17), indicating that termination in *E. coli* is not simply a matter of a steric barrier. Although the termination sites and RTPs are very different in Bacillus subtilis, a similar mechanism of replication termination is used (14).

The mechanism of adenovirus DNA replication in serotypes 2/5 has been well characterized at the level of initiation and elongation (reviewed in (23)). Termination of DNA replication however, has not been studied thus far. Three viral poteins are involved in adenovirus DNA replication: these are the adenovirus DNA polymerase (Ad pol), the precursor terminal protein (pTP), that forms a heterodimer with Ad pol and the DNA binding protein DBP. In addition, transcription factors NFI and Oct-1 stimulate initiation by recruitment of the pTP-pol complex to the origin and topoisomerase I is required for replication of the entire genome. Replication initiates at both origins, located at each end of the linear genome within the inverted terminal repeats. Adenovirus DNA replication uses a protein-priming mechanism to initiate replication. This involves the covalent coupling by Ad pol of the initiating nucleotide to a serine residue of pTP. After initiation at an internal start site, a pTP-trinucleotide intermediate is formed that is relocated at the genome terminus by a so called jumping-back mechanism, whereafter Ad pol starts replication. Elongation of the DNA is via a strand-displacement mechanism for which DBP is essential. At the end of the genome, Ad pol

encounters a covalently linked TP molecule, the processed form of pTP and terminates by an unknown mechanism. After the first round of replication, pTP-DNA is formed which is a perfect template for further rounds of replication. During the viral life cycle pTP is processed to mature TP.

As TP is encountered by the replication machinery at the end of the genome, it may also be involved in the termination of adenovirus replication. Two alternative models can be envisaged for the role of TP for termination of Ad replication. In the first model, termed "blocking" model, the covalently bound TP poses as a non-specific barrier. Ad pol, in the presence of DBP, replicates the entire genome until, after synthesis of the last base, it encounters TP and probably dissociates from the genome. In the second model, termed "interaction" model Ad pol terminates by a specific interaction between Ad pol and TP. In a previous study we have shown that the distance between the polymerase active site and the enzyme surface of Ad pol where the template strand enters is 5 nucleotides (Brenkman et al., in press). This suggests that possibly not all terminal nucleotides can be replicated when TP is encountered, unless TP has access to the template binding groove of Ad pol. However, when templates, lacking up to two terminal nucleotides were used to initiate and subsequently elongate, full length DNA products were synthesized (11). Therefore, synthesis of all nucleotides is not required for the replication of the genome since the jumping back mechanism restores the terminus at a new round of replication.

Here we provide evidence for the "interaction" model. We developed a termination assay, using purified TP-DNA and show that all terminal nucleotides are synthesized without any intermediates. However, when primer-template DNA was replicated by Ad pol with streptavidin bound as a terminal protein, all but 5 terminal nucleotides were replicated. Therefore, these results suggest that i) the jumping-back mechanism is probably not required in restoring the genome termini that resulted from termination, in a new round of replication and ii) the priming part of TP enters the template binding groove to allow synthesis of all terminal nucleotides possibly via a specific interaction. These findings allow us to propose a mechanism for termination of Ad replication.

EXPERIMENTAL PROCEDURES

DNA templates, nucleotides and substrates

All oligonucleotides, unlabeled nucleotides, [α-^{32}P] dNTPs (3000 Ci/mmol), and [γ-^{32}P]ATP (5000Ci/mmol) were purchased from Amersham Pharmacia Biotech. Streptavidin was purchased from USB. Tbio5'(5'-bioATCCAAAATAAG GTATATTATTGATGATG-3') represents the first 29 nucleotides of the template strand of the adenovirus 5 genome and includes a terminal biotin linker, T30-50 (5'-CTCATTATCATATTGGCTTCA -3') represents nucleotides 30 to 50 of the template strand and D20 (5'-CATCATCAATAATATACCTT-3') is the complementary (displaced) strand of T5'bio. Labeling of the oligonucleotides was performed with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-^{32}P]ATP. The hybrid molecule D20/Tbio5' was obtained by boiling oligonucleotides in 60 mM Tris-HCl, pH 7.5, 200 mM NaCl, followed by slow cooling to room temperature. All oligonucleotides used were purified by 10% polyacrylamide-1xTBE gel electrophoresis. Ad 5 TP-DNA was isolated as described (3).

Proteins and buffers

Wild type Ad pol was expressed from a baculovirus expression system and purified to near homogeneity as previously described (1). ΔN-DNA binding protein and NFI were purified as described (5,6). pTP was a gift from R.N. de Jong. 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.

Replication assay

DNA replication assays were performed on TP-DNA treated with Xho I in a volume of 25 µl in a buffer containing 25 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 500 nM [α-^{32}P]dCTP, 40 µM dATP, dTTP and dGTP, 15 ng Ad pol, 9 ng pTP and 1 µg DBP in the absence or presence of indicated amounts of NFI. After incubation for 45 min. at 37°C, reactions were stopped by addition of 2.5 µl stop mix (40% sucrose, 1% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol). Reaction products were separated on a 1% agarose gel using 0.5x TBE, 0.1% SDS as running buffer. Gels were partly dried and quantified using a PhosphorImager (Molecular Dynamics).

Termination Assay

1 µg of TP-DNA was digested for 3 hrs. with the restriction enzyme Ssp I (Pharmacia) to generate TP-containing DNA fragments of 339 and 1303 bps, respectively. After digestion, 10 units of λ-exonuclease (USB) were added to the reaction mixture in presence of λ-exonuclease buffer (2.5 mM $MgCl_2$,50 µg BSA, 6.7 mM HEPES, pH 7.5) for 1 hr. at 37°C in order to digest the template strand of both TP-DNA molecules. λ-exonuclease degrades the 5'-phosphorylated strand of dsDNA but cannot digest the TP-containing displaced strand since this strand is protected by the TP molecule. 2 µl of the resulting single-stranded TP-DNA (ssTP-DNA) was hybridized to a 5-fold molar excess of non-phosphorylated oligonucleotide T30-50 by a 5 min. incubation at 37°C. For the termination assay, 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 $MgCl_2$, 0.1

mM dNTP's (dCTP, dGTP, dTTP), 10 μCi α-^{32}P dATP, 28.5 nM Ad pol and the hybrid ssTP-DNA/T30-50. After incubation at 37°C for 10 min., the reactions were stopped by adding formamide loading buffer (98% formamide, 0.5 M EDTA, pH 8.0, 0.025% bromophenol blue, 0.025% Xylene Cyanol). Samples were analyzed on 8 M urea-20% polyacrylamide gel electrophoresis followed by autoradiography or analysis by phoshorimaging.

RESULTS

Replication on TP-DNA

To study replication termination on viral DNA, TP-DNA was purified as decribed (3) and the quality was tested in an *in vitro* replication assay in the presence of Ad pol, pTP and DBP (Figure 1, lane 1). Digestion with the restriction enzyme XhoI and replication resulted in the specific labeling of the two B/C fragments that contain the origin. In addition, two ssDNA products resulting from second rounds of replication that involves strand displacement can be observed (arrows), indicating very efficient replication. At low concentrations of the pTP-pol complex, NFI can stimulate Ad replication initiation (16,18). To test this, increasing amounts of NFI were added to the reaction mixtures resulting in a stimulated replication (Figure 1, lanes 2-3). These results indicate that TP-DNA was purified without denaturation of TP and thus can be used for the termination assay.

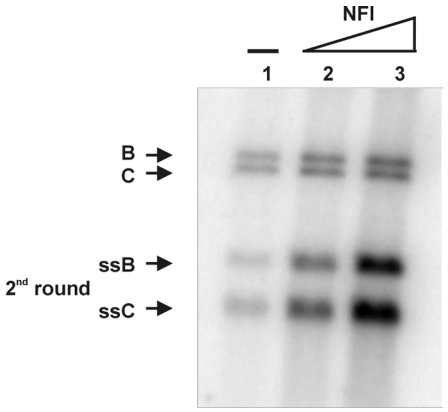

Figure 1. Ad DNA replication on TP-DNA. Ad replication was performed on purified TP-DNA, digested bythe restriction enzyme Xho I. An increasing amount of NFI was added to the reaction mixture in lanes 2 (20ng) and 3 (40ng). The origin-containing fragments from the first and second round of replication are indicated by arrows (B/C).

