Molecular architecture of the preinitiation complex in adenovirus DNA replication

Moleculaire architectuur van het preïnitiatie complex
in adenovirus DNA replicatie

(met een samenvatting in het Netherlands)

Proefschrift

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Crowded DNA molecules containing origin of adenovirus replication visualised by scanning force microscopy (SFM)

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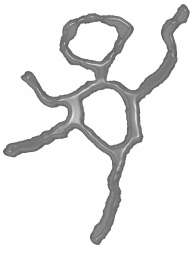

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Introduction

I. Biology of adenovirus

In 1954 a new family of DNA viruses, *Adenoviridae* (adenoviruses, Ad) was independently described by two groups (60, 62). This discovery initiated a field of research focused on understanding the mechanisms underlying different aspects of the viral life cycle. Up till now many different Ad serotypes have been described that infect humans and other species (6). They are classified into six subgroups named A to F. The human Ad serotypes 2 and 5 from subgroup C (Ad2, Ad5 or Ad2/5) became experimental models for Ad research and thus are the most widely analysed.

Adenoviruses as pioneers in molecular biology

Due to the small size of the viral genome the number of proteins that it encodes is limited. Therefore, viruses largely use proteins of the host cell for their basic processes after infection. Moreover, to obtain the optimal environment for reproduction, they modify various processes of the host cell for their own benefit. This interference involves interactions between viral and host proteins, and molecular mechanisms are often restricted to only a few proteins. Therefore, Ads became a good experimental system to study many cellular processes like transcription regulation, translation, DNA replication, repair and recombination as well as cell cycle control and apoptosis (34). Furthermore, the discovery of alternative RNA splicing in the adenovirus system provided the first evidence for the regulation of eukaryotic gene expression at the level of RNA processing. More than a decade ago Ads were chosen to be well suited vectors for the delivery of genetic material into human cells in gene therapy applications. However, after some complications during clinical trials the research has focused more on improvement of the adenovirus-based vectors and search for new therapeutic solutions. Many features of Ads, such as the ability to infect a broad range of human cells, low pathogenecity in humans, and the ability to accommodate a foreign DNA segment up to 7.5 kb, make them attractive DNA carriers and thus they are still among the most commonly used vectors for gene therapy.

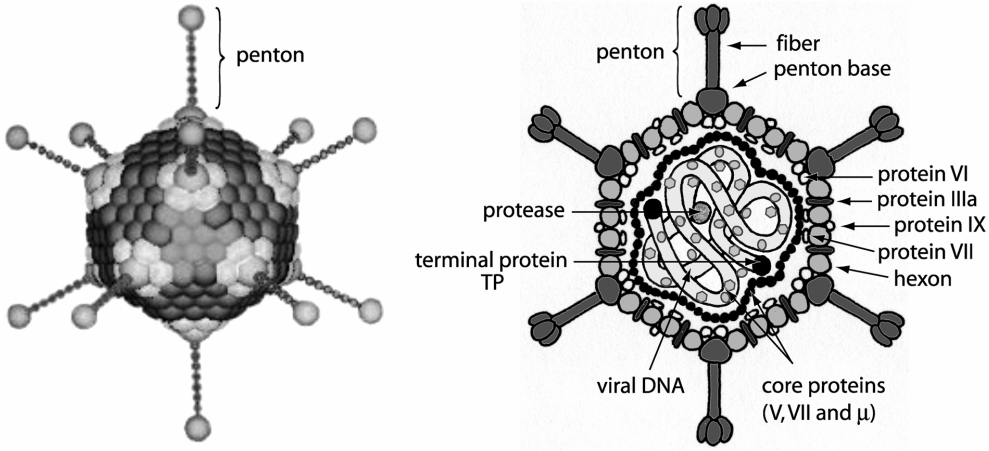

Figure 1. The structure of an adenovirus particle
The left panel represents a three-dimensional view of the Ad particle. The outer capsid is icosahedrally shaped and contains 12 vertices protruding from each apex. Within the outer capsid there is an inner core that consists mainly of the viral genomic DNA and several proteins responsible for its tight compaction. The protein composition of the viron is presented in more details on the right panel and is also described in the text.
The figure is partially adapted from http://people.cornellcollege.edu/jcardon/courses/viruses/figures.html

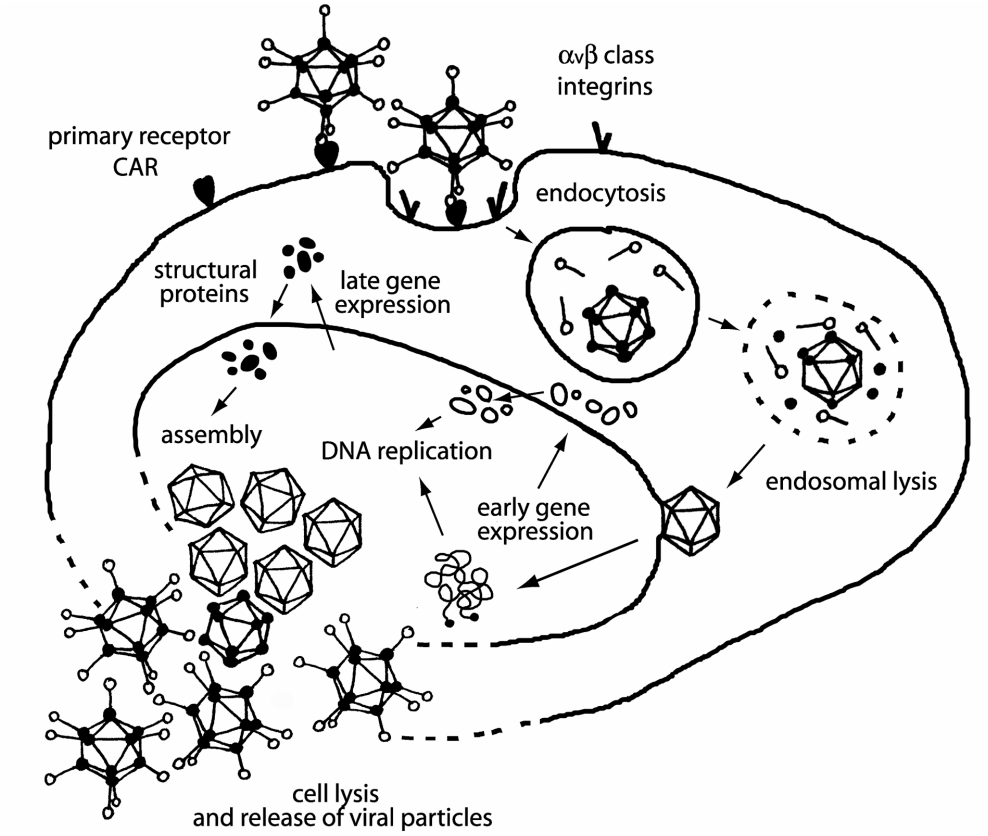

Figure 2. The infection pathway of adenovirus
Adenovirus enters the host cell by interaction of its fiber knob with the primary receptor, the coxsackie-adenovirus receptor (CAR). Interaction of the viral penton base with the cellular $\alpha_v\beta$ integrins initiates endocytosis of the viral particle. After endosomal lysis, virus escapes to the cytoplasm, translocates its genomic DNA into the nucleus and starts transcription of the early genes, which is followed by DNA replication and transcription of the late genes. Finally, synthesis of the structural proteins leads to the assembly of the great amount of nascent virions that trigger cell disruption and virus release. The figure is based on (64).

The structure of adenovirus

A combination of cryoelectron microscopy and X-ray crystallography data allowed a detailed three-dimensional reconstruction of the structure of the Ad particle (Figure 1) (reviewed in (109))(115, 116). The Ad particle has a maximum diameter of 1400 Å and it consists of an outer capsid and an inner core. The outer capsid is icosahedrally shaped and contains 12 vertices (pentons) protruding from each apex. The capsid is mainly built from the hexon proteins and is stabilized by several structural proteins like proteins IIIa, VI, VII and IX. The pentons are composed of a penton base anchored in the capsid and a protruding fiber protein. The globular domain at the end of the fiber is responsible for recognition of the cellular receptor leading to attachment of Ad to the target cell. The inner core of the virus contains 36-kb (for Ad5) of linear double-stranded genomic DNA with two terminal proteins (TP) covalently bound to each end as well as a few copies of the viral protease. DNA is tightly packed within the core by proteins V, VII and μ.

The adenovirus infection cycle

Ads infect mainly the respiratory tracts, gastrointestinal tracts and the eyes causing a number of pathological syndromes (reviewed in (61)). These illnesses are very common, and most people have been already infected with at least one type of Ad at the age of 15. Since most of Ad infections are asymptomatic, they are usually not diagnosed. However, a preexisting Ad infection is a major problem for patients with a defected immune system (AIDS) or for patients undergoing immunosuppresion treatment (transplantation), since at such conditions the virus tends to reactivate.

Ad infection of the host cell starts with binding of the viral fiber knob to the primary receptor, the coxsackie-adenovirus receptor (CAR) on the cell surface (Figure 2). Next, interaction of an Arg-Gly-Asp (RGD) motif of the penton base with the cellular $\alpha_v\beta$ integrins triggers a clathrin-mediated endocytosis of the virus, which subsequently leads to a partial disassembly of the viral capsid in the endosome. Soon after endocytosis, virus disrupts the endosome and escapes to the cytosol (76). The viral capsid is then transported to the membrane of the nucleus, where it docks through specific interactions with proteins of the nuclear pore complex (NPC) (48). The capsid is finally disassembled and viral DNA is translocated through the nuclear pore into the nucleoplasm, which is the ideal environment for viral RNA synthesis and DNA replication. Transcription of the early viral genes gives rise to proteins required for efficient viral DNA replication and subsequent expression of its late genes. Furthermore, viral proteins synthesized during the early phase of infection are also used against the anti-viral defense mechanism of the host cell. Finally, synthesis of the structural proteins in the late phase initiates assembly of the inner core and the outer capsid of the nascent virion. The large amount of the newly synthesized viral particles causes disruption of the infected cell and release of the viruses, allowing infection of the neighbouring cells.

II. The replication of adenovirus DNA

The replication of Ad DNA is a crucial step in the virus life cycle. Reconstitution of this process *in vitro* from purified proteins allowed its detailed molecular analysis and for a long time it served as the main model for studies of eukaryotic DNA replication. Up till now, other models that reflect the eukaryotic DNA replication more accurately have been developed (17, 33, 89). However, replication of Ad DNA is still an important issue in gene therapy procedures, like multiplication of therapy vectors or construction of the conditionally replicating vectors. Therefore, it is essential to investigate the molecular mechanism of Ad DNA replication.

The main players of the Ad5 DNA replication

There are three viral proteins involved in Ad5 DNA replication, Ad5 DNA polymerase (pol), precursor terminal protein (pTP) that serves as a primer, and DNA binding protein (DBP). In addition, two cellular transcription factors, Nuclear Factor I (NFI) and Octamer binding protein (Oct-1) can highly stimulate the replication process. For elongation of the entire genome Ad5 also uses a cellular DNA topoisomerase I (NFII), that is dispensable for elongation of short DNA fragments (83). The optimal assembly and stabilization of the preinitiation complex (PIC) on the origin is an important step in replication and it is achieved by various protein-protein and protein-DNA interactions.

Ad5 DNA polymerase

Ad5 DNA polymerase is a 140 kDa protein of 1198 aa that belongs to the pol α family (B family) (87) (Figure 3). It contains both DNA polymerase and proofreading 3'-5' exonuclease activity (39, 73). The N-terminal part of pol harbors three motifs associated with exonuclease activity (Exo-I, II and III), and the C-terminal part contains conserved motifs involved in DNA polymerase activity (Pol-I to Pol-V, and Pol-VII). Additionally, pol possesses residues and motifs that are conserved, specifically between

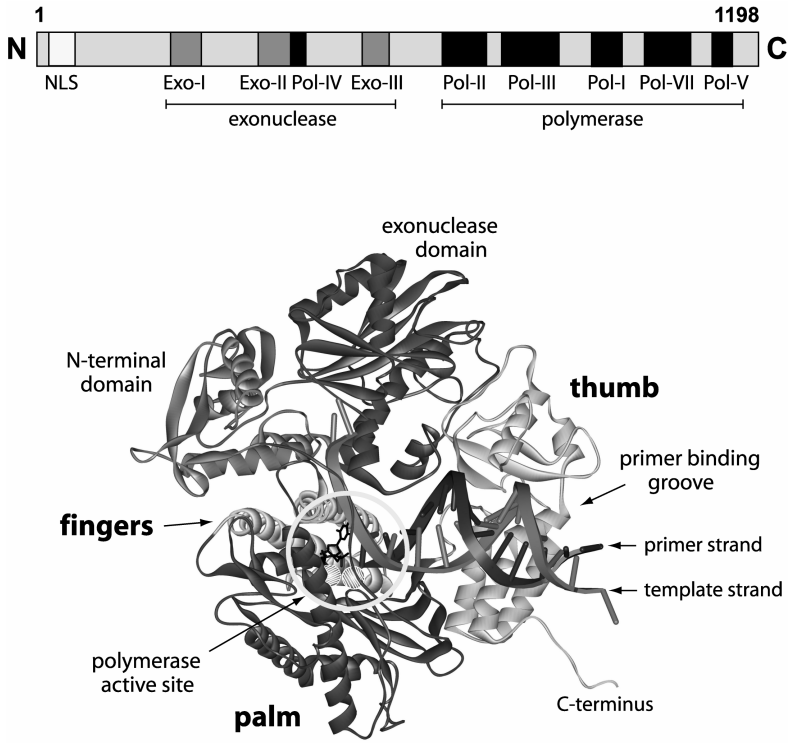

Figure 3. Domain organization of Ad5 DNA polymerase

The upper panel shows a schematic overview of the tertiary structure of the RB69 DNA polymerase from the pol α family (41) (1IG9 in PDB database), which serves as a good model for the study of Ad5 DNA polymerase. The palm, thumb and fingers domains as well as the exonuclease and N-terminal domains are marked. Primer-template DNA enters through the primer binding groove and presents the hydroxyl group of the 3' terminus of the primer strand in the active site of pol (encircled). Since the structure represents the replicating complex captured just before primer extension, an incoming dTTP nucleotide is bound

DNA polymerases that use protein as a primer (35, 140). Similar to other DNA polymerases, the catalytic site of pol is composed of conserved regions located in the Pol-I, Pol-II and Pol-III motifs. During polymerization pol uses a two-metal-ion mechanism, which involves binding of two Mg^{2+} ions by two conserved Asp residues of the active site, D685 and D1014. Mutational analysis of these two residues proved that they are critical for catalysis (73).

The crystal structures of DNA polymerases from different families show that although their primary sequence homology is limited, they share a similar overall architecture. The best studied polymerase from the α family is RB69 DNA polymerase (RB69 pol), and the structures of RB69 pol alone and also of RB69 pol in the replicating complex with primer-template DNA and the incoming nucleotide, have been revealed (41, 113, 139). RB69 pol serves as a good model for the study of the α family of polymerases, such as Ad5 pol. RB69 pol consists of three domains assembled in a shape resembling a right hand with the palm, thumb and fingers (Figure 3). The palm domain is responsible for binding of the catalytic Mg^{2+} ions. The thumb domain binds the primer-template DNA and is also involved in switching from the polymerization to the exonuclease mode during proofreading. The fingers bind the incoming nucleotide and then rotate by 60° towards the palm domain, thus delivering the nucleotide to the pairing template base and closing the active site of pol.