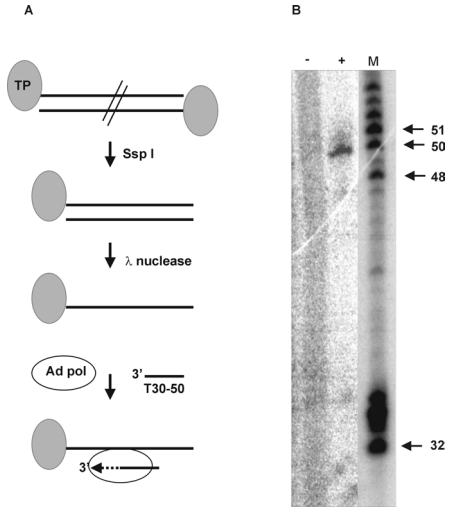

Figure 2. Termination of Ad DNA replication (A) Schematic overview of the termination assay. (B) The termination assay was performed as described in Experimental Procedures. TP-DNA was digested with restriction enzyme Ssp I and treated with with λ-exonuclease to create ssTP-DNA. Subsequently, oligonucleotide T30-50 was hybridized to ssTP-DNA and incubated with dNTPs in the absence (lane 1) or presence (lane 2) of Ad pol for 10 min. at 37°C. The sizes of a DNA marker (M) in nucleotides are indicated by arrows (in nucleotides). Exposure of the marker lane was 5 times shorter than for lanes 1-2 to avoid overexposure of the gel.

Ad pol replicates all terminal bases on TP-DNA

An initial step to study the role of TP in termination of Ad replication is to determine if all terminal nucleotides can be replicated by Ad pol. For this, a termination assay was designed by using the purified TP-DNA. A scheme of the termination experiment is illustrated in figure 2A. Full length TP-DNA was digested with restriction enzyme Ssp I, resulting in two small TP containing DNA fragments of 339 and 1303 bp. The resulting mix of DNA and TP-fragments were then degraded with λ exonuclease. The TP-containing DNA fragments are protected from degradation as λ exonuclease specifically degrades phosphorylated, double-stranded DNA in the 5'-3' direction. A non-phosphorylated oligonucleotide T30-50, complementary to nucleotides 30-50 from the TP-containing strand, was then hybridized to the remaining

ssTP-DNA and elongation was studied upon addition of dTTP, dGTP, dCTP, radiolabeled dATP and Ad pol.
Replication by Ad pol of T30-50 should lead to products of 50 nucleotides (all terminal nucleotides synthesized) or shorter products. As can be observed in figure 2B, T30-50 was elongated to a single band of 50 nucleotides in the presence of Ad pol (lane 2 versus lane 1). The observed result indicates that Ad pol replicates all terminal nucleotides. The efficiency of this reaction is low, possibly due to the inability in this assay to remove Ssp I and λ-exonuclease as TP would otherwise denature. Furthermore, residual viral DNA is likely present that could inhibit the reaction and hybridization of T30-50 had to be carried out at 37°C to avoid denaturation of TP.

Termination of Ad DNA replication is specific for TP-DNA

The previous experiment does not distinguish between the proposed "blocking" and "interaction" model for the role of TP in termination of Ad DNA replication. Therefore, termination of Ad replication with a non-related protein at the terminus was studied. A primer (D20)-template (Tbio5') was used with streptavidin, instead of TP bound at the terminus of the template strand (Figure 3A). 5'-labeled oligonucleotide D20 (20-mer) was hybridized to Tbio5', an oligonucleotide complementary to D20 with a 5'overhang of 9 nucleotides and a terminal biotin molecule. Streptavidin is a tetrameric protein (4x 15 kDa) with a similar molecular mass as TP and binds with very high affinity to biotin (Kd ≅ 10^{-15} M). In the absence of streptavidin, Ad pol could replicate all nucleotides (Figure 3B, lane 2). Under the conditions used, a non-templated nucleotide is added opposite the biotin molecule, leading to a 30-mer product (Brenkman et al., in press). However, when streptavidin was added together with Ad pol and dNTP's, replication resulted in a main accumulation product of 25 nucleotides (Figure 3B, lane 4). This result is in contrast to termination on TP that resulted in replication of all nucleotides. This finding therefore suggests that replication by Ad pol on DNA with a protein coupled to its 5'-terminus is specific for TP. The result obtained is in agreement with our previous findings that the distance between the

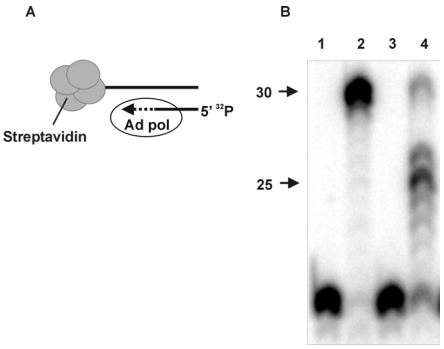

Figure 3. Termination of DNA replication on terminal streptavidin protein. (A) Cartoon of the experiment. (B) 5'-Labeled D20 was hybridized to Tbio5' and replication was studied in a mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 1 mg/ml BSA, 10 mM $MgCl_2$, 0.1 mM of each dNTP's and 28.5 nM Ad pol in the absence (lane 2) of presence (lane 4) of streptavidin (7 nM) for 10 min. at 37°C. The starting material is shown in lanes 1, 3 and replication products are indicated by arrows.

polymerase active site and the surface of the polymerase where the template strand enters is 5 nucleotides (Brenkman et al., in press) and suggests that part of TP needs to interact at the polymerase active site for replication of all terminal nucleotides. The role of TP in termination of Ad DNA replication is therefore in accordance with the proposed "interaction" model.

DISCUSSION

Termination of Ad replication invokes the encounter with TP, covalently attached to the terminus of the linear genome, by the replication machinery. Here we show that TP does not form a simple roadblock that marks the end of the genome ("blocking" model), but that termination of Ad replication specifically requires TP, possibly via an interaction with Ad pol ("interaction" model). Our findings show similarities with the mechanism by which several bacteria and plasmids terminate their DNA replication. The mechanism of termination of DNA replication e.g. in both *E. coli* and *B. subtilis* involves a specific interaction between the replication machinery and the RTP (14,17). Mutational analysis of Tus in combination with crystal data on the Tus-DNA complex revealed a critical loop in Tus, that is responsable for a direct interaction with

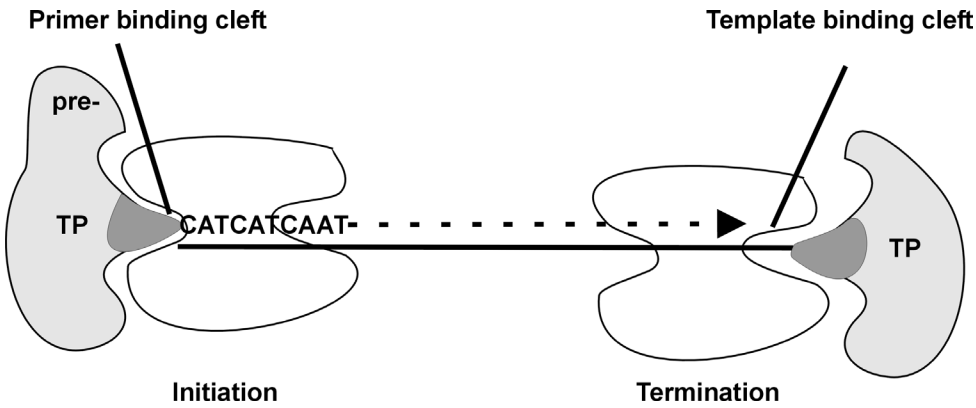

Figure 4. Model for Ad initiation and termination. Cartoon of initiation and termination of Ad replication. For initiation, pTP (pre-TP) is shown binding with the priming part (grey area) at the polymerase active site. When Ad pol reaches the end of the genome, it encounters the same priming part of TP, but in the opposite orientation. This part might interact with the template binding groove of Ad pol, providing an explanation for the specific requirement of TP to replicate all terminal nucleotides.

DnaB (17). TP might have a similar loop that could fit into Ad pol.

For initiation of Ad replication, pTP binds at the region of Ad pol where the duplex part of the primer-template leaves the polymerase (Brenkman et al., in press). The priming part of pTP, including serine 580 to which the first nucleotide is covalently attached needs to be located at the polymerase active site. Mutational analysis of the Ad pol active site has indicated that several amino-acid residues, responsable for DNA binding, are also responsable for pTP binding (13). Moreover, a partial digestion study on the TP/polymerase complex in ϕ29, that uses a similar replication mechanism as Ad, indicated that both duplex DNA and TP gave a similar protection pattern, suggesting that both primers, TP and DNA, fit in the double-stranded DNA-binding groove and protect the same regions of ϕ29 DNA polymerase (22). Therefore it could be argued that this part of pTP mimicks DNA in order to fit at the DNA binding region (Figure 4). Such a conformation of TP might therefore allow a fit at the template binding groove, providing entrance of all terminal bases at the polymerase active site (Figure 4) and giving an explanation why replication terminates after synthesis of all nucleotides on TP-DNA but not on streptavidin-DNA. Indeed, when Ad pol reaches the end of the linear genome, it is this part of pTP that is encountered first when the terminal nucleotides are synthesized (Figure 4). The distance from the polymerase active site to the enzyme surface where the template enters the polymerase has been measured for both Ad pol and ϕ29 DNA polymerase and spans, 5, respectively 4 nucleotides (Brenkman et al., in press, 4). In addition, a minimal distance of 2 nucleotides can be observed in the crystal structure of the replicating complex of RB69 DNA polymerase, a polymerase belonging to the same family as Ad pol and ϕ29 DNA polymerase (8). These findings all indicate that part of TP enters the template binding groove for replication of all terminal nucleotides. A direct interaction between TP and the template binding groove of Ad pol has not been demonstrated sofar (24) suggesting that the interaction with Ad pol is either weak or transient. It would be interesting to learn if mutations in the priming part of pTP might impair termination although these studies are difficult since they require mutated TP-DNA to be made *in vitro*.

Ad pol terminates genome replication by synthesis of all nucleotides. It is therefore unlikely that the jumping-back mechanism in a subsequent round of replication plays a role in recovering any non-replicated terminal bases that resulted from termination of DNA replication. Furthermore, the presence of TP is not a prerequisite for termination of replication

as replication is also terminated in the absence of TP (Figure 2B). This suggests that TP has a primary role other than termination of Ad DNA replication, although it is specifically adapted to enable replication of all terminal bases by Ad pol. This is in contrast to the RTPs in prokaryotes that are necessary for termination and thereby could prevent plasmid instability and the generation of rolling circles (12). Several roles for the covalently bound TP have been proposed, e.g. protection of the linear genome against exonucleases (7), attachment to the nuclear matrix, thereby localizing the virus genome (21), unwinding of the origin of replication and stabilization of incoming pTP-pol complex (19,20).

In summary, our results show that TP plays a specific role in termination of Ad DNA replication. This specificity might be the result of an interaction between Ad pol and the priming part of TP, similar to termination in several prokaryotes and a limited number of eukaryotes (2). Many viruses and plasmids with a linear genome contain a covalently bound protein at each end of the genome, that originated from the protein-priming mechanism by which these organisms initiate replication. Therefore, the mechanism for termination of DNA replication described here could be universal among protein-priming organisms.

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Transcription Factor Nuclear factor I Recruits Adenovirus DNA Polymerase via its DNA Interaction Surface

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Transcription Factor Nuclear factor I Recruits Adenovirus DNA Polymerase via its DNA Interaction Surface

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Nuclear factor I (NFI) is a site-specific DNA binding protein involved in regulation of transcription and stimulation of adenovirus (Ad) DNA replication. NFI stimulates initiation of Ad replication by recruitment of Ad DNA polymerase (pol) to the core origin of DNA replication through a direct protein-protein interaction and by inducing structural changes in the DNA upon binding to its site present in the auxiliary origin. Previous studies further demonstrated that NFI could stabilize binding of the pTP-pol complex to the origin. These results were interpreted as the existence of a physical interaction between NFI and Ad pol on the origin. Moreover, insertion of one or two nucleotides between the core origin and the NFI binding site severely inhibits stimulation of Ad replication. Here, we have examined the fate of the NFI-Ad pol interaction when NFI is bound to the origin recognition site. Interestingly, by immunoprecipitation assays with purified Ad pol and NFI we show, that Ad pol is released once NFI is bound to its recognition site. To examine the possibility that the DNA and Ad pol interaction surfaces of NFI are overlapping, we constructed site-directed mutants of NFI. All mutants were purified to near homogeneity and screened for DNA and Ad pol binding, resulting in the identification of a distinct class of mutants affected in both functions. Thus, these results suggest that the DNA and Ad pol interaction surfaces of NFI are overlapping, although the identification of mutants that were affected only in one activity demonstrated that the overlap is partial. Based on these results a new model is proposed for recruitment of the pTP-pol complex to the origin by NFI.

INTRODUCTION

NFI is a sequence-specific transcription factor involved in regulation of cellular and viral gene expression (reviewed in (11)). In addition, NFI is one of an increasing number of transcription factors that are also involved in DNA replication (reviewed in (25)). These transcription factors are capable of enhancing DNA replication by facilitating rate-limiting steps in the initiation process. NFI is necessary for the replication of the adenovirus types 2 and 5 (12). The mechanism of adenovirus DNA replication is one of the best characterized eukaryotic replication systems due to the relatively small amounts of proteins involved and the availability of a well characterized *in vitro* replication assay, thereby providing an optimal tool to study the role of transcription factors in DNA replication.

Adenoviruses have linear genomes of approximately 36 kB, containing two origins of replication located within each inverted terminal repeat. Each origin contains a core and an auxiliary region. Three viral proteins are involved in adenovirus DNA replication: adenovirus DNA polymerase (Ad pol) and precursor-terminal protein (pTP) which together form a pTP-pol complex, and the DNA binding protein DBP. After binding of the pTP-pol complex to the core origin, Ad replication initiates by a protein-priming mechanism in which a dCTP is coupled to a serine residue of pTP. Initiation is catalyzed by Ad pol and stimulated by the transcription factors NFI and Oct-1 (reviewed in (8,24)). NFI stimulates initiation of DNA replication by binding to a consensus NFI-binding site located within the auxiliary origin (15,19). This stimulation is dependent on the Ad pol concentration, with maximal stimulation at low amounts of Ad pol,

as exists *in vivo* (18). Moreover, mutation of the NFI binding site prevents efficient initiation of Ad replication *in vivo* (12).

Deletion analysis of NFI identified two functional domains. The variable C-terminal domain of NFI is involved in transcriptional activation or repression but is dispensable for stimulation of Ad replication (16). For this, the highly conserved N-terminal domain (approximately 220 aa) that encodes the DNA binding and dimerization function is sufficient (5,16). The interaction domain of human NFI with Ad pol has also been mapped to this part of the N-terminal domain, aa 68-150 (5)

A dual mechanism for stimulation of Ad replication by NFI has been proposed. The first mechanism involves a direct interaction between Ad pol and NFI (3,5,17). This interaction facilitates recruitment and positioning of the pTP-pol complex at the Ad origin, leading to assembly of a multiprotein initiation complex (17). In the second mechanism NFI induces structural changes in the Ad origin, as was demonstrated by DNA bending assays and hydroxylradical footprinting (17,28). The contribution of each mechanism to stimulation of initiation remains unclear.

Recruitment and stabilization of the pTP-pol complex via the direct NFI interaction can be achieved in two ways (17). NFI could interact with the pTP-pol complex in solution and recruit it to the origin. Alternatively, NFI is already bound to the origin and then associates with the pTP-pol complex, thereby stabilizing it at the origin. To further study this, we examined the fate of the NFI-Ad pol interaction after binding to origin DNA. Here we show that when NFI binds to its DNA recognition site, Ad pol is released suggesting that NFI interacts with Ad pol in solution and delivers it to the origin. Unexpectedly, our results further demonstrate that DNA binding and Ad pol binding are overlapping. These findings allow us to propose a new model for recruitment of the pTP-pol complex to the origin of adenovirus DNA replication.