Ad pol forms a tight heterodimer with pTP (pTP/pol complex), and this stable interaction is essential for the initiation process in which pol covalently couples the first dCMP residue to pTP (16, 26, 37, 114, 117). Mutagenesis studies of pol demonstrated that the residues involved in pol binding to pTP are widely distributed along the protein sequence. They could, however, form an interacting domain in a three-dimensional structure (73, 87, 104). But there is limited homology in the primary structure between Ad5 pol and RB69 pol, and because RB69 pol does not interact with a priming protein, it is difficult to locate putative pTP interacting domains based on the structure of RB69 pol. Ad5 pol also interacts with NFI, but the residues of pol that are involved in NFI binding have not been clearly established. Based on the pTP/pol-NFI interaction it was proposed that NFI recruits pol or the pTP/pol complex to the origin and stabilizes the PIC (10, 16, 80).

Ad5 precursor Terminal Protein - pTP

pTP is a 75 kDa protein of 671 aa and its most important function is to serve as a primer in Ad5 DNA replication (Figure 1 in Chapter 4). The serine residue at position 580 (Ser580) of pTP is crucial for this process and mutation of this residue leads to an abolished initiation (93). Within the primary sequence of pTP there are three protease cleavage sites that are used by the viral cysteine protease p23 to generate mature TP in a later infection stage (141, 143). First, the two intermediate iTP sites are cleaved and then the TP site is cleaved, resulting in TP protein of 322 aa. pTP also contains a strong nuclear localization sequence (NLS) that permits the efficient nuclear import of not only pTP, but also pTP complexed with pol (148).

pTP has been shown to interact with several viral or cellular proteins and, since multiple regions of pTP are involved in these interactions, it is likely that pTP has a compact or globular structure. The most important and also the strongest interaction of pTP is the formation of a heterodimer with pol (16, 37, 114, 117). Protease cleavage experiments and mutational studies showed that a large portion of the pTP surface is involved in pTP binding to pol (12, 87, 104, 142). Additionally, pTP forms dimers on short 20 bp DNA fragments and also in solution as shown by gel shift and crosslinking studies (23). This observation suggests that interaction between pTP and TP covalently bound to the viral DNA (parental TP) could stabilize the formation of the PIC. There is also evidence that parental TP alone could additionally stabilize the PIC formation by induction of changes in the DNA structure at the terminus of the origin, like local destabilization of DNA or even limited unwinding (97). This is in agreement with the observation that TP-DNA is replicated more efficiently than protein-free DNA (96, 127). Another interacting partner for pTP is Oct-1 that recruits pTP or the pTP/pol complex to the origin and stabilizes the PIC (20, 24, 133). Similarly to the interaction with pol, residues involved in Oct-1 binding are spread throughout the sequence of pTP (11). Furthermore, an interaction of pTP with the CAD (carbamyl phosphate synthetase) protein, which is a nuclear matrix enzyme involved in pyrimidine synthesis, ensures proper attachment of the viral DNA within the nucleus of the infected cell (2). Indeed, Ad5 DNA replication takes place in discrete foci in the nucleus (8, 95). Studies on the DNA binding properties of pTP reveal that pTP binds single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) with very limited sequence specificity (23, 122). However, when complexed with pol, pTP/pol binds with increased specificity to a conserved DNA region at positions 9-18 in the Ad5 origin of replication (103, 122) (Figure 5).

Ad5 DNA Binding Protein - DBP

DBP is ubiquitously expressed during infection and therefore it was the first non-structural viral protein discovered in infected cells (131). DBP is a 59 kDa protein of 529 aa. Its main role is to stimulate initiation and facilitate DNA elongation during Ad DNA replication. However, DBP is also involved in transcription control, mRNA stability, transformation, recombination, virus assembly

and determination of the host range (59, 130). The N-terminal domain (residues 1-173) contains a nuclear localization signal (NLS) and it is not well conserved among different Ad serotypes. The C-terminal domain (residues 173-529) is well conserved and it is mainly involved in DNA binding. DBP binds ssDNA as well as dsDNA and RNA without clear sequence specificity (131). The C-terminal domain has a compact and globular structure with a protruding C-terminal arm that can hook into a neighbouring DBP molecule, thus enabling multimerization (126). This allows protein chain formation on ssDNA, which is proposed to be the driving force for DNA unwinding during elongation (30, 129). This process is independent of ATP hydrolysis showing that DBP does not have helicase activity (149). By binding to ssDNA DBP also protects it against nuclease degradation. Another role of DBP in elongation is the removal of DNA secondary structures that could act as roadblocks for pol during DNA synthesis. During elongation, DBP also enhances the processivity of pol and changes its sensitivity to nucleotide analogues (39, 72, 79).

DBP can also stimulate the initiation of replication and the level of stimulation depends on the pTP/pol concentration, with the highest effect at low pTP/pol amounts (118, 128). The mechanism of stimulation of initiation by DBP is not clear. Since no direct interaction between DBP and pol has been detected so far, it is likely achieved indirectly by modification of the DNA structure that leads to stabilization of PIC. Previously the footprinting analysis showed that DBP stimulates binding of NFI and/or pol to the origin by stabilization of their DNA binding (118, 119, 128). Kinetics studies additionally revealed that DBP decreases the K_m for dCTP incorporation by pol in the initiation (79).

Nuclear Factor I - NFI

NFI is a 55 kDa cellular transcription factor of 499 aa (Figure 4). It was originally discovered based on its ability to stimulate adenovirus (Ad) DNA replication *in vitro* (82). Later NFI was also found to play an important role in transcription regulation of a large variety of cellular and viral genes (50). The conserved N-terminal domain of NFI (residues 1-220) is required for DNA binding and protein dimerization and the C-terminal domain (residues 220-499) is involved in transcription activation (47, 78). For the stimulation of adenovirus DNA replication only the DNA binding domain is sufficient (16, 78).

NFI binds as a dimer to the consensus sequence 5'TGGA(N_5)GCCAA3' located in the adenovirus 5 (Ad5) origin of replication between nucleotides 25 and 38 (Figure 5), and this sequence is essential for optimal viral replication (57, 58, 71, 84). It represents the optimal binding sequence as determined by mutagenesis studies of various NFI binding sites and also by modelling using the SELEX-SAGE method (52, 53, 106). The sequence of the five nucleotide linker (N_5) located between the two recognized regions does not contribute significantly to the affinity of NFI binding (51). In addition to the NFI binding site, also nucleotides adjacent to this site are important for NFI binding to DNA and for its ability to stimulate replication (1, 28, 145). Moreover the position of the NFI binding site with respect to the core origin is important. Insertion of one or two nucleotides between the pTP/pol binding site and the NFI binding site severely inhibits NFI stimulation of replication (1, 10, 18, 145). Contact point and footprinting analysis showed that almost all contacts of the NFI dimer are clustered at the same side of the DNA helix, mainly in the major groove (29). It is likely that binding of the NFI dimer to the origin is symmetrical, since inversion of its binding site does not influence the ability of NFI to stimulate replication (29, 150).

Adenovirus makes use of NFI to greatly enhance its DNA replication process up to 50 fold (25). An intriguing question is thus, what is the mechanism of NFI stimulation? It was demonstrated that NFI influences the kinetics of replication by increasing the V_{max} of initiation, without affecting the K_m. This observation suggests that NFI does not increase the activity

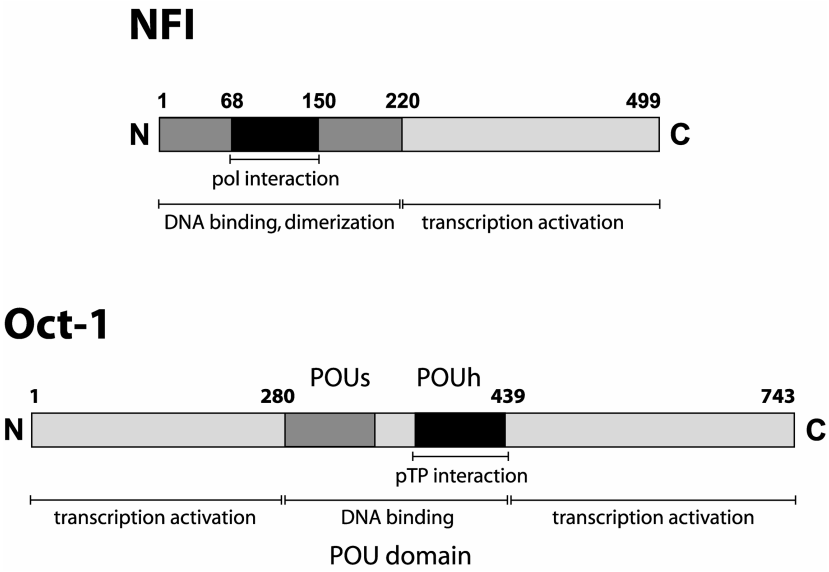

Figure 4. Schematic overview of the primary sequences of NFI and Oct-1
The schemes show the domains of NFI and Oct-1 that are involved in DNA binding and transcription activation. The DNA binding domain of each protein is sufficient for stimulation of Ad5 DNA replication (47, 78, 136). The regions responsible for NFI interaction with pol and Oct-1 interaction with pTP are also marked. Numbers above each scheme refer to the amino acid position within the primary sequence of NFI or Oct-1.

of the initiation complex but rather increases the number of active initiation complexes (80). There are two models that might explain how this is achieved. The first model proposes that binding of NFI to the origin induces changes in the DNA structure, which stabilize DNA binding of other proteins and facilitate the PIC formation (80, 81, 150). This is in agreement with the observation that mutations of the NFI binding site that affect the DNA binding properties of NFI, concomitantly affect the ability to stimulate replication (49). Moreover, studies of Mul *et al.* showed that NFI can stabilize binding of the pTP/pol complex to the origin (80). The second model (the recruitment model) postulates that NFI recruits pol or the pTP/pol complex to the origin through a direct NFI-pol interaction, thereby increasing the number of initiating complexes. NFI directly interacts with pol in solution and for this interaction the DNA binding domain of NFI is sufficient (9, 10, 16). The interaction surface was mapped to the NFI region between residues 68 and 150. The highest stimulation of *in vitro* Ad5 replication by NFI is achieved at physiological (low) pol concentrations (80, 81). It is most likely that the first model contributes the most to the stimulation of replication by NFI and this will be discussed in more details in Chapter 2 and Chapter 3.

Octamer binding protein - Oct-1

Oct-1 is a second cellular transcription factor used by adenovirus to stimulate its DNA replication up to 8 fold (25, 98, 105). It is a 83 kDa protein of 743 aa (Figure 4) that is mainly involved in transcription regulation of a variety of genes (38, 40, 94). Oct-1 has a centrally located bipartite DNA binding domain (POU domain), which is flanked by two transcription activation domains. Similarly to NFI, the DNA binding domain is sufficient for the stimulatory function of Oct-1 in Ad5 DNA replication (136). The POU domain consists of a POU specific domain (POUs) that binds DNA with high specificity but low affinity, and a POU homeodomain (POUh) that binds DNA with low specificity but high affinity (31, 120, 135).

The crystal structure of the POU domain bound to DNA shows that POUs and POUh fold into two separate domains connected with a flexible linker. The POUs domain consists of four α-helices connected by short loops, whereas the POUh domain is formed from three α-helices (68).

Oct-1 binds as a monomer to the recognition sequence 5'TATGATAATG3' at position 39-48 in the Ad5 origin (98, 137)(Figure 5) and the presence of this binding site in the origin stimulates viral replication (56). The POUs domain preferentially binds the 5'-halve and the POUh domain binds the 3'-halve of the Oct-1 recognition sequence. The Oct-1 interaction with DNA involves many contact points, mainly in the major groove, that are located on different sides of the DNA helix (68, 99). POUs and POUh domains recognize DNA with a helix-turn-helix motif (68). There are also strict spacing requirements for the position of the Oct-1 recognition sequence with respect to the pTP/pol binding site, since increasing or decreasing the distance between the core origin and the auxiliary origin by two nucleotides severely affects the stimulatory function of Oct-1 (18).

The mechanism of stimulation of Ad5 DNA replication by Oct-1 resembles the NFI mode of action. Oct-1 also influences the kinetics of replication by increasing the V_{max} of the initiation, without affecting the K_m. As shown by circular permutation assays and ligase mediated cyclization, binding of Oct-1 to the origin DNA induces a DNA bend, which facilitates the PIC assembly (138). In agreement with this notion, Oct-1 stabilizes binding of the pTP/pol complex to the origin by lowering its dissociation rate (133). Similar to NFI, protein-protein interactions are also involved in the Oct-1 mechanism of stimulation. Oct-1 directly interacts with pTP, suggesting that it recruits pTP or the pTP/pol complex to the origin (20, 133). The Oct-1 interaction surface with pTP confines mostly to the POUh domain, and also represents an interaction surface of Oct-1 with DNA (11, 24). Since pTP or DNA binding to Oct-1 is mutually exclusive, the present model of recruitment proposes that the Oct-1-pTP complex is brought to the origin by the POUs domain. Subsequently, the Oct-1-pTP interaction is lost after the POUh domain binds to the origin DNA (24).

Initiation of DNA replication using protein as a primer

To start DNA replication, DNA polymerases require a primer. It is usually a DNA or an RNA molecule, which is complementary to the DNA template and provides the 3' hydroxyl group for the initiation of DNA synthesis. Some bacteriophages, plasmids and viruses utilize a protein as a primer and the first nucleotide is then attached to the hydroxyl group of the amino acid serine, threonine or tyrosine of the priming protein (107, 108). A versatile group of bacteriophages transfecting *B. subtilis* (ϕ29, ϕ15, PZA, Nf, or GA-1) use protein priming to start replication of their genome. Among them, phage 29 (ϕ29) is the most extensively studied example (77). Protein priming is also used by phage PRD1 infecting *E. coli* or *S. typhimurium*, phage Cp-1 infecting *S. pneumoniae*, and also by a variety of linear plasmids isolated from bacteria, yeast, fungi, and higher plants (reviewed in (108)). Also animal viruses like adenovirus, hepadnavirus or parvovirus, were demonstrated to start replication with a protein primer. Among them the protein priming mechanism of Ad has been the most extensively investigated.

During initiation of Ad5 DNA replication, pTP presents the hydroxyl group of Ser580 to the polymerase active site and pol catalyzes the covalent coupling of the first dCMP residue. However, Ad5pol can also use DNA as a primer and it is likely that the same domain of Ad5 pol is used for interaction with DNA or with pTP (14, 27, 65, 77, 108, 125). The crystal structure of RB69 pol reveals details of pol interaction with the primer-template DNA (41). The structure shows that the residues of the highly conserved $K_{705}K_{706}R_{707}Y_{708}$ consensus sequence located in motif VII hold the primer-template DNA in the correct position. K705 and R707 bind the template strand, whereas K706

and Y708 bind the primer stand. Interestingly, in almost all protein-priming DNA polymerases, residues corresponding to K705 and R707 are not conserved, resulting in a KxY consensus sequence (7). Mutagenesis study of Ad5 pol showed that residues K1078 and Y1080 (corresponding to K706 and Y708 of RB69 pol, respectively) are important for Ad5 initiation activity and DNA binding (73). Similarly, the Y500 residue of ϕ 29 pol corresponding to Y708 of RB69 pol is also important for its initiation activity (7). These results demonstrate that the KKRY/KxY consensus sequence is involved in binding of the primer/template for both DNA-priming and protein-priming polymerases. Moreover, since mutation of Y1080 of Ad5 pol strongly affects the interaction of pol with pTP, it suggests that this consensus sequence could be also involved in correct positioning of the protein primer (73). Since the sequence of the KxY motif is limited in protein-priming DNA polymerases compared to DNA-priming polymerases, it suggests that the use of a protein primer requires a different arrangement of the interactions between pol and the primer.

Studies on the molecular architecture of Ad5 pol show that pTP binds at the entrance of the primer binding groove of pol (13), which according to the structure of RB69 pol is also the binding site for the DNA primer (41). In order to present Ser580 of pTP in the active site of pol during initiation, pTP has to insert one of its domains into the primer binding channel of pol, but the exact arrangement of this interaction is not known. Attempts to obtain the structure of the pTP/pol complex have been unsuccessful up to now. Previously, is was shown that pTP influences the catalytic properties of pol, since the exonuclease activity of pol is decreased when it is in complex with pTP (65). Moreover, the change in the active site of pol was observed after dissociation of pTP upon transition from initiation to elongation (66). These data show that pTP is not a simple primer but rather an integral part of the initiating pTP/pol complex.

The origin of Ad5 DNA replication

The genome of Ad5 is a 35935 bp linear and double-stranded DNA containing TP proteins covalently attached to each 5' DNA end. Ad5 has two origins of DNA replication of about 50 bp that are located in the 103 bp long inverted terminal repeats (ITR) at each end of the genome (Figure 5). Deletion studies showed that the first 18 bp of the origin are essential for Ad5 DNA replication (58).

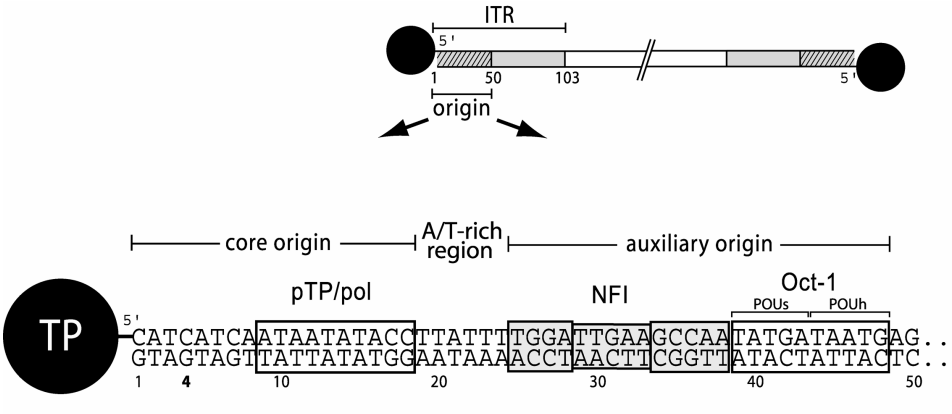

Figure 5. The origin of Ad5 DNA replication

Two 50 bp Ad5 origins of replication are located in the inverted terminal repeats (ITR) of 103 bp at each end of the Ad genome. TP is covalently bound to each 5' DNA end. The origin consists of a highly conserved core origin with the pTP/pol binding site and an auxiliary origin containing the consensus sequences recognized by NFI and Oct-1 (boxed). The binding half-sites for the POU specific domain (POUs) and POU homeodomain (POUh) are indicated. The core origin and the auxiliary origin are separated by a 6 bp long A/T-rich region. Replication is initiated opposite the fourth nucleotide of the template strand.

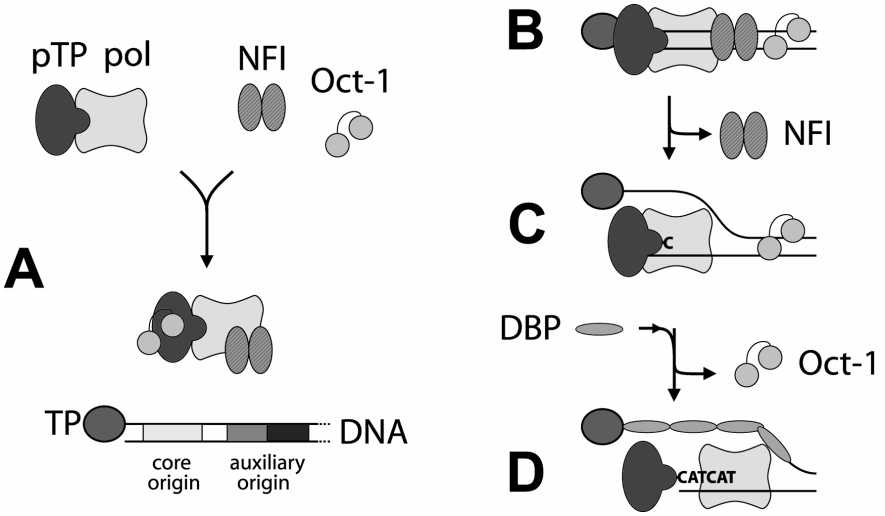

Figure 6. Model of adenovirus DNA replication
Replication of Ad DNA requires three viral proteins, DNA polymerase (pol), precursor terminal protein (pTP), which serves as a primer, and DNA binding protein (DBP). Two cellular transcription factors, NFI and Oct-1, are able to greatly enhance the replication. First the preinitiation complex is formed on the origin (A, B). The core origin is recognized by the pTP/pol complex and the auxiliary origin is bound by NFI and Oct-1. DNA replication is initiated by covalent coupling of the first dCTP residue to a Ser580 hydroxyl group of pTP. At this step NFI dissociated from the DNA. (C). Subsequently, pol dissociates from pTP and elongates DNA with the support of DBP that unwinds and protects DNA. Oct-1 is displaced from the DNA when the replication fork passes (D).

In this region, at position 1-6 there is a GTAGTA triplet repeat, which is important for the correct initiation of replication (67). Moreover, a highly conserved core origin is located at position 9-18 that is recognized by the pTP/pol complex. The core origin is followed by the auxiliary origin containing the binding sites for NFI (position 25-38) and Oct-1 (position 39-48). The core origin and the auxiliary origin are separated by a 6 bp long A/T-rich region, which is conserved among different Ad serotypes.

The model of Ad5 DNA replication

In order to start Ad5 DNA replication first the pre-initiation complex consisting of viral and cellular proteins is formed on the origin (25, 26)(Figure 6). Then the replication process is initiated by covalent coupling of the first dCMP nucleotide to the Ser580 hydroxyl group of pTP by pol, opposite a G residue located at position 4 of the template strand. This is followed by addition of two more nucleotides, A and T, generating a pTP-CAT intermediate.

Next, the newly synthesized CAT trinucleotide jumps back from positions 4-6 to positions 1-3 at the beginning of the template strand (26, 67). The jumping-back mechanism allows correction of possible errors caused by a decreased accuracy of pol during the first steps of initiation and it is likely to be universal for protein-primed DNA replication systems (67, 107, 108). After jumping-back, pol dissociates from pTP and processively elongates DNA (66). The elongation process is facilitated by DBP, which unwinds double-stranded DNA and stabilizes single-stranded DNA that is displaced in the process of replication (30). NFI leaves the preinitiation complex early during initiation, whereas Oct-1 dissociates from DNA during elongation after at least 26 nucleotides have been replicated (19, 133). When replication of the entire viral genome is completed, covalently attached pTP is cleaved by a viral protease to its mature form TP, which stays attached to each DNA end for the remaining part of the viral life cycle (141, 143). This form of viral DNA is also referred to as TP-DNA.

III. Protein-induced DNA bending

A DNA molecule is structurally flexible and certain DNA regions can adopt an energetically preferred conformation, generating an intrinsic DNA bend, the curvature of which is determined by the nucleotide sequence (22, 69). This was demonstrated for the first time in studies of minicircle DNA from the kinetoplast of *Leishmania tarentolae* (146). In addition to the intrinsic bend, a DNA bend can be also induced as the result of the interaction between a protein and DNA. This involves intercalation of specific protein residues into the minor or the major groove of DNA (144). This manipulation results in DNA distortion observed as narrowing or broadening of a particular DNA groove. The DNA bend is described by a certain angle value and is defined as the angle by which a segment of DNA departs from its linear form. It can be analysed by techniques based on aberrant migration of bent DNA in polyacrylamide gels, like circular permutation analysis and phasing analysis (132). Another method, the cyclization analysis, is based on the observation that a DNA bend induced in a linear DNA increases the rate of its circularization by DNA ligase. More advanced techniques like scanning force microscopy (SFM) and crystallization allow visualization of protein-DNA complexes and direct measurement of DNA bend angles. Protein-induced DNA bending plays an important role in many aspects of DNA metabolism. It is involved in packaging of DNA into nucleosomes, regulation of gene expression as well as DNA replication, repair and recombination (54, 55, 63, 102, 132, 134).

This paragraph will focus only on protein-induced DNA bending in transcription regulation and in DNA replication. The first part will demonstrate that the magnitude of a protein-induced DNA bend (the value of an angle) is determined by the amino acid sequence of a protein or the nucleotide sequence of DNA. The second part will present the functional significance of DNA bending for transcription regulation and DNA replication. Moreover, examples from various systems will be presented, demonstrating that specifically the magnitude and the direction of a protein-induced DNA bend are functionally important.

The DNA and protein sequence determines the protein-induced DNA bend

There are numerous examples of proteins involved in transcription regulation and DNA replication, that induce a DNA bend (132). The magnitude of this bend widely extends from a slight to a severe kink induced by proteins like the bacterial integration host factor IHF (180°), eukaryotic transcription factors LEF-1 (lymphoid enhancer-binding factor 1, 130°) and Sox2 (80°) (44, 101, 110), and it is determined by several factors. One determinant is the nucleotide sequence of the DNA binding site that is recognized by a DNA-bending protein, since certain mutations of the binding site lead to decrease or increase of the protein-induced bend angle. This was demonstrated for several proteins, including yeast mitochondrial RNA polymerase, eukaryotic TATA binding protein (TBP), bacterial catabolite gene activator protein (CAP) and eukaryotic transcription factors LEF-1 and Sox2 (43, 44, 75, 88, 110, 111). A second factor determining the magnitude of a protein-induced DNA bend is the presence of specific DNA regions upstream or downstream from the binding site, that act synergistically with a protein-induced DNA bend. An example is the yeast regulatory protein Reb1p that induces a DNA bend in the promoter of the yeast profilin gene where the presence of a neighbouring dA-dT sequence is important for Reb1p-induced DNA bending (3). Also sequences outside the binding site of CAP protein affect the degree of the CAP-induced DNA bend (74). Another example is T-antigen (T-ag) from simian virus 40 (SV40), which is involved in viral DNA replication. The DNA bend induced by T-ag in the origin is located within a triplet of 21-bp repeats, but when only one or two of the triplets are present, bending does not occur (85). A third important determinant of a protein-induced DNA bend angle is the amino acid sequence of a binding protein and it was shown that specific amino

acids contribute to DNA bending. The N48Q mutation of Sox2 and the V34A mutation of the yeast transcription factor Mcm1 as well as mutations of IHF decrease the DNA bend angle (15, 36, 45, 110).

The functional significance of the protein-induces DNA bending

Although introduction of a bend in DNA requires energy input, such DNA distortions are necessary and have significant functional consequences for transcription regulation and DNA replication. Several studies show convincingly that the reduction of a protein-induced DNA bend leads to functional defects. Decrease of a DNA bend angle induced by the transcription factors Sox2, LEF-1 and Mcm1, consequently affects the ability of these proteins to activate transcription (15, 44, 75, 110). Also, the magnitude of the DNA bend induced by yeast mitochondrial RNA polymerase correlates with the transcriptional activity (111). Another example is the IHF protein, which binds to the phage λ early P_L promoter from the maltose operon and activates transcription (45). Mutations of IHF that reduce its ability to bend DNA also lead to a decrease of transcriptional activity. Moreover, studies of SV40 DNA replication showed that inhibition of DNA bending by T-ag, concomitantly reduces the replication efficiency (85). From all these observations an intriguing question emerges. How does protein-induced DNA bending contribute to transcription regulation and DNA replication?

DNA bending shapes the nucleoprotein architecture of the PIC

During initiation of transcription or during DNA replication, a preinitiation complex (PIC) consisting of many proteins is assembled on a relatively short DNA stretch of a core promoter or an origin. Since all these proteins would not fit on a linear DNA molecule, a specific nucleoprotein architecture of the PIC is required in order to form the optimally functional complex (90, 124). At this stage, protein-induced DNA bending plays an important role in shaping the DNA structure, which involves one or more DNA bends. When multiple proteins bind and consequently more DNA bends are present, their orientation with respect to each other is functionally important and it depends on the DNA spacing between the two induced bends. A good example is the function of the viral EBNA-1 proteins during the Epstein-Barr virus (EBV) DNA replication. Bashaw *et al.* showed that the precise 21 bp spacing between two binding sites bound by the viral EBNA-1 protein in the origin is essential for replication, since an increase or decrease of this spacing by 1 or 2 bp completely impairs replication (4). Each EBNA-1 dimer binds DNA at the same side of the helix and induces a DNA bend. The spacing of 21 bp, positions the two DNA bends in helical phase, resulting in a larger combined DNA bend. Therefore, any change of the spacing alters the direction of these DNA bends relative to each other, resulting in a distorted architecture of the replication complex and abolished replication (42). Another example is the PLS1 plasmid-encoded RepA protein, which was shown to activate or repress transcription depending on the position of the RepA-induced DNA bend relative to the DNA bend induced by RNA polymerase (91). Similarly, the specific direction of the DNA bend induced by Sox2 also leads to the opposite effects on transcription (110).

DNA bending facilitates protein-DNA and protein-protein interactions

Another essential function of DNA bending is to facilitate and stabilize protein-DNA and protein-protein interactions, and it can be achieved on different levels of interaction. The first level involves proteins interacting over a short distance within the core promoter or the core origin, whereas the second level involves the DNA-bound regulatory proteins that are located further apart. The replication of bovine papilloma virus (BVP) illustrates the first case. Viral transcription factor E2 stimulates binding of the viral E1 protein to the origin DNA, and thus the E1-E2 complex binds the origin cooperatively (46, 70). The E1-E2 complex

induces a sharp DNA bend and DNA mutations that inhibit DNA bending also affect the cooperative binding of E1-E2 complex to the origin. Also in transcription regulation, the eukaryotic DNA-bending protein HMG1 facilitates binding of the human progesterone receptor to the progesterone response elements in the promoter (86). Furthermore, the yeast transcription factor Mcm1 binds to the P'Q-element present in the promoter of the α-specific genes of the α-type cells. Its interaction with the α1 protein significantly increases the Mcm-1-induced DNA bend, thus promoting transcriptional activation (15).

When the important regulatory proteins are located further apart, they can be brought in close proximity by formation of a DNA loop, which is facilitated by DNA bending (21, 90). This subsequently promotes additional protein-protein or protein-DNA interactions. A good illustration of such a mechanism is the regulation of the transcriptional activity by the p4 protein from *B. subtillis* phage ϕ29. Activation of transcription occurs only when the DNA between the p4 binding site and the RNA polymerase binding site is bent and has the optimal length allowing interaction between p4 and RNA pol (112). Formation of a DNA loop that facilitates interaction with RNA pol was also observed for the human heat-shock transcription factor 2 (HSF2), for the regulatory protein Jun and for the bacterial enhancer protein NtrC (5, 121, 147).

DNA bending destabilizes the DNA structure

Another important function of protein-induced DNA bending is destabilization or strand separation of a local DNA region (123). It is proposed that energy invested in DNA bending facilitates DNA opening, and once the DNA is open it will bend more easily (100). Formation of the "open" transcription complex involves bending and unwinding of the promoter DNA (92). Also the origins of replication often contain a less stable A/T-rich DNA region, and it is likely that DNA bending can facilitate unwinding of DNA in this region (32).