EXPERIMENTAL PROCEDURES

DNA templates

All oligonucleotides and [γ-^{32}P]ATP (5000Ci/mmol) were purchased from Amersham Pharmacia Biotech. The intact NFI binding site (dsNFI) consists of 5'-GCACGTTTTGGATTGAAGCCAATATGACGCA and its complementary strand. The sequence to which NFI binds is underlined. T20 (5'-AATCCAAAATAAGGTATATT) represents the first 20 nucleotides of the bottom (template) strand of the adenovirus 5 genome. D20 (5'-CATCATCAATAATATACCTT) is the complementary (displaced) strand of T20. Oligonucleotide YC (5'-CCCGCCGCCCGAATTCGC) and its complementary strand YG are a GC rich probe. Labeling of the oligonucleotides was performed with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-^{32}P]ATP. The hybrid molecules dsNFI, D20/T20 and YC/YG were obtained by boiling oligonucleotides in 60 mM Tris-HCl, pH 7.5, 200 mM NaCl, followed by slow cooling to room temperature. All oligonucleotides used were purified by 10% polyacrylamide-1xTBE gel electrophoresis.

Construction of NFI mutants

Site-directed mutagenesis was performed using the Quickchange method from Stratagene. The forward oligonucleotides for the PCR mutagenesis were for RK-WH: 5'-CTGGCCAAGTTA**TGGCAT**GATATCCGACCC, RK-WL: 5'-CTGGCCAAGTTA**TGGCTA**GATATCCGACCC YR-LE: 5'-ATCCGACCCGAG**TTAGAA**GAGGATTTTGT T YR-WL: 5'-ATCCGACCCGAG**TGGCTA**GAGGATTTT GTT, T-D: 5'-GTTCTTACAGTT**GAT**GGGAAAAAACCT, with changes marked in boldface type.

Proteins and buffers

Wild type Ad pol was expressed from a baculovirus expression system and purified to near homogeneity as previously described (4). The his-tagged DNA binding domain of rat NFI (NFI-BD) was purified as described (10). pTP was a gift from R.N. de Jong. Expression and purification of the NFI mutants was as described (10). The buffer used for dilution of these proteins contained 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mg/ml bovine serum albumin (BSA) and 20% glycerol.

Co-immunoprecipitation assay

150 µl of pre-swollen 10% protein G-sepharose were washed twice with IP buffer containing 25 mM HEPES, pH 7.5, 0.1% NP-40, 4% glycerol and 150 mM NaCl. Then, 1µg of anti-penta His-antibody (Qiagen) was added to the beads and incubated for 1 hr. at 4°C under constant rotation. In a separate tube, 2 µg Ad pol and 400 ng wild-type or mutant NFI-BD were incubated in 200 µl IP buffer, 125 mM NaCl at 37°C in the presence or absence of DNA as indicated in the figure legend. After 1 hr., the protein mixture was added to the beads and incubated for 1 hr. at 4°C under constant rotation. Then, the beads were centrifuged for 3 min. at 1500 r.p.m. and washed twice in IP buffer with 150 mM NaCl. After the last wash, the supernatant was removed and 20 µl Laemmli sample buffer was added. Samples were boiled for 3 min. and loaded onto a SDS-polyacrylamide gel. Proteins were detected by Western blotting by using a polyclonal antibody to Ad pol (4) and NFI (17).

DNA binding assay

Gel retardation was performed using 5'-labeled dsNFI. The binding reaction (20 µl) contained 25 mM HEPES, pH 7.5, 4 % Ficoll, 1 mM EDTA, 4 mM DTT, 0.1 mg/ml BSA, 80 mM NaCl, 0.05 ng probe and the indicated amounts of each protein. The addition of poly (dI-dC)- (dI-dC) is described in the legend of the figures. After incubation for 60 min at 4 °C samples were loaded and separated on a 10 % polyacrylamide-1x TBE gel at 4 °C. Gels were dried, autoradiographed, and quantified using a Phosphor Imager.

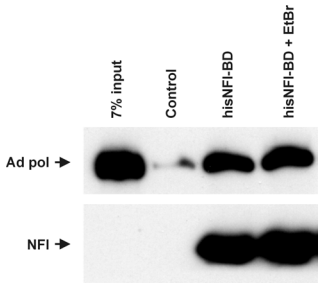

Figure 1. Co-immunoprecipitation of Ad pol by NFI-BD is not mediated by DNA. A co-immunoprecipitation assay was performed for Ad pol by NFI-BD in the absence or presence of EtBr. In the control lane, the pulldown assay was performed in the absence of NFI-BD. The NFI-BD input is shown in the lower panel.

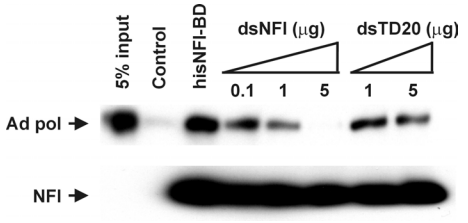

Figure 2. Ad pol and DNA compete for NFI-BD binding. The co-immunoprecipitation assays were preformed as described (Figure 1.) in the presence of increasing concentration of the NFI DNA binding site (dsNFI) or the duplex first 20 origin bases (TD20) that lacks the NFI binding site.

RESULTS

NFI directly interacts with Ad pol

The his-tagged N-terminal domain of rat NFI (NFI-BD) was tested for interaction with Ad pol in a co-immunoprecipitation assay. Complexes were immobilized on Protein G beads coupled to an anti-his antibody and analyzed by Western blot analysis using a specific anti-Ad pol antibody. About 5% of Ad pol bound to NFI-BD (Figure 1), whereas the control experiment without NFI-BD showed only background binding. This result is in agreement with previous interaction studies on Ad pol and NFI-BD (3,5,17). To exclude that the interaction between NFI-BD and Ad pol is mediated by DNA, a co-immunoprecipitation assay was performed in the presence of EtBr at a concentration of 50 μg/ml, that interferes strongly with the DNA binding of proteins (14). Co-immunoprecipitation of Ad pol was not inhibited by addition of EtBr (Figure 1), indicating that the observed NFI-BD-Ad pol interaction is not mediated by DNA.

Ad pol dissociates from DNA-bound NFI-BD

To examine the fate of the NFI-Ad pol interaction when NFI binds at its recognition site at the Ad origin, an NFI-Ad pol complex was challenged with increasing amounts of dsNFI, that contains the NFI consensus sequence $TGG(N)_{6\text{-}7}GCCAA$. As can be observed in figure 2, already in the presence of 0.1 μg dsNFI, the NFI-BD-Ad pol interaction strongly decreased. More than 1 μg of dsNFI reduced the interaction to background levels. At this point it is however unclear whether NFI-BD binds the DNA thereby dissociating the Ad pol-NFI-BD complex or whether Ad pol binds DNA, thereby freeing NFI-BD, or both. Therefore, Ad pol was co-immunoprecipitated with NFI-BD in the presence of TD20, the duplex Ad origin, to which NFI-BD cannot bind, but Ad pol can (data not shown). As can be seen in figure 2, NFI-BD remained associated with Ad pol in the presence of TD20. A slight reduction in Ad pol binding is observed at high concentrations of TD20, suggesting that by binding of Ad pol to TD20 some NFI is released (Figure 2). Alternatively, it has been observed that at high concentrations of DNA, NFI-BD can bind DNA non-specifically (data not shown, (10)). To address this question, a co-immunoprecipitation was performed in the presence of increasing amounts of YC/YG, a GC-rich duplex to which Ad pol has strongly reduced binding affinity as compared to TD20 (data not shown). Ad pol binding reduced to a similar extent as compared to challenging with TD20, indicating that the reduction is caused by DNA binding of NFI-BD thereby dissociating the Ad pol-NFI-BD complex (data not shown). In conclusion, these results indicate that once NFI binds to its DNA binding site, Ad pol is released. This suggests that recruitment *in vivo* of Ad pol through the direct interaction with NFI requires formation of the NFI-Ad pol complex in solution rather than NFI already bound to the Ad origin. Interestingly, these results further suggest that Ad pol and DNA are competing for overlapping interaction surfaces of NFI-BD. Alternatively, NFI-BD binding to DNA could induce a conformational change, re-

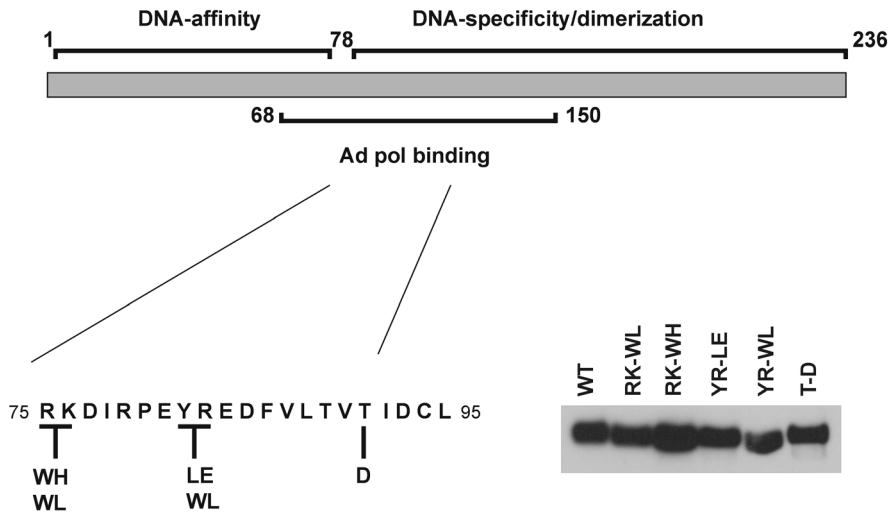

Figure 3. Generation of NFI-BD mutants. The upper panel shows the organization of NFI-BD. Site-directed NFI-BD mutants were generated between aa 75-95 as has been depicted in the panel bottom left. In the panel bottom right, equal amounts of purified wild-type and mutant NFI-BD were detected by Western blotting. Only for mutant RK-WH, 2 times more protein was loaded.

sulting in dissociation of Ad pol, a possibility that needs to be excluded.