IV. The outline of the thesis

The aim of the experiments described in this thesis is to learn more about the mechanism of Ad5 DNA replication, specifically concentrating on two early initiation events: first, the assembly of the preinitiation complex (PIC) on the origin DNA, and second, the mechanism of protein priming. Ad uses the cellular transcription factors, NFI and Oct-1, to enhance its DNA replication. Chapter 2 raises the question how NFI performs its function. Using scanning force microscopy (SFM) it is demonstrated that NFI induces a 60° bend upon binding to the origin DNA. Moreover, this DNA bend is functionally important, since mutations of the A/T-rich region preceding the NFI recognition sequence reduce the bend angle and lead to a loss of NFI-stimulated replication. The SFM analysis is continued in Chapter 3 showing that Oct-1 induces a 42° bend in the origin DNA. When NFI and Oct-1 are bound simultaneously to the origin, they induce a 82° bend. The SFM data combined with the functional replication assays demonstrate that the two DNA bends induced by NFI and Oct-1 alone are oriented towards each other, which leads to the synergistic stimulation of replication in the presence of both proteins. Chapter 3 is followed by an Addendum in which the effect of the mutation in the A/T-rich region on DNA bending and stimulation by Oct-1 is examined.

The protein-primed initiation is an important step during Ad5 DNA replication. In Chapter 4 the role of pTP in initiation is analysed by mutagenesis of the conserved Asp578 and Asp582 residues of pTP located in the priming region close to the Ser580. The presented data demonstrate that pTP contributes to the kinetics of initiation. Since there are more negatively charged residues close to Ser580, the Addendum of Chapter 4 investigates the importance of other acidic residues from the priming region of pTP. Finally, in Chapter 5, a model for PIC assembly and protein priming is presented and future perspectives for the Ad5 DNA replication research are discussed.

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Chapter

2

Bending of adenovirus origin DNA by Nuclear Factor I as shown by scanning force microscopy is required for optimal DNA replication

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Bending of adenovirus origin DNA by Nuclear Factor I as shown by scanning force microscopy is required for optimal DNA replication

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Nuclear Factor I (NFI) is a transcription factor that binds to the Adenovirus type 5 origin of replication and recruits the Ad DNA polymerase, thereby stimulating initiation of DNA replication *in vitro*. Using scanning force microscopy we demonstrate that NFI induces a 60° bend upon binding to the origin. The A/T-rich region preceding the core recognition sequence of NFI influences the DNA bend angle, since substitution of A/T basepairs by G/C basepairs severely decreases bending. Mutations in the A/T-rich region do not affect binding of NFI to DNA. However, mutations that reduce the protein induced bend lead to a loss of NFI stimulated replication, indicating that DNA bending is functionally important. In contrast, basal initiation or DNA binding of the polymerase is not impaired by these origin mutations. We conclude that binding of NFI to the Ad5 origin causes structural changes in DNA that are essential for the stimulatory function of NFI in replication. We propose that NFI induced origin bending facilitates the assembly of a functional initiation complex.

Introduction

Replication of Ad5 DNA is well characterised and can be fully reconstituted *in vitro* using purified proteins. There are three viral proteins involved in this process, precursor terminal protein (pTP) that serves as a primer, adenovirus DNA polymerase (pol) and DNA binding protein (DBP). Two cellular transcription factors, Nuclear Factor I (NFI) and Octamer binding protein (Oct-1) are able to greatly enhance this replication process (14). Ad5 contains a 36 kb double stranded linear genome with terminal proteins (TP) covalently attached at the 5' ends. The origins of DNA replication are located at both ends of the genome in 103 bp long inverted terminal repeats (ITR). The core origin at position 9-18 is a conserved region bound by pTP and DNA polymerase, which form a stable heterodimer in solution and bind DNA as a complex. The core origin is followed by the auxiliary origin recognised by transcription factors NFI and Oct-1 (nucleotides 25-38 and 39-47, respectively). The core origin and the auxiliary origin are separated by a 6 bp long A/T-rich region (Figure 1).

NFI is a transcription factor that was originally discovered based on its ability to stimulate adenovirus (Ad) DNA replication *in vitro* (33). It was later found to be part of a family of related proteins that play an important role in transcription regulation of a large variety of cellular and viral genes (20). Deletion studies show that the conserved N-terminal domain of NFI (residues 1-220) is required for DNA binding and protein dimerisation. The C-terminal domain (residues 220-499) is involved in transcription activation (19, 30). The DNA binding domain is necessary and sufficient for the stimulation of adenovirus DNA replication by NFI, whereas the transcription activation domain is dispensable for this function (8, 30). NFI binds as a dimer to the consensus sequence TGG(C/A)(N_5)GCCAA

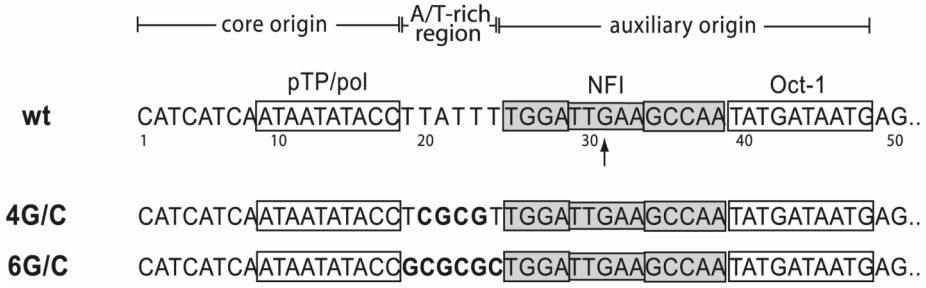

Figure 1. DNA sequence of the wild type Ad5 origin and the 4G/C and 6G/C mutants
The Ad5 origin consists of the core origin containing the pTP/pol binding site and the auxiliary origin containing the consensus sequences recognised by the transcription factors NFI and Oct-1 (boxed). The A/T-rich region is located between the core origin and the NFI binding site. The 4G/C origin mutant was constructed by replacing the TATT sequence at positions 20-23 with CGCG and the 6G/C origin mutant by replacing the TTATTT sequence at positions 19-24 with GCGCGC. The mutated nucleotides are shown in bold. The arrow indicates the middle of the NFI consensus sequence at position 31.

located at the adenovirus 5 (Ad5) origin of replication between nucleotides 25 and 38 (28, 34) (Figure 1). This sequence is essential for optimal viral replication *in vivo* (25, 26) and represents a perfect match to the optimal binding sequence determined by mutagenesis studies of NFI binding sites (21, 22) and by the SELEX-SAGE modelling method (38). Contact point analysis shows that almost all contacts of the NFI dimer are clustered at one side of the DNA helix, mainly in the major groove (16). NFI influences the kinetics of replication by increasing the V_{max} of initiation, without affecting the K_m, suggesting that NFI does not increase the activity of the initiation complex but rather the number of active initiation complexes (31). In agreement with these observations, it is postulated that NFI recruits polymerase to the origin *via* a direct interaction with polymerase. Additional experiments confirm such an interaction in solution (5, 8). The highest stimulation of *in vitro* Ad5 replication by NFI is observed at physiological DNA polymerase concentrations (31, 32). The position of the NFI binding site with respect to the core origin is also important. Insertion of one or two nucleotides between the pTP/pol binding site and the NFI binding site severely inhibits NFI stimulation of replication *in vitro* and *in vivo*, suggesting that an interaction exists between NFI and the pTP/pol complex on the origin (2, 5, 9, 47). In addition to the NFI binding site, nucleotides adjacent to this site are also important for NFI binding and for its ability to stimulate replication (2, 15, 47). Studies of Mul *et al.* show that NFI can stabilise binding of the pTP/pol complex to the origin (31). Furthermore, binding of NFI to the origin might induce structural changes in the DNA thereby stimulating replication (31, 32, 48). Protein-induced changes in DNA structure such as bending play an important role in many aspects of DNA metabolism, like packaging DNA into nucleosomes, regulation of gene expression, DNA replication, repair and recombination. DNA bending facilitates the assembly of multi-protein complexes and consequently protein-protein and protein-DNA interactions (45)

In this study we used scanning force microscopy (SFM) to show that NFI induced a bend of 60° in DNA upon binding to the Ad5 origin. In addition, the A/T-rich region preceding the NFI binding site was required for formation of a functional bend DNA-protein complex. This A/T-rich region was essential for NFI stimulation of Ad5 DNA replication. We propose that optimal replication only occurs when all the proteins and the bent origin DNA are properly assembled in the preinitiation complex.

Materials and Methods

Construction of origin mutants

Origin mutants were constructed by site directed mutagenesis performed on the PHRI plasmid using the Quickchange method from Stratagene. PHRI is a pUC9 derivative containing the first 103 bp of the Ad5 origin of replication. EcoRI digestion of this plasmid leads to a 2.9 kb DNA fragment with the origin located at the end, which makes the fragment suitable as a template for replication studies (26). Oligonucleotides for the PCR based mutagenesis were:
4G/C: 5'CAATAATATACCT**CGCG**TTGGATTGAAGC3',
5'GCTTCAATCCAA**CGCG**AGGTATATTATTG3',
6G/C: 5'CAATAATATACC**GCGCGC**TGGATTGAAGC 3',
5'GCTTCAATCCA**GCGCGC**GGTATATTATTG3' with the mutations marked in bold. The presence of the mutation was confirmed by DNA sequence analysis.

Proteins and buffers

For all experiments the DNA binding domain of rat NFI type A_1 (NFI-BD; position 5 to 242) was used. Of the 499 aa long NFI protein, the N-terminal 238 aa contain the DNA binding domain. The NFI-BD will be further referred to in the text as NFI. NFI, adenovirus DNA polymerase, pTP and DBP were expressed using the baculovirus expression system and purified to near homogeneity as previously described. (7, 13, 32, 44). The dilution buffer contained 25 mM HEPES-KOH pH 7.5, 150 mM NaCl, 15% glycerol, 5 mM DTT and 0.5 µg/µl BSA.

Scanning force microscopy

For the SFM studies of NFI-DNA complexes a 711bp NdeI/AflIII DNA fragment of the PHRI plasmid was used. This fragment contains the Ad5 origin of replication with the middle of the NFI consensus sequence located at 35% of the DNA length, starting from position 249. The fragment was purified from a 1% agarose gel using the QIAEX II Gel Extraction Kit (Qiagen) and resuspended in 10 mM HEPES-KOH pH 8.0. The binding of NFI to this fragment was confirmed by an electrophoretic mobility shift assays (EMSA) (data not shown). The SFM depositions were performed as follows. NFI (4.5 pmol) was incubated with the DNA fragment (0.19 pmol) for 15 minutes on ice in replication buffer (see below). The final mixture was diluted 20-30 times in deposition buffer (5 mM HEPES-KOH pH 7.8, 5 mM $MgCl_2$) and deposited onto freshly cleaved mica. After 1 min the surface of the mica was washed with 3 ml of HPLC grade water and dried in a stream of air. The complexes were imaged in the tapping mode using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). The DNA contour length and the DNA bend angle were measured using the Image SXM v 1.69 software, a NIH Image version modified for use with SXM images by Steve Barrett, Surface Science Research Centre, University of Liverpool, UK. For each measurement we analysed 100-150 molecules. The obtained data were analysed in Sigma Plot and Matlab.

Protein-DNA interactions (EMSA)

For protein-DNA binding studies TD50 was used, a double stranded oligonucleotide consisting of T50 and D50 that represent the first 50 nucleotides of the template and displaced strand of the Ad5 origin, respectively.
T50:5'CTCATTATCATATTGGCTTCAATCCAAAATAAG GTATATTATTGATGATG3',
D50:5'CATCATCAATAATATACCTTATTTTGGATTGAA GCCAATATGATAATGAG3'. In the case of mutated origins, oligonucleotides of the same length but containing the desired mutations were used. For the preparation of TD50, the D50 oligonucleotide was end-labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP (4500 Ci/mmol) in a standard kinase buffer. D50 was subsequently hybridised with T50 and the labelled TD50 was purified on a 10 % polyacrylamide gel. The DNA binding assays were performed in EMSA experiments as follows. NFI or Ad5 DNA polymerase were incubated with DNA for 30 min on ice in binding buffer (25 mM HEPES KOH pH 7.5, 10 mM $MgCl_2$, 4 mM DTT, 1 mM EDTA, 0.1 mg/ml BSA, 4% Ficoll) For the NFI binding studies 80 mM NaCl and 1 µg of poly(dI-dC)-(dI-dC) was used to exclude non-specific DNA-protein interactions. The Ad5 polymerase binding studies were performed at 50 mM NaCl in the absence of competitor DNA to optimise binding to the core origin (41). TD50-protein complexes were separated on a 10 % polyacrylamide gel in TBE buffer at 4°C and the intensity of the bands was quantified using a Storm 820 phosphorimager. In the case of 711 bp probe protein-DNA complexes were separated on a 1% agarose gel.

In vitro DNA replication and initiation assays

In vitro DNA replication was performed using the indicated amounts of Ad5 DNA polymerase, Ad5 pTP, Ad5 DBP and NFI-BD in a 20 µl reaction mixture containing replication buffer (25 mM HEPES-KOH pH 7.5, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT), 40 µM dATP, dTTP, dGTP, 0.7 µM dCTP and 4 µCi of [α-^{32}P] dCTP (3000 Ci/mmol). As a template, 100 ng PHRI plasmid linearised with EcoRI or 30 ng XhoI digested viral TP-DNA were used. TP-DNA is a 36 kb linear Ad5 genome with the terminal protein (TP) covalently attached to both DNA ends. TP-DNA was isolated from Ad5 virions as previously described (10). XhoI digestion generated seven fragments, two of which (6.2 kb fragment B and 5.8 kb fragment C) contain the origin. The replication mixtures were incubated for 45 min at 37°C and stopped by the addition of 2 µl stop mix (40% sucrose, 1% SDS, 0.1% bromophenol blue and 0.1% xylene cyanol). The replication products were separated on a 1% agarose gel containing 0.1% SDS in 0.5×TBE/0.1%SDS buffer. The replicated bands were quantified using a Storm 820 phosphorimager.

In vitro initiation was assayed using the indicated amounts of Ad5 DNA polymerase and Ad5 pTP, in a 20 µl reaction mixture containing initiation buffer (20 mM HEPES-KOH pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 40 ng/µl BSA) and 4 µCi of [α-^{32}P] dCTP (3000 Ci/mmol). As a template, 10 ng TD50 representing the first 50 bp of the Ad5 origin DNA was used. Reactions were incubated for 45 min at 37°C and stopped by the addition of EDTA to a final concentration of 80 mM. Initiation products were precipitated with 20% trichloroacetic acid (TCA), washed with 5% TCA, dissolved in a sample buffer and analysed on 7.5% polyacrylamide/SDS gel. The intensity of the initiation bands was quantified using a Storm 820 phosphorimager.

Results

NFI induces DNA bending upon binding to the Ad5 origin

To study changes in the origin DNA structure induced by NFI we used a 711 bp ds DNA fragment containing the first 103 bp of the Ad5 inverted terminal repeat (ITR) from the left end of the virus genome. This fragment contains the 50 bp origin, located at about one third of the 711 bp DNA fragment starting from position 218. The middle of the consensus sequence recognised by NFI (Figure 1) is located 31 bp upstream from the beginning of the origin, at position 249 (35% of the 711 bp DNA fragment from one side). The remaining part of the fragment contains no obvious NFI recognition sequences. For our experiments we used the DNA binding domain of NFI (NFI-BD, aa 5-242), which will further be referred to as NFI. The DNA binding domain of NFI is sufficient for dimerisation and the stimulation of adenovirus replication (8, 30). To study NFI-DNA complexes by SFM the 711 bp DNA fragment was incubated with NFI, at a 24 times molar excess of NFI over DNA, for 15 minutes under conditions similar to the ones used for the replication assays.