Two site-directed NFI-BD mutants are affected in both DNA and Ad pol binding

Since no structural information is available for NFI or NFI-BD, we used mutational analysis to study the overlapping binding domains of NFI-BD. Five site-directed NFI-BD mutants were designed (Figure 3). These mutants were selected based on a study of Armentero and colleagues who demonstrated that at least aa 79-88 are involved in both DNA binding and polymerase interaction (2). Mutants were cloned, expressed and purified to near homogeneity (Figure 3.) as described (10). All mutants were assayed for interaction with Ad pol in the co-immunoprecipitation assay (Figure 4 and Table I). Binding to Ad pol was compared with the wild-type and control lanes. Only mutant YR-WL bound Ad pol wild-type like as can be observed in figure 4. The other four mutants showed reduced Ad pol interaction and mutants RK-WH and T-D lost their interaction with Ad pol almost completely (Figure 4, table I). All mutants were further tested for specific binding to the dsNFI binding site. As shown in figure 5 and summarized in Table I, both mutations in RK retained DNA binding, whereas Ad pol binding was lost for mutant RK-WH but reduced for mutant RK-WL. On the other hand mutant YR-WL showed wild-type Ad pol binding but reduced DNA binding. Mutant RK-WL showed increased DNA binding activity (figure 5), but this was due to the double amount of this mutant used in the assay (figure 3). Interestingly, two mutations were affected in both DNA binding and Ad pol interaction. While mutant YR-LE displayed reduced Ad pol and DNA binding, mutant T-D was severely affected in both properties (Table I). Higher concentrations of mutant T-D showed that DNA binding was not lost completely (data not shown). These latter two mutants indicate that

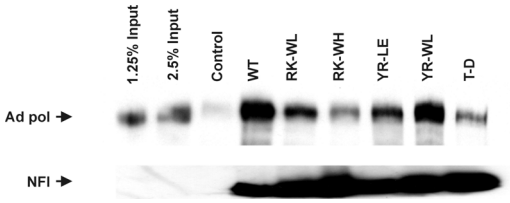

Figure 4. Co-immunoprecipitation assay of Ad pol by NFI-BD mutants. Wild-type NFI-BD and all mutants were screened for interaction with Ad pol.

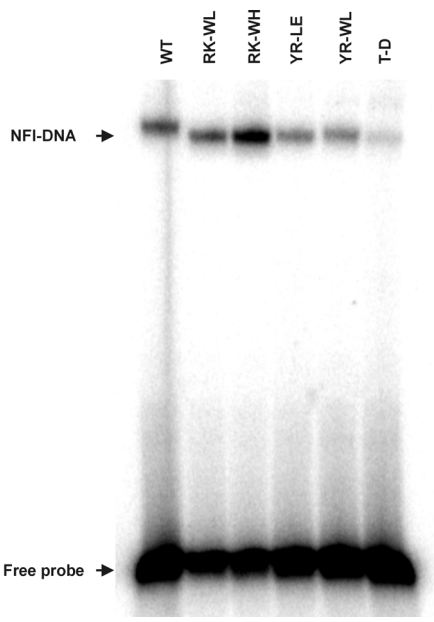

Figure 5. DNA binding of NFI-BD mutants. A DNA binding assay was performed with wild-type NFI-BD and the mutants on dsNFI, containing one intact binding site for NFI, in the presence of 1 μg of poly (dI-dC)- (dI-dC) per lane. For mutant RK-WH, 2 times more protein was added as compared to the other lanes (see text for more details).

Table I.
DNA and Ad pol binding of NFI-BD mutants.

Mutant	DNA binding (%)	Pol binding (%)
Wt	100	100
RK-WL	80	40
RK-WH	200	10
YR-LE	50	30
YR-WL	50	100
T-D	10	10

the same residues are involved in both DNA and Ad pol binding in support of overlapping binding sites on NFI-BD. Based on the phenotypes of the other NFI mutants, the overlap is likely partial as mutants were found that lost only one of the activities. A large scale mutational analysis of the NFI-BD aa region 75-115 is currently underway to further determine the amount of overlap. Moreover, by screening mutants with only Ad pol interaction lost, like RK-WH, for the ability to stimulate Ad replication we could directly identify the contribution of recruitment by NFI-BD for Ad replication stimulation.

DISCUSSION

One way by which NFI stimulates Ad replication initiation is by recruitment and positioning of the pTP-pol complex on the Ad origin through a direct interaction between NFI and Ad pol resulting in stabilization of the pre-initiation complex (3,5,17). Two models for NFI stimulation via this direct interaction have been proposed (17). The first model proposes that NFI interacts with the pTP-pol complex in solution thereby recruiting it to the origin. In the second model NFI is already bound to the origin and then associates with the pTP-pol complex thereby stabilizing it. Our results suggest that when NFI binds to its recognition site, Ad pol is released (Fig. 2). Moreover, The NFI DNA binding site specifically competed with Ad pol for interaction with NFI, suggesting that DNA and Ad pol binding are overlapping. From five site-directed NFI-BD mutants, two (YR-LE and T-D) were affected in both DNA binding and Ad pol interaction (Figure 4, 5, Table I) confirming the overlapping interaction surfaces. These findings are rather surprising as previous data indicated that Ad pol and NFI continued to interact after binding DNA. For example, insertion of one or several nucleotides between the pTP-pol binding site in the core origin and the NFI binding site severely inhibits stimulation of Ad replication by NFI both *in vitro* (1,27) and *in vivo* (3). This has been interpreted as the existence of a physical interaction between Ad pol and NFI during initiation. Our data clearly argue against a physical interaction once NFI has bound to its site. A new model for recruitment of the pTP-pol complex therefore needs to be proposed (Fig. 6). In this model, NFI already interacts with the pTP-pol complex in solution. Ad pol binds at the DNA interaction surface of NFI, but once NFI has found its high affinity binding site on the origin, Ad pol dissociates and binds to the core origin (Figure 6).

This model for recruitment of Ad pol by NFI immediately raises the question how the pTP-pol complex is stabilized on the origin in the absence of a protein-protein interaction. Furthermore, how could insertion of one nucleo

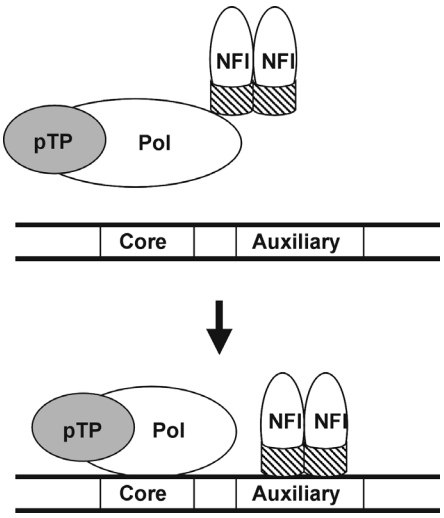

Figure 6. Model for recruitment of the pTP-pol complex by Ad pol. The pTP-pol complex is shown with pTP shaded in gray. NFI is presented as a dimer with the DNA interaction surfaces hatched. The Ad origin, consisting of the core and the auxiliary origin is depicted in bold lines.

tide between the pTP-pol complex and the NFI binding site lead to an inhibition of replication stimulation? A possible explanation is that NFI induces structural changes in the DNA that could lead to an optimal binding of pTP-pol to the core origin. Indeed, such changes in DNA structure induced by NFI have been reported (17,28). Further evidence for this mechanism of stimulation comes from a deletion analysis of the NFI-BD that revealed that the DNA binding domain could be divided in two subdomains. The first, spanning aa 1-78, is responsible for high affinity DNA binding, with low specificity, whereas the other domain (aa 75-182) is involved in specific DNA binding, with low affinity. As the first domain could not interact with Ad pol, but was still able to stabilize the pTP-pol complex on the origin and stimulate replication, these results were explained by induction of structural changes in the DNA (10). Another point that needs to be addressed is how NFI could still recognize the origin when Ad pol is complexed to its DNA interaction surface. To answer this point, it is important to know the extent of overlap between Ad pol and the DNA binding surface. This is unclear at present due to the lack of a crystal structure for NFI and Ad pol. Importantly, mutants RK-WL and RK-WH were only affected in Ad pol binding whereas mutant YR-WL was only affected in DNA binding (Figure 4, 5, Table I). These results suggest that the overlapping interaction surfaces are partial. Indeed, two mutants in region 79-88 were capable of DNA binding and dimerization but lost the ability to recruit the pTP-pol complex (2). DNA binding competition studies with NFI-BD and Ad pol are underway to determine if Ad pol inhibits binding of NFI to DNA.

Two other aspects of NFI-DNA recognition need further discussion. One aspect is the need for NFI-BD to dimerize in order to bind DNA (2). NFI binds DNA symmetrically to one side of the DNA helix (9), reflecting the putative structural symmetry of the NFI dimer binding to its recognition site. Correspondingly, inversion of the DNA binding site does not affect stimulation of replication (9,28). This symmetry of the NFI dimer allows most likely the binding of two Ad pols instead of one, as that would require two identical contacting surfaces on Ad pol. At low concentrations of the pTP-pol complex, present early in infection, NFI likely binds only one Ad pol molecule. Such a situation would leave one monomer of NFI free to contact the DNA (Figure 6), thereby facilitating recognition of its origin site. Late in infection, when pTP-pol concentrations are higher, the need for NFI may be overcome since NFI stimulation is dependent on the pTP-pol concentrating as determined *in vitro* (18). Whether one or two Ad pols can bind a NFI dimer awaits to be determined.

The other aspect comes from the organization of the protein. As discussed, the NFI binding domain could be divided in two subdomains, aa 1-78 and 75-182. Mutational analysis of NFI-BD has demonstrated that aa 68-150 (corresponding to aa 71-153 in the rat protein) are responsible for Ad pol binding (5), thus overlapping largely with the subdomain responsible for high specificity , but low affinity binding. As the first 78 aa are responsible for high affinity, but low specificity DNA binding and cannot interact with Ad pol (10), this region could play an important role in initial recognition of the NFI binding site.

Recently, we identified a strikingly similar mechanism for recruitment of pTP by Oct-1 (7). Oct-1 is the second cellular transcription factor involved in a 6-8 fold stimulation of Ad

replication (21). The central bipartite POU DNA binding domain of Oct-1 is sufficient for stimulation of Ad replication (26). The DNA binding domain of Oct-1, consisting of a homeodomain and a specific domain was shown to recruit pTP via its homeodomain by overlapping DNA and pTP interaction surfaces. In agreement with this, DNA binding and pTP binding were mutually exclusive (7).

The adenovirus serotype 2/5 uses two ubiquitously expressed cellular transcription factors NFI and Oct-1 to increase the number of initiation events. However, there are about 75.000 NFI binding sites within the human genome and a large number of enhancers and promotors contain binding sites for Oct-1 (8,11) to which the pTP-pol complex could also be recruited. Therefore this could lead to a logistical nightmare for the virus when all the pTP-pol complex would be sequestered to these sites. Indeed, as has been shown in hamster cells, a low pTP concentration leads to the lack of Ad12 DNA replication, whereas overexpression resulted in *de novo* synthesis of full-length Ad12 DNA (13). Our findings however, demonstrate that the pTP-pol complex dissociates from NFI after it binds DNA thereby providing a possible escape from being trapped on the wrong genomic site. Only in the context of the viral genome, the pTP-pol complex will bind the core origin for which it has an increased specificity (23). Here, binding of the pTP-pol complex could be further stabilized by the structural changes induced by NFI and Oct-1 that allow an optimal fit of the pTP-pol complex at the origin. In addition, the presence of genome-bound TP stabilizes the pTP-pol complex by inducing changes in the origin structure (20) or by interacting directly with pTP (R.N. de Jong et. al., manuscript in preparation). Finally, the viral DBP has been found to stimulate NFI binding to the Ad origin by increasing the association rate and decreasing the dissociation rate (6,22). As the recruitment of replication proteins by transcription factors has been described for many viruses (25), it would be interesting to learn if these viruses use a similar recruitment principle.

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7

Summarizing Discussion

Central in adenovirus (Ad) replication is the viral DNA polymerase, Ad pol. In addition to replication of the entire linear genome, it also catalyzes initiation that occurs at two distinct origins, each located within the inverted terminal repeat of the Ad genome. It is therefore not surprising that a large number of protein-protein interactions and protein-DNA interactions are involved to achieve successful duplication of the genome. This thesis has focused on these interactions from the perspective of Ad pol and this chapter summarizes and discusses our findings. Together with other available data, these findings have led to the presentation of a model for Ad replication as is schematically outlined in figure 1.

Early in infection, Ad pol is found always in complex with the viral protein primer pTP. The pTP-pol complex forms a strong heterodimer (Fig. 1A). Deletion and mutational analysis of both pTP and Ad pol have shown that there are numerous interactions across the proteins although a precise mapping is lacking {Parker, Botting, et al. 1998 560 /id}{Roovers, van der Lee, et al. 1993 647 /id}{Roovers, Overman, et al. 1991 646 /id}{Chen, Mermod, et al. 1990 1125 /id}. The crystal structure of the replicating complex of RB69 DNA polymerase {Franklin, Wang, et al. 2001 1773 /id}, belonging to the same family of DNA polymerases as Ad pol (Chapter 1) was used as a working model for the structure of Ad pol. By using oligonucleotides with a biotin group incorporated we showed that pTP is bound at the primer binding cleft of Ad pol (Chapter 4, Fig. 1A).

The resulting pTP-pol complex needs to bind at the core origin in order to initiate replication. Adenoviruses belonging to serotype 2/5 use two abundant cellular transcription factors, NFI and Oct-1, to recruit and stabilize the pTP-pol complex at the origin, resulting in a 200-fold stimulation of replication initiation. NFI and Oct-1, for which a high affinity binding site is located at the auxiliary origin (Fig. 1B) stimulate Ad replication by two distinct mechanisms. NFI can directly interact with Ad pol and Oct-1 can interact with pTP, thereby attracting the pTP-pol complex to the Ad origin (Fig. 1A, B). In addition, both proteins could induce structural changes in the origin, thereby allowing optimal binding of the pTP-pol complex. In Chapter 6, the mechanism of recruitment by NFI was further studied. It was demonstrated that when NFI binds to the origin, Ad pol dissociates (Fig. 1B) indicating that NFI already interacts with the pTP-pol complex in solution (Fig. 1A). Recently, a similar mechanism for recruitment of pTP via Oct-1 has been described {de Jong, Mysiak, et al. 2002 1916 /id}, suggesting that the pre-initiation complex contains pTP-pol, NFI and Oct-1 (Fig. 1A). The contribution of each mechanism by which NFI and Oct-1 can stimulate Ad replication remains however unclear.

When NFI and Oct-1 bind at the auxiliary origin, the pTP-pol complex dissociates (Chapter 6) and can bind at the core origin, for which it has an increased specificity {Temperley & Hay 1992 787 /id}. Numerous interactions ensure here that the pTP-pol complex is optimally positioned and stabilized for initiation of replication. The pTP-pol complex is stabilized by NFI and Oct-1 possibly through structural changes in the origin induced by these proteins (Chapter 6). Binding of the pTP-pol complex to the origin is further stabilized by TP that changes the origin structure thereby allowing optimal binding {Pronk, Stuiver, et al. 1992 1341 /id}{Pronk & van der Vliet 1993 1342 /id}. In addition, an interaction between TP and pTP has recently been detected (de Jong et. al., submitted) similar to what has been described for the phi 29 replication mechanism {Serna-Rico, Illana, et al. 2000 1918 /id}. DBP is also involved in stabilization of the pTP-pol complex, albeit indirectly by increasing the association rate but decreasing the dissociation rate of NFI {Cleat & Hay 1989 1136 /id}{Stuiver & van der Vliet 1990 752 /id}. DBP is furthermore involved in unwinding of the DNA (Fig. 1B).