NFI was observed bound specifically to DNA at the position of the origin. The position of NFI bound to DNA was determined by measuring the length of the DNA from the centre of the protein in a protein-DNA complex, to each end of the DNA (Figure 2). The binding position was then expressed as the ratio *r* of the length of the shorter DNA arm divided by the total DNA length (contour length) with a theoretically calculated value of $r = 0.35$ for the middle of the NFI consensus sequence in the origin. 150 molecules were measured and the position of the protein was on average of 0.36 ± 0.09, which confirms that NFI is bound to the origin (Table I). Very few NFI molecules (3/150) were bound to non-specific sites on the DNA.

DNA bend angle is defined as the angle by which a DNA segment departs from linearity. The bend angle induced at the Ad5 origin by NFI was determined by measuring the angle between two 20 nm long straight segments of DNA on each side of NFI followed by subsequent calculation of the angle by which DNA deviates from straight. As a control the intrinsic bending of the origin was determined in the absence of NFI by measuring the DNA bend angle at 35% of the DNA length from both ends. Since such a measurement resulted in a mixture of origin and non-origin angles, we measured the DNA bend angle of a random DNA segment at the middle and subtracted this distribution from the mixed distribution to get accurate data on intrinsic bend angle of the origin DNA. Mean bend angles with standard deviations were determined from Gaussian curve fitting according to the equation (1) in Schulz et al. (39). From the fitting defined in equation (2), which corrects for different directions of bending, we obtained similar results with at most a 3º difference in the mean bend angle (data not shown). The NFI-bound DNA was compared with the unbound DNA and a clear increase of the bend angle was observed (Figure 2A, Table I). The average bend induced by NFI at the Ad5 origin, was $60° \pm 19$. A DNA bend of $17° \pm 7$ was present in the absence of NFI showing that the Ad5 origin DNA is intrinsically bent.

There was evidence of additional slight DNA distortion caused by NFI binding. DNA contour length was analysed by tracing the DNA molecules from one end to the other in the absence and in the presence of NFI. For the 711 bp DNA fragment values of 226 ± 19 nm without NFI, and 217 ± 26 nm with NFI were obtained (Figure 2A, Table I). A slight ~ 9 nm shortening of protein-bound DNA molecules corresponding to ~ 28 bp is observed. It is unlikely that the observed DNA shortening is caused by DNA wrapping around NFI, since only 28 bp are involved. Alternatively, we suggest that for the Ad5 origin DNA shortening is rather the result of DNA compaction caused by NFI binding.

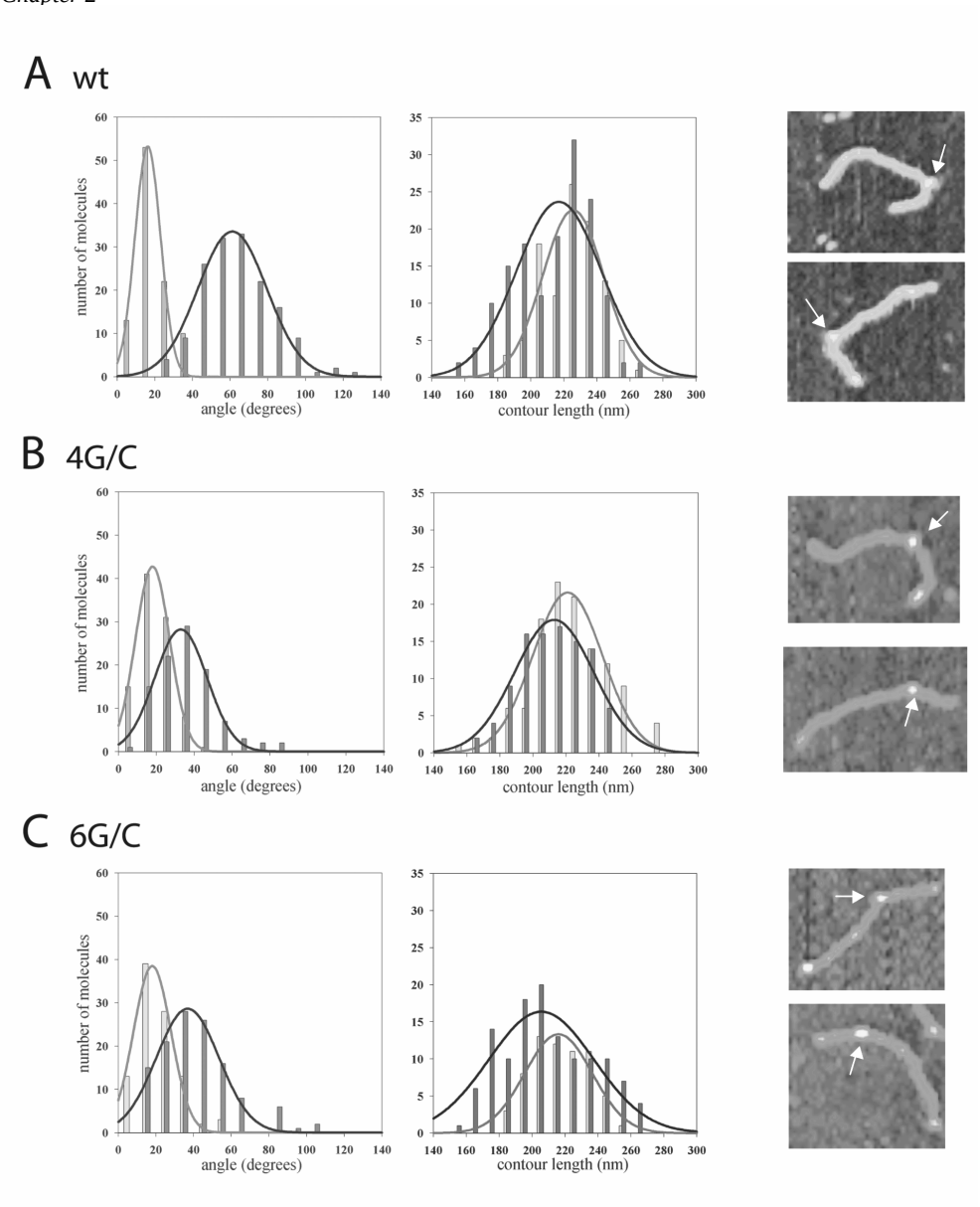

Figure 2. DNA bend angle and contour length distributions based on SFM measurements
(A) DNA fragment with the wild type origin (B) DNA fragment with the 4G/C origin (C) DNA fragment with the 6G/C origin. The two panels show the SFM data as histograms representing the DNA bend angle distribution (left) and the DNA contour length distribution (right). The distribution of the protein-free DNA molecules is represented with light grey bars and the distribution of the NFI-DNA complexes with dark grey bars. The grey and black lines represent the Gaussian fitting of the distribution as defined in equation (1) of Schulz et al. (39). A 711 bp DNA fragment was used containing the 103 bp of Ad5 origin of replication with the middle of the NFI consensus sequence at position 249 of the fragment. For each experiment 100-150 molecules were analysed. The mean values with standard deviations are presented in Table I. At the right zoomed SFM images of the representative NFI-DNA complexes are shown. An arrow indicates NFI bound to DNA.

Table 1. Summary of the SFM data

Origin DNA	Protein position *r*	Angle (degrees)	Contour length (nm)
Wild type			
DNA	-	17 ± 7	226 ± 19
DNA-NFI	0.36 ± 0.09	60 ± 19	217 ± 26
4G/C			
DNA	-	18 ± 9	221 ± 21
DNA-NFI	0.37 ± 0.04	33 ± 14	213 ± 24
6G/C			
DNA	-	18 ± 10	216 ± 20
DNA-NFI	0.36 ± 0.04	37 ± 17	206 ± 32

NFI-induced bending is influenced by an A/T-rich region in the Ad5 origin

The NFI binding site in the Ad5 origin is preceded by the A/T-rich region which might be involved in DNA bending (Figure 1). To test if mutations in the A/T-rich region alter the DNA bend angle induced by NFI, two origin mutants were generated: the 4G/C mutant by replacing the TATT sequence at positions 20-23 with CGCG and the 6G/C mutant by replacing the TTATTT sequence at positions 19-24 with GCGCGC (Figure 1). Introduction of GC pairs in place of AT pairs raises the energy required for strand separation of this part of the DNA. The 4G/C and 6G/C substitutions do not change the consensus NFI and pTP/pol binding sequences in the origin and moreover, the correct spacing between the core origin and the NFI binding site is maintained.

To analyse the effect of the 4G/C and 6G/C mutants on DNA-NFI complex formation with SFM we used again the 711 bp DNA fragment, but with the mutated origins. The experiments were performed in the absence and in the presence of NFI (Figure 2BC, Table I). Binding of NFI to the 4G/C origin caused clear bending of DNA, but the angle was smaller (33° ± 14) compared with the bend induced in the wild type origin (Figure 2BC). A similar effect was seen with the origin containing the 6G/C mutation, where NFI induced a bend of 37° ± 17 (Figure 2BC). For both mutants the position of NFI on the DNA corresponded with correct origin binding (Table I). Based on these results we conclude that substitution of AT pairs with GC pairs in the A/T-rich region results in reduced bending of DNA by NFI.

In the absence of NFI the intrinsic bend of the 4G/C origin has an 18° ± 9 angle, and the 6G/C origin is bent by 18° ± 10 (Figure 2BC, Table I). This shows that the mutations in the A/T-rich region do not significantly alter the intrinsic bend angle of the origin. However, it cannot be excluded that the mutations induce small changes in DNA curvature that are not possible to detect by SFM analysis because of the resolution of the method (49). The contour length of the 4G/C and the 6G/C DNA molecules was also measured in the presence and absence of NFI (Figure 2BC, Table I). Similar results as for the wild type DNA were found. Again a slight but consistent shortening of the protein-bound DNA molecules was observed, which was ~ 8 nm (~ 26 bp) and ~ 10 nm (~ 33 bp) for 4G/C and 6G/C origin, respectively.

Binding of NFI to the mutated Ad5 origins is not significantly altered

Since the 4G/C and 6G/C mutations in the A/T-rich region are introduced close to the NFI consensus sequence, they might influence the DNA binding strength of NFI (Figure 1). Therefore, we performed electrophoretic mobility shift assays (EMSA) using the first 50 base pairs of the Ad5 origin as a template (TD50) containing the wild type or the mutated A/T-rich region. Addition of NFI resulted in the formation of a single DNA-NFI complex (Figure 3).

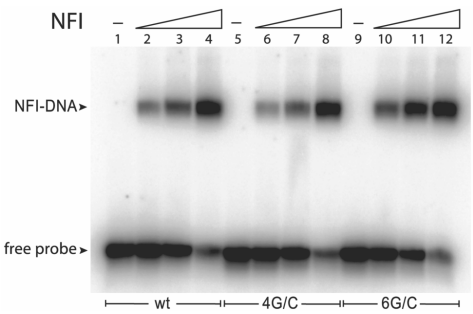

Figure 3. DNA binding of NFI is not significantly affected by the mutations

The binding ability of NFI to the origins containing 4G/C and 6G/C mutations was studied in EMSA experiments using wild type or mutated ds TD50 probes containing the first 50 nucleotides of the Ad5 origin of replication. The position of the NFI-DNA complexes and free probe are marked with arrowheads. Radiolabelled TD50, wt (lanes 1-4), 4G/C (lanes 5-8) and 6G/C (lanes 9-12) were incubated with 30 ng (lanes 2, 6, 10), 60 ng (lanes 3, 7, 11) and 120 ng (lanes 4, 8, 12) of NFI. Lanes 1, 5 and 9 represent free probe.

DNA binding affinities were measured for three different NFI concentrations. At the highest NFI concentration the binding affinity of NFI to the mutated origins 4G/C and 6G/C were 100% and 105%, respectively compared to the wild type affinity (100%).

The stimulation of Ad5 replication by NFI on mutated origins is impaired

To analyse if the AT to GC conversions in the A/T-rich region affected the stimulation of replication by NFI we performed *in vitro* replication assays. First we used the natural XhoI digested TP-DNA templates from Ad5 virions. TP-DNA is the 36 kbp long linear viral genome including terminal proteins (TP) covalently attached to each 5' end. When a range of NFI concentrations was used optimal stimulation of TP-DNA replication up to 7 times was observed (Figure 4A). This indicates that the NFI used in the SFM experiments was functionally active.

Since it is not technically possible to create TP-containing mutated templates, we investigated the NFI stimulation ability on mutated TP-free DNA origins. TP-free origin DNA is a less effective template but still supports replication (40, 43). Digestion of the PHRI plasmid with EcoRI generated a linear 2.9 kb DNA fragment with the origin of replication at the end. Under conditions similar to TP-DNA, replication of the PHRI DNA fragment containing the wild type origin was stimulated by NFI up to 5 times (Figure 4B). In previous experiments we determined the optimal NFI concentration for both templates (data not shown). For the TP-DNA template saturation of the NFI binding sites occurred already with 63 ng of NFI, whereas the PHRI template replication was still stimulated at higher concentrations of NFI (up to 250 ng).

Next, we used EcoRI digested PHRI DNA fragments containing the 4G/C or 6G/C mutation in the origin as a template. Interestingly, the replication stimulation of these two DNA fragments by NFI is severely affected (Figure 4B). Since the stimulation by NFI is maximised at low pTP/pol levels (31, 32) we also tested the mutant templates at lower pTP/pol concentration. The stimulation was 32-fold under these conditions with wild type, and it decreased to 6-fold and 3-fold for the 4G/C and 6G/C mutated origins, respectively (data not shown). Based on these results we conclude that the A/T-rich region is necessary for NFI function and replacement of AT bases with GC bases severely impairs the stimulation ability of NFI. Since the DNA binding affinity of NFI is not affected by the mutations, we propose that improper bending of the mutated origins causes severe reduction of stimulation of replication.

It should be noted that in the absence of NFI the basal level of replication of the origins containing the mutations is lower in comparison with wild type origin (65% and 20% of wild type for 4G/C and 6G/C, respectively). The decrease of the basal level of replication may be caused by difficulties in unwinding of the DNA in the process of elongation due to the AT to GC substitutions.