The position of pTP and the measurement of the relative distances (in nucleotides) from the polymerase and exonuclease active sites of Ad pol to the surface of the enzyme allowed us to propose a model for binding of the pTP-pol complex to the origin (Chapter 4). It was shown that approximately 14-15 nucleotides of DNA were covered by Ad pol. Based on these results and the location of a putative Zn-finger domain, it was proposed that the N-terminal 250 aa fold into a domain that specifically binds to the core

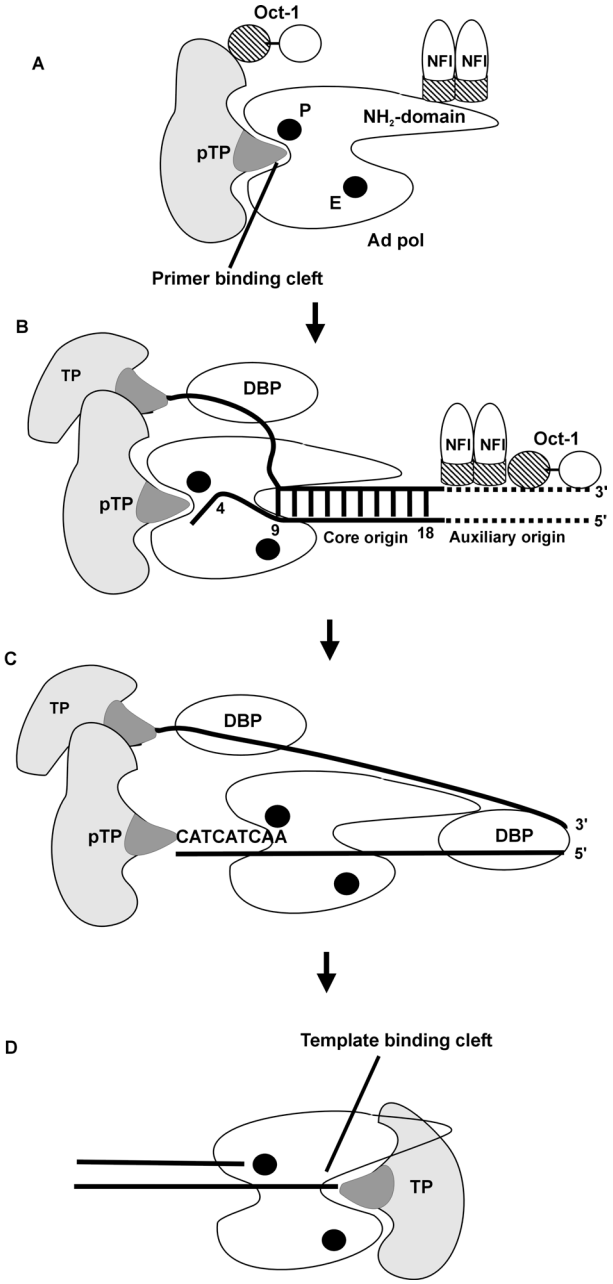

Figure 1. Ad DNA replication model. The mechanism of Ad replication has been schematically depicted in 4 steps. The polymerase (P) and exonuclease (E) active sites of Ad pol are depicted as dark spheres. TP and pTP are shown in gray, with their priming part dark gray. The DNA binding domains of NFI and Oct-1 are hatched. (A) Assembly of the pre-initiation complex in solution. (B) Assembly of the pre-initiation complex on the Ad origin. (C) After initiation and jumping-back, Ad pol and pTP dissociate, enabling Ad pol to elongate, while DBP performs strand displacement synthesis. (D) Termination of Ad replication.

origin bases 9-18 (Chapter 4, Fig. 1B).
Replication is initiated at an internal start site by the covalent linkage of a dCTP molecule to pTP. DBP stimulates initiation by lowering the Km. Since no other DNA binding protein can substitute for DBP, a direct interaction with Ad pol was suggested {Lindenbaum, Field, et al. 1986 1871 /id}. However, no such interaction has been described at present, suggesting that if it exists it is rather weak or transient. After synthesis of the pTP-CAT intermediate, the template strand jumps back to recover the terminal ends and continues to elongate the DNA (Chapter 2, Fig. 1C). Mutational analysis of Ad pol revealed that the highly conserved polymerase motif I/YXGG is involved in stabilization of the template strand at the polymerase active site (Chapter 3) as was later visualized in the crystal structure of the replicating complex of RB69 DNA polymerase {Franklin, Wang, et al. 2001 1773 /id}. Especially the glycine pair is important for stabilization and translocation of the template strand as the G666A/G667A mutant was severely affected in both polymerization and protein-primed initiation and in the transition from initiation to elongation (Chapter 3). These findings argue for a model of the jumping back mechanism in which the template strand, instead of the pTP-CAT intermediate jumps (Chapter 2).
In the elongation phase of Ad replication, Ad pol works in concert with DBP that coordinates the strand-displacement synthesis (Fig. 1C) and with NFIII, a topoisomerase, involved in synthesis of the full length genome (not shown). At the end of the genome, Ad pol encounters the covalently attached TP (Fig. 1D). In chapter 5, the role of TP in termination of Ad replication was studied. It was found that in the presence of TP, all nucleotides are synthesized in contrast to termination on DNA with a non-related protein bound at the 5'-end of the template. These results suggest that TP has a specific interaction with Ad pol for termination of Ad replication. As shown in figure 1D, the priming part of pTP that is located at the primer binding groove of Ad pol for initiation of replication is the same part that is encountered at the template binding groove of Ad pol at termination. Moreover, the distance between the template binding groove and the polymerase active site is five nucleotides (Chapter 4). Therefore, the priming part of TP could enter at the template binding cleft allowing synthesis of all bases and raises the intriguing possibility that it mimics ssDNA.
Ad pol, the key enzyme in Ad replication, needs to have multiple interactions with DNA and various proteins. The results described in this thesis have provided further insight in the mechanism by which Ad pol interacts with DNA, NFI, pTP and TP, leading to a better understanding of origin binding, jumping-back, recruitment and termination of replication than possible hitherto. Further understanding of these processes awaits determination of the crystal structure of the pTP-pol complex and NFI.

Nederlandse Samenvatting

Het adenovirus (Ad) is een DNA bevattend virus dat onder andere humane cellen kan infecteren. Dit betreft vooral cellen van de luchtwegen, wat leidt tot infecties in dit orgaan. In de gastheercel vermeerdert het virus zich. De vermeerdering (replicatie) van het virus is een complex gebeuren, al is dit naar verhouding veel eenvoudiger dan deling van humane cellen. Het mechanisme van Ad replicatie is dan ook een veelgebruikt modelsysteem. Daarnaast worden adenovirussen veelvuldig gebruikt als transportsysteem voor gentherapie. In dat geval moeten deze virussen echter zodanig aangepast zijn dat ze niet meer kunnen repliceren, wat anders kan leiden tot ongewenste verspreiding van recombinante virussen.

Een essentiële stap in de vermeerdering van het virus is de replicatie van het virale DNA. Na infectie van de gastheercel zal het adenovirus zich zo efficiënt mogelijk repliceren. Hiervoor maakt het drie virale eiwitten aan: adenovirus DNA polymerase (Ad pol), precursor terminal protein (pTP), die samen een heterodimeer vormen (het pTP-pol complex) en het DNA binding protein (DBP). Tijdens de start van de replicatie, de initiatie, katalyseert Ad pol de koppeling van het eerste nucleotide aan pTP. Vervolgens wordt gedurende de elongatie-fase het complete virale genoom gerepliceerd door Ad pol. De overgang tussen initiatie en elongatie wordt gekarakteriseerd door een zogenoemd "jumping-back" mechanisme, waarna pTP van Ad pol loslaat. DBP helpt Ad pol bij het repliceren door onder andere de structuur van het DNA te veranderen.

Adenovirussen van serotype 2 en 5 maken tevens gebruik van twee algemene transcriptie factoren van de gastheercel om de replicatie te stimuleren. Deze eiwitten zijn nuclear factor I (NFI) en octamer binding protein 1 (Oct-1). De bindingsplaatsen van NFI en Oct-1 zijn onder andere aanwezig in de startplaats van adenovirus replicatie (de origin) aan het uiteinde van het lineaire genoom. De precieze interacties tussen Ad pol en de verschillende hierboven besproken eiwitten en met DNA zijn essentieel voor efficiënte replicatie. In dit proefschrift zijn een aantal van deze interacties in detail bestudeerd.

In het eerste hoofdstuk worden de verschillende families van DNA afhankelijke DNA polymerasen besproken, waarbij in detail gekeken is naar de familie B polymerasen waartoe Ad pol behoort. Daarnaast wordt het mechanisme van Ad replicatie belicht. In hoofdstuk twee staat het "jumping-back" mechanisme van Ad replicatie centraal. Initiatie van Ad replicatie begint op een interne startplaats op het virale genoom en na de synthese van de eerste drie basen ontstaat een pTP-CAT intermediair. Deze springt vervolgens terug op de eerste drie basen aan het uiteinde van het Ad genoom waarna elongatie volgt. Met behulp van de structuurgegevens van verschillende polymerasen wordt een model gepostuleerd waarin de template strand terugspringt, in plaats van de pTP-CAT intermediair.

In hoofdstuk 3 wordt met behulp van mutagenese het sterk geconserveerde I/YXGG motief in Ad pol gekarakteriseerd. Dit motief blijkt belangrijk te zijn voor de stabilisatie van de DNA template streng in het actieve centrum van het polymerase. Interessant is vooral de rol van het glycine paar in het motief. Terwijl elongatie op een DNA primer mogelijk was, bleek elongatie na initiatie met pTP als primer volledig geremd. Dit betekent dat het defect optreedt in het "jumping-back" mechanisme. De gevonden resultaten sluiten goed aan bij het gepostuleerde model van dit mechanisme (hoofdstuk 2).

In hoofdstuk 4 wordt de DNA binding door Ad pol beschreven aan de hand van oligonucleotiden met een biotine groep. De polymerase en exonuclease experimenten met deze oligonucleotiden laten zien dat beide actieve centra op verschillende plekken in het Ad pol molecule liggen. Tevens blijkt dat Ad pol ongeveer 14-15 nucleotiden DNA kan binden. Deze gegevens zijn in overeenstemming met structuurgegevens van familie B polymerasen en suggereren dat Ad pol een vergelijkbare moleculaire architectuur heeft, ondanks het gebruik van pTP als primer. Daarnaast werd gevonden dat pTP bindt aan de primer bindende groeve van Ad pol. De waargenomen afname van polymerase en exonuclease activiteit van Ad pol in aanwezigheid van pTP suggereert dat DNA en pTP competeren voor dezelfde bindingsplaats.

In hoofdstuk 5 wordt de terminatie van Ad replicatie bestudeerd. Tijdens terminatie moet Ad pol de laatste nucleotiden van het genoom

repliceren in aanwezigheid van het covalent gebonden terminale eiwit, TP, het klievingsproduct van pTP. Aangezien de afstand tussen de buitenkant van het polymerase, waar de template binnenkomt, en het polymerase actieve centrum 5 nucleotiden is (hoofdstuk 4) kan het zijn dat niet alle nucleotiden worden gerepliceerd, omdat TP mogelijk de toegang van de laatste nucleotiden verhindert. Dit hoeft niet te leiden tot een verlies van nucleotiden aangezien Ad pol in de volgende replicatieronde tot 2 nucleotiden kan herstellen via het "jumping back" mechanisme. De resultaten beschreven in dit onderzoek laten echter zien dat bij terminatie alle nucleotiden worden gerepliceerd, mits het covalent gekoppelde eiwit TP is. Dit suggereert dat er een interactie is tussen het deel van TP waar de nucleotiden aan vast zitten en aan de kant van Ad pol waar de template streng binnenkomt. Aangezien hetzelfde deel van TP voor de initiatie in de primer bindings groeve ligt, speculeren wij dat het de DNA structuur nabootst.

In hoofdstuk 6 wordt de plaatsing en stabilisatie van het pTP-pol complex door NFI nader bestudeerd. De beschreven resultaten duiden erop dat Ad pol loslaat wanneer NFI gebonden is aan de Ad origin. Dit betekent tevens dat de contact oppervlakten tussen NFI en Ad pol overlappend zijn. Een mutatie analyse van het NFI DNA-bindende domein bevestigt deze overlap maar laat tevens zien dat deze slechts gedeeltelijk is. De gevonden resultaten hebben geleid tot een nieuw model voor plaatsing en stabilisatie van het pTP-pol complex op de Ad origin. Aangezien er ongeveer 75000 bindingsplaatsen voor NFI op het humane genoom aanwezig zijn, kan NFI Ad pol ook naar één van deze bindingsplaatsen brengen en daarmee Ad pol wegvangen. In het nieuwe model, beschreven in dit hoofdstuk laat Ad pol echter los wanneer het naar het humane genoom gebracht wordt. Ad pol kan dan specifiek aan het adenovirus genoom binden aangezien daar een bindingsplaats voor het pTP-pol complex aanwezig is.

De resultaten beschreven in dit proefschrift hebben geleid tot een beter inzicht in de manier waarop het pTP-pol complex de origin bindt, hoe dit complex geplaatst en gestabiliseerd wordt door NFI, hoe de terminatie verloopt en hoe het "jumping-back" mechanisme werkt.

Curriculum Vitae

Arjan Brenkman werd op 13 maart 1975 geboren te Culemborg. In 1993 behaalde hij zijn atheneum diploma aan het Koningin Wilhelmina College te Culemborg en werd ook begonnen met de studie Medische Biologie aan de Universiteit Utrecht. In augustus 1994 werd het propedeutisch examen Medische Biologie *Cum Laude* behaald. De hoofdvakstage werd bij de vakgroep Pathologie verricht onder supervisie van Prof. J.G. van den Tweel. en Dr. P. Joling. Voor deze stage werd in juni 1997 onder auspiciën van de Commissie Excellent Tracé de Van 't Hoog prijs toegekend. Van april 1997 tot en met april 1998 werd een stage gevolgd bij Novartis Pharma AG, Transplantation Unit, Department of Molecular Biology and Gene Therapy, Basel, Zwitserland onder supervisie van Dr. N.R. Movva en Dr. H.F.J. Dullens. Voor dit afstudeeronderzoek werd onder auspiciën van de Koninklijke Nederlandse Academie van Wetenschappen door N.V. Organon, de Organon Young Research Talent prijs toegekend. In juni 1998 behaalde hij het doctoraal examen *Cum Laude*. In juli 1998 werd aangevangen met het in dit proefschrift beschreven promotie-onderzoek dat werd verricht bij de vakgroep Fysiologische Chemie van de Universiteit Utrecht onder supervisie van Prof. P.C. van der Vliet. Het onderzoek werd afgesloten met een promotie in oktober 2002.

List of publications

Rob N. de Jong*, Peter C. van der Vliet, and **Arjan B. Brenkman***; Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP. *Current Topics in Microbiology and Immunology, in press, 2002*
**These authors contributed equally to this publication.*

Arjan B. Brenkman, Marinus R. Heideman, Veronica Truniger, Margarita Salas and Peter C. van der Vliet; The "(I/Y)XGG" motif of adenovirus DNA polymerase affects template DNA binding and the transition from initiation to elongation. *Journal of Biological Chemistry 276 (32); 29846-29853, 2001*

Arjan B. Brenkman, Elise C. Breure and Peter C. van der Vliet; Molecular Architecture of Adenovirus DNA Polymerase and Location of the Protein-Primer. *Journal of Virology 76 (16); 8200-8207, 2002*

Arjan B. Brenkman, Elise C. Breure and Peter C. van der Vliet; Termination of Adenovirus DNA Replication implicates a Role for the Terminal Protein. *To be submitted.*

Arjan B. Brenkman, Marinus R. Heideman and Peter C. van der Vliet; Transcription Factor Nuclear factor I Recruits Adenovirus DNA Polymerase via its DNA Interaction Surface. *To be submitted.*

B. van Breukelen, **Arjan B. Brenkman**, P. Elly Holthuizen and Peter C. van der Vliet. Adenovirus type 5 DNA binding protein stimulates binding of DNA polymerase to the replication origin. S*ubmitted.*

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