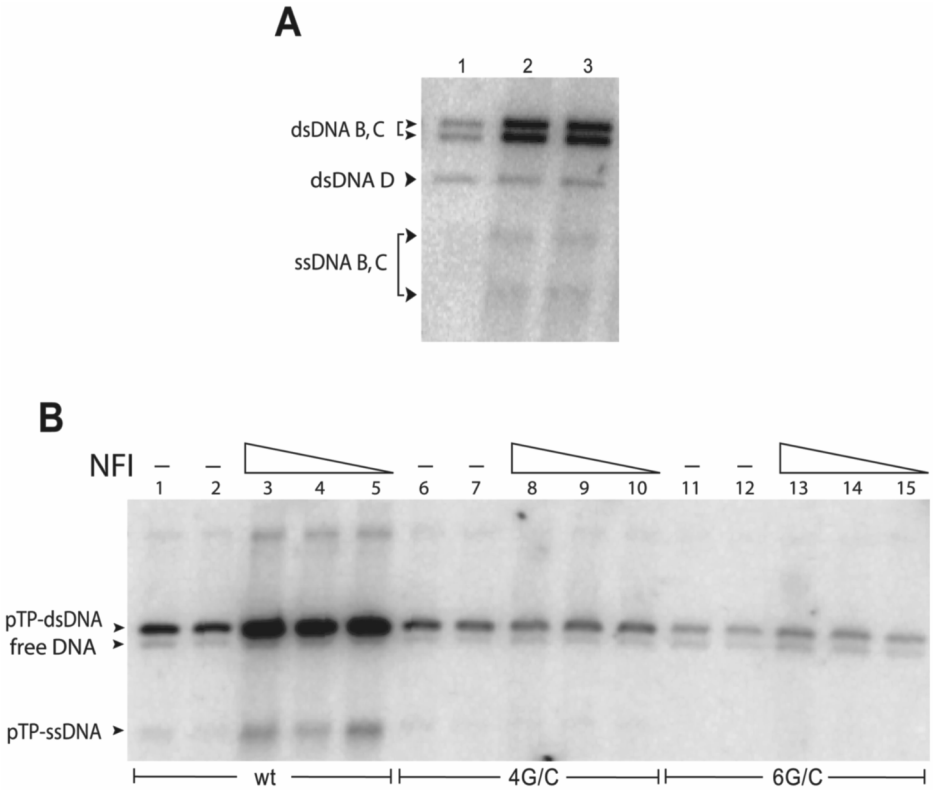

Figure 4. Stimulation of Ad5 replication by NFI on the templates containing 4G/C and 6G/C mutations is impaired
(A) DNA replication assay with Ad5 TP-DNA as a template. 60 ng of polymerase, 37 ng of pTP and 1μg of DBP were incubated with 60 ng of XhoI digested TP-DNA resulting in the synthesis of the two labelled ds origin fragments (dsDNA B,C) and labelled displaced single strands originated from the second and subsequent rounds of replication (ssDNA B,C). The D fragment (dsDNA D) is not replicated, since it doesn't have the origin but it is non-specifically labelled by polymerase. The first lane represents the basal level of replication in the absence of NFI. Lanes 2 and 3 show stimulation of replication by 32 ng and 63 ng of NFI, respectively. The stimulation ability of NFI was determined by comparison of the NFI induced replication signal with the average basal signal.
(B) DNA replication assay on linearised TP-free origin DNA. 100 ng of EcoRI linearised wt-PHRI (lanes 1-5), 4G/C-PHRI (lanes 6-10) and 6G/C-PHRI (lanes 11-15) plasmid were incubated with 15 ng of polymerase, 12 ng of pTP and 1μg of DBP. Replication leads to a pTP containing 2.9 kb ds DNA fragment (pTP-dsDNA) which runs slightly slower than the naked DNA (free DNA). Subsequent rounds of replication lead to pTP coupled to displaced single strands (pTP-ssDNA). Lanes 1, 2, 6, 7, 11 and 12 represent the basal level of replication in the absence of NFI. To stimulate the replication, a range of NFI concentrations was used: 250 ng (lanes 3, 8, 13), 125 ng (lanes 4, 9, 14) and 63 ng (lanes 5, 10, 15).

Binding of polymerase to the mutated Ad5 origins is not affected

The 4G/C and 6G/C mutations in the A/T-rich region are located close to the pTP/pol binding site (Figure 1). Since mutations in this region cause loss of replication stimulation by NFI and additionally reduce the basal level of replication, it was necessary to verify that the ability of polymerase to bind the mutated origins was still intact. Pol/DNA interactions were checked in EMSA assays, using TD50 wild type and the mutant probes (Figure 5). Addition of polymerase to the DNA probe gave rise to protein-DNA complexes containing one, two or three polymerase molecules. This is possibly due to the fact that polymerase alone does not bind the core origin with high specificity (41) and in addition to the specific complexes non-specific

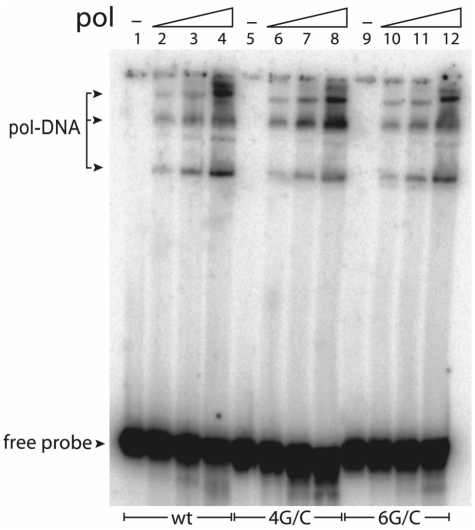

Figure 5. Binding of DNA polymerase to the mutated Ad5 origins is wild type-like
Binding of polymerase to wild type or mutated origins was studied in EMSA experiments using ds TD50 probes. The positions of pol-DNA complexes and free probe are marked with arrowheads. Radiolabelled TD50, wt (lanes 1-4), 4G/C (lanes 5-8) and 6G/C (lanes 9-12) were incubated with 50 ng (lanes 2, 6, 10), 100 ng (lanes 3, 7, 11) and 200 ng (lanes 4, 8, 12) of Ad5 polymerase. Lanes 1, 5 and 9 represent free probe.

complexes are formed. It also cannot be excluded that some of the complexes are the result of polymerase breakdown products bound to DNA. However, no significant changes in polymerase binding to the templates containing mutations were observed compared to the wild type binding. This demonstrates that the reduced basal level of replication and the loss of stimulation by NFI on mutated origins were not caused by impaired polymerase binding.

Function of the pTP/pol complex is not affected by AT to GC mutations

During initiation Ad5 polymerase catalyses the covalent coupling of the first dCTP residue to pTP, which results in the formation of the pTP-dCMP complex (14). This event primes adenovirus DNA replication and allows polymerase to further elongate template DNA. In view of the reduced basal replication level on the mutated origins we analysed the effect of mutations introduced in the A/T-rich region on the ability of polymerase to initiate the replication (Figure 6). The initiation activity of the pTP/pol complex on the 4G/C and 6G/C origins was not significantly affected and resulted in 98% ± 9 and 82% ± 15 of the wild type initiation activity for the 4G/C and 6G/C mutant origins, respectively. Taking into account the slight variations between experiments we conclude that the initiation ability of the pTP/pol complex on the origins containing the 4G/C and 6G/C mutations is similar to that of the wild type. When we used less pTP/pol the amount of initiation product was dependent on the pTP/pol concentration, demonstrating that the pTP/pol concentration was not saturating for the experimental conditions (data not shown). However, the initiation activity of the pTP/pol complex on the mutated origins was still comparable with the wild type origin.

Discussion

Structural properties of the Ad5 origin and NFI induced changes

In this study we show by SFM that NFI induces a 60º bend in the Ad5 origin of replication. The presence of an NFI-induced DNA bend in the origin was not clear from previous biochemical studies. Based on hydroxyl radical footprinting and circular permutation assays, DNA bending by NFI was suggested (48). However, Verrijzer *et al.* did not observe DNA bending induced by NFI, applying also circular permutation analysis (46).

We demonstrate that the 6 bp long A/T-rich region located between the pTP/pol binding site and the NFI binding site in the Ad5 origin (Figure 1), contributes to DNA bending induced by NFI, since substitution of 4 or 6 AT pairs with GC pairs decreases the bend angle from 60° to 33°-37°. In view of these results we propose that the A/T-rich region in the Ad5 origin of replication determines the functional bend introduced by NFI binding.

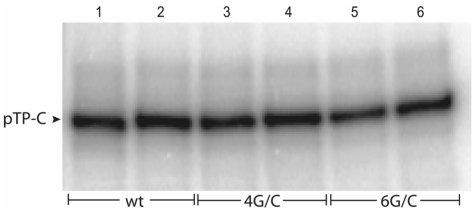

Figure 6. Origins containing the 4G/C and 6G/C mutations support initiation of Ad5 DNA replication
The initiation reaction represents the origin-dependent covalent coupling of the radiolabelled [α-^{32}P]-dCTP to pTP by polymerase, forming pTP-dCMP (pTP-C). 50 ng of polymerase and 40 ng of pTP were incubated with the appropriate template DNA. As a template 10 ng of TD50 was used, wt (lanes 1, 2), 4G/C (lanes 3, 4) and 6G/C (lanes 5, 6).

A/T-rich regions occur commonly in replication origins, but in most cases they are implicated in facilitated unwinding. Studies on the replication complex assembly demonstrate that protein binding to the origin DNA influences the DNA structure (4, 6, 18, 35). It is very likely that the NFI induced DNA bend in the Ad5 origin results in strand separation and/or destabilisation of the A/T-rich region. This might result in stimulation of DNA replication. Energy invested in bending can be used to facilitate DNA opening and on the other hand, once the DNA is open, it will become more flexible and will bend more easily (37). Therefore, we propose that the bending of origin DNA by NFI results in the accumulation of energy in the form of backbone strain, which can be subsequently relieved by base pair opening. Conversely, the low energy requirements for melting of the A/T-rich region might facilitate NFI induced DNA bending.

SFM studies also revealed an intrinsic 17° ± 7 bend of the Ad5 origin (Table I). Previous studies showed that origin residues 20 to 22 are protected from hydroxyl radical damage in the absence of protein and that the mobility of origin containing DNA is slightly different in a circular permutation assay (48), which might well be explained by the presence of the bend in DNA. Intrinsic, sequence-dependent DNA bending is a common feature present in different promoters. The presence of an A/T stretch (16 T's out of 20 bases) in the yeast profilin promoter generates a 25° natural bend (3). A DNA bend of 8-11° is also present in the phage T7 promoter (16 A/T's out of 21 bases) (42). The Ad5 origin contains 20 A/T's out of the first 25 bases (Figure 1), which is very likely to determine the intrinsic DNA bending.

Functional importance of DNA bending for adenovirus replication

We also demonstrate the functional importance of the A/T-rich region for stimulation of adenovirus DNA replication by NFI. We show that replacement of 4 or 6 AT pairs with GC pairs in the A/T-rich region abolishes the ability of NFI to stimulate the Ad5 DNA replication (Figure 4B). Previous mutation studies of the A/T-rich region gave variable results in replication efficiency (2, 15, 47). Our results exclude that the defect in replication stimulation of the mutant origin sequences was caused by impaired NFI or polymerase binding (Figures 3 and 5) or that it is due to impaired initiation activity of pTP/pol (Figure 6). Since the mutations in the A/T-rich region decrease the DNA bend angle induced by NFI, we postulate that the defect in replication stimulation on mutated origins is due to the inability to form a functional nucleoprotein structure at these origins with a smaller bend angle.

Nucleoprotein architecture of the replication complex

So far several pair-wise interactions among the proteins involved in Ad5 DNA replication have been demonstrated. The polymerase and pTP form a tight heterodimer (17). In solution polymerase interacts directly with NFI, and Oct-1 interacts directly with pTP (5, 8, 11, 13). In addition, based on the dimerisation of pTP, an interaction between pTP and the terminal protein was postulated (12). Formation of such a multi-protein complex with the web of protein-protein interactions on a linear 50 bp origin requires a specific nucleoprotein architecture. Our SFM data demonstrate that NFI induced a bend of 60° in DNA upon binding to the Ad5 origin.

We propose that there are two protein induced DNA bends. The first sharp bend (60°) is induced by NFI and the second bend of 37° (46), located at position 40-43 is induced by Oct-1. Based on the available data we can not determine the orientation of these two bends with respect to each other. We postulate that the resulting nucleoprotein architecture facilitates optimal protein-DNA binding and co-operative protein-protein interactions within the replication complex, which will result in a functionally efficient complex. Assembly of specific nucleoprotein structures including protein-induced DNA bending is a common feature in the regulation of transcription as well as in DNA recombination and repair (23, 24, 27). Moreover, in transcription a relation between an A/T-rich stretch and DNA bending is suggested for the regulation of the yeast profilin gene (3).

This study provides evidence that NFI stimulates Ad5 replication by inducing a strong origin DNA bend. Furthermore we suggest that DNA bending might allow efficient strand separation, which could also lead to stimulation of replication. We assume that both functions contribute to the stimulation of Ad5 replication, but it remains to be determined if they are independent of each other. It is likely that the ability of NFI to stimulate replication by inducing a bend in DNA represents a general mechanism of NFI function. Since NFI is also involved in the transcription regulation of various genes (20), it may induce DNA bending in order to perform its regulatory function in transcription as well. In this respect it is noteworthy that several NFI regulated promoters contain an A/T-rich stretch in front of an NFI binding site (1, 29, 36).

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Chapter

3

NFI and Oct-1 bend the Ad5 origin in the same direction leading to optimal DNA replication

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NFI and Oct-1 bend the Ad5 origin in the same direction leading to optimal DNA replication

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Two cellular transcription factors, Nuclear Factor I (NFI) and Octamer binding protein (Oct-1), bind simultaneously to their recognition sequences in the Ad5 origin of replication thereby enhancing initiation. Using scanning force microscopy we have previously shown that NFI induces a 60° bend in the origin DNA. Here we demonstrate that Oct-1 induces a 42° bend in the origin DNA. Simultaneous binding of NFI and Oct-1 induces an 82º collective bend suggesting that both bends are oriented towards each other. In functional replication assays we further demonstrate that this extensive DNA bending leads to a synergistic enhancement of DNA replication. We propose that collective DNA bending induced by NFI and Oct-1 facilitates the optimal assembly of the preinitiation complex and plays an important role in the stimulatory mechanism of NFI and Oct-1 in replication.

Introduction

Adenovirus serotype 5 (Ad5) contains a 36 kb double stranded linear DNA genome with terminal proteins (TP) covalently attached at the 5' ends. The origins of DNA replication are located at each end of the DNA in 103 bp long inverted terminal repeats (ITR). Three viral proteins are involved in Ad5 DNA replication, precursor terminal protein (pTP) that serves as a primer, adenovirus DNA polymerase (pol) and DNA binding protein (DBP)(reviewed in (1-5)). pTP and pol form a stable heterodimer in solution and bind as a complex to the conserved core origin at position 9-18. The core origin is followed by the auxiliary origin that is recognised by two cellular transcription factors, Nuclear Factor I (NFI) and Octamer binding protein (Oct-1)(6). NFI and Oct-1 considerably enhance the replication process with the highest effect at low (physiological) pol concentrations (7,8).

NFI binds as a dimer to the consensus sequence 5'TGGA(N_5)GCCAA3' located in the Ad5 origin at position 25-38 (9-12) (Fig. 1). This sequence is essential for optimal replication (13,14). Contact point analysis demonstrates that almost all contacts of the NFI dimer are clustered at one side of the DNA helix, mainly in the major groove (15,16). The conserved N-terminal domain of NFI is required for DNA binding and protein dimerization and is sufficient for the stimulation of Ad DNA replication (17,18). The C-terminal domain is involved in transcription regulation (18).

Oct-1 binds as a monomer to the recognition sequence 5'GATAATG3' position 39-48 in the Ad5 origin (Fig. 1) (19,20) and the presence of this sequence in the origin stimulates viral replication (21). The Oct-1 interaction with DNA involves many contact points, mainly in the major groove, that are located on different sides of the DNA helix (22,23). Oct-1 consists of a centrally located bipartite DNA binding domain (POU domain) flanked by two transcription activation domains. The POU domain is sufficient for the stimulatory function of Oct-1 in Ad5 DNA replication (24). It consists of a POU specific domain (POUs) that binds DNA with high specificity but low affinity, and a POU homeodomain (POUh) that binds DNA with low specificity but high affinity (25-27).

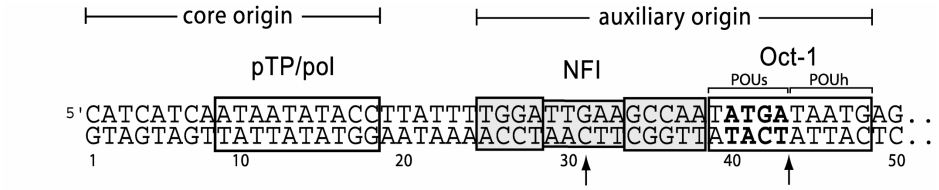

Figure 1. The origin of Ad5 DNA replication

The Ad5 origin consists of a highly conserved core origin with the pTP/pol binding site and an auxiliary origin containing the consensus sequences recognised by the NFI and Oct-1 (boxed). The arrows indicate the middle of the NFI consensus sequence at position 31 and the middle of the Oct-1 recognition sequence between position 43 and 44. The binding half-sites for the POU specific domain (POUs) and POU homeodomain (POUh) are indicated. The ATGA sequence (in bold) within the Oct-1 binding site represents the apparent centre of DNA bending induced by Oct-1 as demonstrated by biochemical assays (28).

There are strict spacing requirements for the position of the NFI and Oct-1 recognition sequences with respect to the core origin bound by the pTP/pol complex. Insertion or deletion of one or two nucleotides between the core and the auxiliary origin severely inhibits the stimulatory function of NFI or Oct-1 (29-32). NFI and Oct-1 have a similar mode of action resulting in stimulation of replication. They stabilise binding of the pTP/pol complex to the origin (7,33). They also influence the kinetics of replication by increasing the V_{max} of initiation, without affecting the K_m, suggesting that they do not change the activity of the initiation complex, but rather increase the number of active initiation complexes (7). In solution NFI interacts with pol and Oct-1 interacts with pTP suggesting that NFI and Oct-1 recruit the pTP/pol complex to the origin (17,29,33-35).

In a previous study using scanning force microscopy (SFM) we demonstrated that NFI induces a 60° bend in the Ad5 origin DNA that is important for the stimulation of replication (36). In this study we show that Oct-1 induces a 42° DNA bend in the origin. Moreover, we demonstrate that simultaneous binding of NFI and Oct-1 resulted in an 82° collective bend, indicating the two bends are in the same direction. Also, stimulation of replication is greatly enhanced when both proteins bind simultaneously compared with the individual stimulation of replication by NFI or Oct-1 alone. We propose that the assembly of the preinitiation complex involves additive bending by NFI and Oct-1 of the origin DNA, thereby facilitating protein-protein and protein-DNA interactions resulting in a maximised stimulation of replication.

Materials and Methods

Proteins

For all experiments the DNA binding domain of rat NFI type A_1 (NFI-BD consisting of 5 - 242 aa out of 499 aa) and the DNA binding domain of Oct-1 (POU domain consisting of 280 - 439 aa out of 743 aa) was used. The NFI-BD will be further referred to in the text as NFI and the Oct-1 POU domain will be referred to as Oct-1. NFI, adenovirus DNA polymerase, pTP and DBP were expressed using the baculovirus expression system and the proteins were purified to near homogeneity as previously described (8,28,35,37). Oct-1 was expressed in bacteria as a GST-tagged (GST-Oct-1, 47 kDa) or 6×His-tagged (his-Oct-1, 20 kDa) fusion protein and purified as previously described (33,35). The protein dilution buffer contained 25 mM HEPES-KOH pH 7.5, 150 mM NaCl, 15% glycerol, 5 mM DTT and 0.5 µg/µl BSA.

Scanning force microscopy

For the SFM studies of Oct-1-DNA or NFI-Oct-1-DNA complexes a 717 bp DNA fragment was used. This fragment contains the first 103 bp of the Ad5 inverted terminal repeat (ITR) from the left end of the virus genome with the Ad5 origin of replication starting from position 209. The Oct-1 binding site (Fig. 1) is located between 39 and 48 bp downstream from the beginning of the origin, at position 247 and 256 of the fragment (35% of DNA fragment length). The middle of the auxiliary origin (Fig. 1) is located at 34% of the fragment. The DNA fragment was obtained by PCR using the PHRI plasmid (14) with the following primers: forward 5' - GTGAAATACCGCACAGATGCGTAAGGAG - 3', reverse 5' - CTTTTGCTCACATGTTCTTTCCTGCGTTATC - 3'. The PCR fragment was purified from a 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and resuspended in 10 mM HEPES-KOH pH 8.0. For analysis of a DNA bend induced by Oct-1 alone, GST-Oct-1 of 47 kDa was used to facilitate detection in SFM images, since his-Oct-1 (20 kDa)

was difficult to detect (data not shown). However, for the study of the DNA bend induced by NFI and Oct-1 together we did use his-Oct-1 to avoid possible steric hindrance from the GST tag and also because the GST tag apparently interferes with the stimulatory effect of Oct-1 on replication (35). We assume that the bend angles induced by his-Oct-1 and GST-Oct-1 are the same. The SFM depositions were performed as follows. NFI (4.5 pmol) and/or Oct-1 (17 pmol his-Oct-1 or 10 pmol GST-Oct-1) was incubated with the DNA fragment (0.19 pmol) for 20 minutes on ice in replication buffer (see below). The final mixture was diluted 20-30 times in deposition buffer (5 mM HEPES-KOH pH 7.8, 5 mM $MgCl_2$) and deposited onto freshly cleaved mica. After 1 min the surface of the mica was washed with 3 ml of HPLC grade water and dried in a stream of air. The complexes were imaged in the tapping mode using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). The DNA contour length and the DNA bend angle were measured using the Image SXM v 1.69 software, a NIH Image version modified for use with SXM images by Steve Barrett, Surface Science Research Centre, University of Liverpool, UK. For each measurement we analysed 70-120 molecules. The data obtained were analysed in Sigma Plot.

Protein-DNA interactions (EMSA)

For the protein-DNA binding studies TD50 was used, a double stranded oligonucleotide consisting of the first 50 nucleotides of the template (T50) and displaced (D50) strand of the Ad5 origin.

T50: 5'CTCATTATCATATTGGCTTCAATCCAAAATAA GGTATATTATTGATGATG3', D50: 5'CATCATCAATAAT ATACCTTATTTTGGATTGAAGCCAATATGATAATGAG 3'. For the preparation of TD50, the D50 oligonucleotide was end-labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP (4500 Ci/mmol) in a standard kinase buffer. D50 was subsequently hybridised with T50 and the labelled TD50 was purified from a 12 % polyacrylamide gel. DNA binding was determined by electrophoretic mobility shift assay (EMSA) as follows: 0.86 pmol of NFI or/and 2 pmol of his-Oct-1 was incubated with DNA for 60 min on ice in binding buffer (25 mM HEPES KOH pH 7.5, 4 mM $MgCl_2$, 0.4 mM DTT, 4 % Ficoll, 80 mM NaCl) including 1 µg poly (dI-dC)-(dI-dC) as a non-specific DNA competitor. TD50-protein complexes were separated on a 5 % polyacrylamide gel in TBE buffer at 4°C and the intensity of the bands was quantified using a Storm 820 phosphorimager at the linear range of the signal.

In vitro DNA replication

In vitro DNA replication was performed using 9 ng Ad5 DNA polymerase, 9 ng Ad5 pTP, 1 µg Ad5 DBP and the indicated amounts of NFI and his-Oct-1 in a 15 µl reaction mixture containing replication buffer (25 mM HEPES-KOH pH 7.5, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT), 40 µM dATP, dTTP, dGTP, 0.7 µM dCTP and 3 µCi of [α-^{32}P] dCTP (3000 Ci/mmol). As a template, 60 ng of XhoI digested viral TP-DNA was used. TP-DNA is the 36 kb linear Ad5 genome with the terminal protein (TP) covalently attached to each DNA end that was isolated from Ad5 virions as previously described (38). XhoI digestion generated seven fragments, two of which (6.2 kb fragment B and 5.8 kb fragment C) contain the origin and are replicated. The replication mixtures were incubated for 45 min at 37°C and stopped by the addition of 2 µl stop mix (40% sucrose, 1% SDS, 0.1% bromophenol blue and 0.1% xylene cyanol). The replication products were separated on a 1% agarose gel containing 0.1% SDS in 0.5×TBE/0.1%SDS buffer. The replicated bands were quantified using a Storm 820 phosphorimager at the linear range of the signal. The level of stimulation by NFI and/or Oct-1 was calculated as the ratio between the replication signal (the sum of the ds and the ss B and C bands) in the presence of NFI and/or Oct-1, and the average basal signal.

Results

Oct-1 induces a 42° DNA bend in the Ad5 origin of replication

In order to study Oct-1-induced changes in the origin DNA structure by SFM, a 717 bp double stranded DNA fragment containing the Ad5 origin of replication was used. The middle of the Oct-1 binding site (Fig. 1) is located at 35% of the fragment length from one end. The remaining part of the DNA contains no obvious Oct-1 recognition sequences. Oct-1 and the 717 bp DNA fragment were incubated at a 53 fold molar excess of Oct-1 over DNA in conditions similar to those used for the functional replication assays.

SFM images showed that Oct-1 bound specifically to DNA at the position of the origin (Fig. 2C). Very few Oct-1 molecules were bound to other sites on the DNA. The position of Oct-1 bound to DNA was determined by measuring the length of the DNA from the centre of the protein in a protein-DNA complex, to each end of the DNA. The binding position was then expressed as the ratio r of the length of the shorter DNA arm divided by the total DNA length (contour length) with a theoretically calculated value of $r = 0.35$ for the middle of the Oct-1 consensus sequence in the origin. The protein position was on average 0.35 ± 0.05 (70 molecules measured), which confirms that Oct-1 is specifically bound to the origin (Table 1). Additionally, the DNA contour length of the 717 bp DNA fragment was analysed by tracing the DNA molecules from one end to the other in the absence and in the presence of Oct1. There were no obvious differences in DNA contour length observed upon Oct-1 binding (Fig. 2B, Table 1), showing that Oct-1 does not induce DNA shortening by DNA compaction upon binding or DNA wrapping around Oct-1.

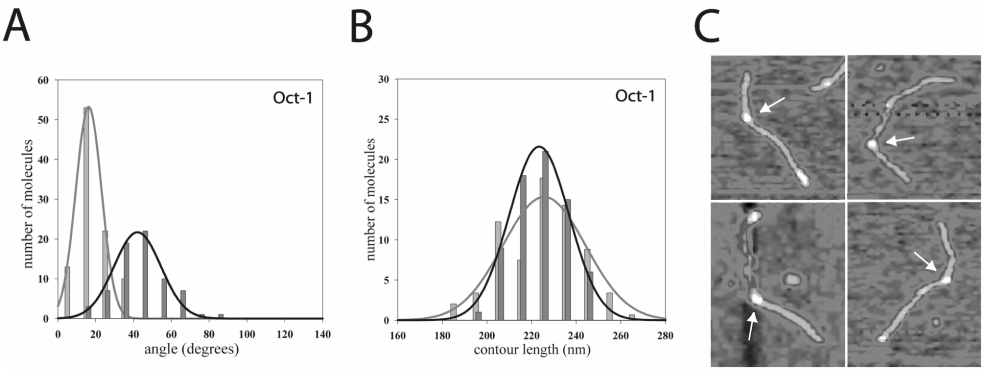

Figure 2. Oct-1 induces a 42° bend when bound to the Ad5 origin DNA
Two histograms represent the DNA bend angle distributions (A) and contour length distributions (B) of the protein-free DNA molecules (light grey bars, data taken from Mysiak *et al* (36)) and the Oct-1-DNA complexes (dark grey bars) measured by SFM. The grey and black lines represent the Gaussian fitting of the distribution as defined in equation 1 of Schulz *et al* (39). A 717 bp DNA fragment was used that contains the Ad5 origin of replication with the middle of the Oct-1 recognition sequence at 35% of the DNA fragment. The mean values with standard deviations are presented in Table 1. (C) SFM images of the representative Oct-1-DNA complexes. An arrow indicates Oct-1 bound to DNA.

The DNA bend angle is defined as the angle by which a DNA segment departs from linearity. The bend angle induced at the Ad5 origin by Oct-1 was determined by measuring the angle between two 20 nm long straight segments of DNA on each side of Oct-1 followed by subsequent calculation of the angle by which DNA deviates from straight. Gaussian curve fitting according to equation 1 in Schulz *et al* (39) was used to determine the average values of bend angles with standard deviations. The intrinsic bending of the origin was determined in the absence of Oct-1 as described in Mysiak *et al* (36). The Ad5 origin DNA showed an intrinsic 17° ± 7 DNA bend and after addition of Oct-1 a clear shift in DNA bend angle distribution was observed resulting in the average bend of 42° ± 12 (Fig. 2A, Table 1).

Simultaneous binding of NFI and Oct-1 to the Ad5 origin increases DNA bending

Previously we determined that NFI induces a 60° bend in the Ad5 origin DNA (36). In order to study the collective DNA bending induced by NFI and Oct-1 bound simultaneously to the origin we incubated the 717 bp DNA fragment with NFI and Oct-1 under conditions used for the replication assays. Since NFI binds to the origin with a higher affinity than Oct-1 (25,40,41) we used a 12 fold molar excess of NFI dimers over DNA and a 90 fold excess of Oct-1 over DNA. The protein position on the 113 protein-DNA complexes analysed, corresponded well with the auxiliary origin (experimental value r = 0.36 ± 0.03, theoretical value r = 0.34 calculated for the middle of the auxiliary origin, since we now look at the binding of NFI and Oct-1) (Fig 3D, Table 1). Also the protein binding was specific since very few molecules were bound outside of the origin region. We also analysed the DNA contour length of the 717 bp DNA fragment in the presence of NFI and Oct-1 and no noticeable differences were observed compared with the contour length of the protein-free DNA (Fig. 3B, Table 1). As presented in Fig. 3A the average DNA bend induced in the origin in the presence of NFI and Oct-1 together was 73° ± 20, which is more that the individual bends induced by NFI or Oct-1 alone, indicating that NF1 and Oct-1 together induce additional bending in the origin DNA.

One should consider the fact that among the protein-DNA complexes analysed some DNA

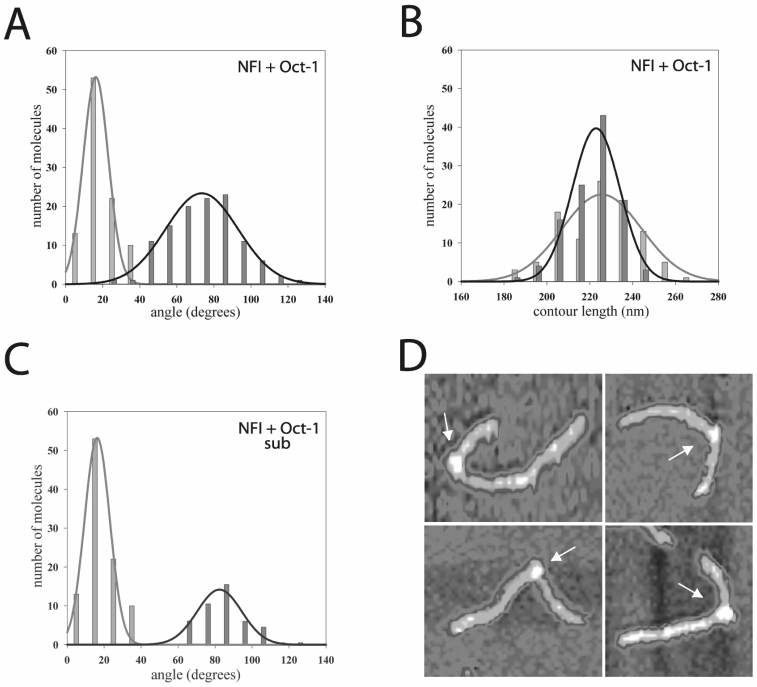

Figure 3. The DNA bend angle increases when both NFI and Oct-1 are bound to the Ad5 origin DNA
The SFM data were collected in the presence of both NFI and Oct-1. The histograms represent DNA bend angle distributions (A) and contour length distributions (B) of the protein-free DNA molecules (light grey bars, data from Mysiak *et al* (36)) and protein-DNA complexes (dark grey bars). The grey and black lines represent the Gaussian fitting of the distribution. The mean values with standard deviations are presented in Table I. (C) The distribution of DNA bends induced by NFI alone (based on (36)) was subtracted from the NFI-Oct-1 distribution. (D) SFM images of the representative protein-DNA complexes. An arrow indicates proteins bound to DNA.

molecules are bound by NFI alone or Oct-1 alone. In these SFM images it was not possible to distinguish between the different protein-DNA complexes. Due to the small size of Oct-1 used in this experiment (see Materials and Methods), we assumed that Oct-1-DNA complexes are not clearly distinguishable from free DNA and therefore were not selected for analysis. Indeed, the distribution of bend angles induced by Oct-1 alone does not appear to contribute to the bend angle distribution of NFI and Oct-1 together, since in Fig. 2A the distribution is centred at 42º, whereas in Fig 3A the distribution is almost empty below 50º. Therefore, the distribution measured in the presence of NFI and Oct-1 is made up of two overlapping distributions. One is the distribution of bend angles induced by binding of NFI alone, and the other is the distribution of bend angles induced by NFI and Oct-1 together. Based on the DNA binding data presented below, we assumed that 68% of the complexes are bound by NFI alone under these conditions. In order to eliminate the contribution of DNA bending angles induced by binding of NFI alone, this distribution (based on (36)) of 77 molecules was subtracted from the distribution obtained in the presence of both NFI and Oct-1 (113 molecules, Fig. 3C). The remaining distribution represents the DNA bend angle induced by NFI and Oct-1 simultaneously bound to DNA. Similar analysis was performed previously in the study of the photolyase-DNA complexes (42,43). The average angle obtained from this corrected distribution was 82° ± 12 (Fig. 3C, Table 1), indicating that the bends are additive.

Table I. Summary of the SFM data

	Protein position (r)	Angle (degrees)	Contour length (nm)
Bare origin [a]	-	17 ± 7	226 ± 19
Origin + NFI [a]	0.36 ± 0.09	60 ± 19	217 ± 26
Origin + Oct-1	0.35 ± 0.05	42 ± 12	223 ± 13
Origin + NFI + Oct-1	0.36 ± 0.03	73 ± 20, 82 [b] ± 12	223 ± 11

[a] Data from Mysiak *et al* (36)

[b] The value was calculated by subtraction of the distribution of DNA bending angles induced by NFI alone (based on (36)) from the distribution obtained in the presence of both NFI and Oct-1

NFI and Oct-1 bind the Ad5 origin DNA simultaneously

The Ad5 origin DNA binding sites recognized by NFI and Oct-1 are located next to each other (Fig. 1). In order to confirm independently that NFI and Oct-1 can bind the origin simultaneously and analyse if their binding is cooperative we performed electrophoretic mobility shift assays (EMSAs) using a double-stranded TD50 DNA fragment representing the first 50 base pairs of the origin (Fig. 4). Two concentrations of the DNA probe were used, 170 fmol (Fig. 4, lanes 1-4) and 35 fmol (Fig. 4, lanes 5-8). Addition of NFI resulted in the formation of a single NFI-DNA complex (Fig. 4, lanes 2, 6) and addition of Oct-1 gave rise to a single Oct-1-DNA complex (Fig. 4, lanes 3, 7). When both proteins were incubated with the DNA probe, in addition to single NFI-DNA and Oct-1-DNA complexes, a third supershifted band appeared on the gel (Fig. 4, lanes 4, 8). This complex represents both proteins simultaneously bound to a single DNA molecule, demonstrating that NFI and Oct-1 do not exclude each other from binding to the Ad5 origin, despite the close proximity. When 170 fmol of DNA probe was used the simultaneous binding of NFI and Oct-1 to the origin did not seem to be very cooperative, since the supershifted complexes represented 7 % of all protein-DNA complexes (Fig. 4, lane 4). When 35 fmol of DNA probe was used, which is similar to conditions used for the SFM analysis, the amount of supershifted complexes increased to 22 % (Fig. 4, lane 8), showing that an increase of the protein-DNA ratio promotes simultaneous binding of NFI and Oct-1. This validates formation of the NFI-Oct-1-DNA complexes and allows to estimate the proportion of NFI-DNA complexes (68 %) in the SFM experiments that included both proteins.

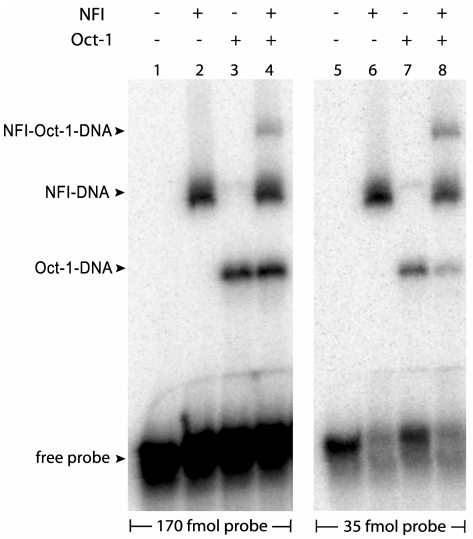

Figure 4. NFI and Oct-1 bind the Ad5 origin of replication simultaneously

Binding of NFI and Oct-1 to the Ad5 origin was studied by EMSA using the TD50 probe containing the first 50 nucleotides of the Ad5 origin. 0.17 pmol (lanes 1-4) and 35 fmol (lanes 5-8) of TD50 probe was used. The position of the protein-DNA complexes and free probe are marked with arrowheads. Lanes 1 and 5 represent free probe.

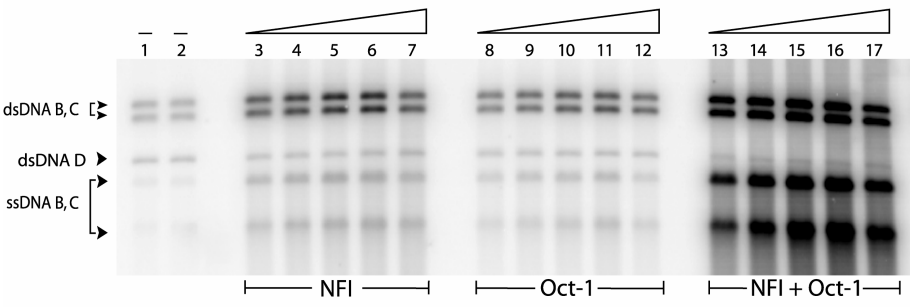

Figure 5. NFI and Oct-1 stimulate Ad5 DNA replication
The *in vitro* replication assays were performed using Ad5 XhoI-digested TP-DNA as a template. As a result of replication two double-stranded DNA fragments containing the origin (dsDNA B and C) and single-strands originated from the second and subsequent rounds of replication (ssDNA B and C) are generated. The D fragment (dsDNA D) is lacking an origin, but is nonspecifically labelled by polymerase. The first two lanes represent the basal level of replication in the absence of NFI and Oct-1. To stimulate replication 50 ng, 75 ng, 110 ng, 165 ng and 250 ng of NFI (lanes 3-7 and 13-17) or 53 ng, 55 ng, 80 ng, 120 ng and 180 ng of Oct-1 (lanes 8-12 and 13-17) were added. Lanes 13-17 represent replication in the presence of both NFI and Oct-1. The level of stimulation was determined by comparing the replication signal (the sum of the ds and ss B and C bands) in the presence of NFI and/or Oct-1 with the average basal signal. A second independent experiment gave similar results.

Simultaneous stimulation of replication by NFI and Oct-1 is cooperative

To analyse if simultaneous binding and presumably additive DNA bending of the Ad5 origin by NFI and Oct-1 enhances their stimulatory effect on replication we performed *in vitro* replication assays. As a template the natural XhoI-digested TP-DNA isolated from Ad5 virions was used. TP-DNA is the 36 kb long linear double stranded viral genome containing terminal proteins covalently attached to each 5' end. XhoI digestion generated seven fragments, but only two of them contain the origin and are replicated (DNA fragments B and C). First we analysed the ability of NFI or Oct-1 alone to stimulate replication. In order to obtain the maximal stimulatory effect, low pTP and pol concentrations (9 ng pTP and 9 ng pol) were used (6-8). Addition of NFI (Fig. 5, lanes 3-7) stimulated replication maximally 9-fold at 165 ng and addition of Oct-1 (Fig. 5, lanes 8-12) stimulated replication maximally 5-fold stimulation at 120 ng. The presence of NFI and Oct-1 resulted in a large stimulation of replication, up to 83-fold at 165 ng of NFI and 120 ng of Oct-1 (Fig. 5, lanes 13-17). The reaction was very efficient as evidenced by the large amount of single-stranded DNA fragments, displaced during the replication process, indicative of second and subsequent rounds of replication. Theoretically, the level of combined stimulation by NFI and Oct-1 can be calculated from the multiplication of the stimulation levels of NFI and Oct-1 alone and thus would be $9 \times 5 = 45$. The 83-fold stimulation observed indicates that NFI and Oct-1 act synergistically in this reaction. Similar levels of synergistic stimulation of replication were observed for other concentrations of NFI and Oct-1 (Fig. 5). The molar excess of NFI and Oct-1 over DNA in these reactions was similar to SFM and EMSA experiments favouring simultaneous binding of both transcription factors and additive DNA bending.

Discussion

Protein induced DNA bending of the Ad5 origin of replication

In this study, using SFM, we demonstrate that the two DNA bends induced in the Ad5 origin of replication by NFI and Oct-1 are additive, since the collective angle induced by both proteins simultaneously bound to the origin increased up to 82°, compared to the individual angles of 60° and 42° induced by NFI and Oct-1,

respectively. Based on biochemical assays and on the co-crystal structure, the Oct-1-induced DNA bend is estimated to be 30-37° with the apparent centre of DNA bending located in the left part of the recognition sequence contacted by POUs (Fig. 1)(23,28). However, if both NFI and Oct-1 bends were in the same plane, a calculated theoretical value of the collective bend would be 102°. Since NFI and Oct-1 bind very close to each other (Fig. 1) it is possible that they influence each other's ability to bend DNA resulting in a lower value of the collective angle. Alternatively, both DNA bends may not be exactly in the same plane and projection of a three-dimensional arrangement of DNA bends onto a two-dimensional surface may influence the value measured by this method.

Contact point analysis of NFI and Oct-1 binding to the Ad5 origin reveals that both proteins bind to the DNA major groove and that NFI binds on the opposite side of the Ad5 origin DNA than the POUs domain of Oct-1 (15,16,22,23). It is also suggested that the POUs domain of Oct-1 bends DNA by compression of a major groove (28). Up till now the mechanism of DNA bending by NFI is not known. Given the distance between the binding sites of NFI and Oct-1, we propose that in order to gain an additive effect of the collective DNA bending, NFI will most likely bend DNA by broadening of the major groove.

NFI and Oct-1 interaction with the origin DNA

NFI and Oct-1 bind the Ad5 origin in close proximity to each other (Fig. 1)(15,20,22). Using EMSA we showed that despite the close spacing, NFI and Oct-1 can bind to the origin together and they do not exclude each other (Fig. 4). Since both proteins have the ability to stimulate binding of the pTP/pol complex to DNA (7,33), possibly by inducing a DNA bend, they could also enhance their simultaneous binding to DNA. However we did not observe an increased binding affinity of NFI or Oct-1 when both proteins were incubated together with the origin DNA (Fig. 4), suggesting that these transcription factors do not bind the origin cooperatively.

In the early stage of infection the amount of a viral DNA in infected cells is minimal, whereas viral DNA replication needs to be enhanced rapidly (6). Since both transcription factors, NFI and Oct-1 are ubiquitously expressed, the concentration ratio between these proteins and viral DNA in the early stage of infection would be very high. We demonstrated that under such conditions NFI and Oct-1 can simultaneously bind to the origin (Fig. 4), collectively inducing an 82° bend in the origin DNA and thereby contributing to optimal stimulation of DNA replication (Fig. 5). Moreover, at the early stages of infection *in vivo* pTP/pol concentration is low, which is similar to the condition in which NFI and Oct-1 have the highest stimulatory effect *in vitro* (6-8,44).

Arrangement of the preinitiation complex

This study reveals that two DNA bends induced in the Ad5 origin by NFI and Oct-1 are oriented towards each other resulting in an increased DNA curvature of 82°. Assembly of the preinitiation complex involves several protein-protein interactions among the proteins involved in Ad5 DNA replication (17,29,35,45-47). Since all these proteins interact with an origin consisting of only 50 bp (Fig. 1), extensive DNA bending by NFI and Oct-1 would facilitate the formation of an optimal nucleoprotein structure of the preinitiation complex. The resulting specific arrangement of protein-protein and protein-DNA interactions is presumably required for optimal stimulation of replication. We have previously shown that the ability of NFI to stimulate replication depends specifically on the degree of DNA bending, since the introduction of mutations in the origin leading to a DNA bend angle smaller than 60° reduced this activity (36). Protein-induced DNA bending is an important architectural feature of specific nucleoprotein complexes involved in several different DNA replication processes as well as in the regulation of transcription and DNA repair (48-54).

In addition to DNA bending by NFI and Oct-1, there are other changes induced in the structure of the origin DNA. DBP changes

the DNA structure in a way that apparently facilitates binding of NFI and the pTP/pol complex to the origin (55-58). During initiation the core origin has to be unwound since pol uses single-stranded DNA as a template for initiation while the displaced strand is protected by DBP (59,60). Another distortion is the intrinsic 17° bend of the origin (16,36). Moreover, the high content of A and T bases is likely to facilitate DNA destabilisation, unwinding or even bending (61). Indeed, the A/T-rich stretch located between the core origin and auxiliary origin is essential for an optimal bend angle induced by NFI (36), although it is not involved in DNA bending and stimulation of replication by Oct-1 (data not shown).

The precise position of the NFI and Oct-1 recognition sequences with respect to the core origin (Fig. 1) is very important, since insertion or deletion of only one or two nucleotides in front of the auxiliary origin abolishes the stimulatory effect of NFI or Oct-1 (29-32). We propose that such insertions or deletions would change the direction of the DNA bend induced by NFI or Oct-1 in relation to the core origin DNA bound by the pTP/pol complex. Consequently this change in bend direction could disrupt the optimal architecture of the replication complex and reduce the stimulatory effect of NFI or Oct-1 on replication. The direction of protein-induced DNA bending was shown to play an important role in other examples of replication and transcription (62,63).

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Addendum

The A/T-rich region in the Ad5 origin of replication is not involved in the origin DNA bending and subsequent stimulation of replication by Oct-1

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The A/T-rich region in the Ad5 origin of replication is not involved in the origin DNA bending and subsequent stimulation of replication by Oct-1

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Binding of NFI and Oct-1 to their recognition sequences in the Ad5 origin of replication induces a DNA bend. The A/T-rich region preceding the NFI and Oct-1 binding sites is important for NFI function, since substitution of four A/T pairs with G/C pairs (4G/C) reduces the NFI-induced DNA bend and consequently abolishes the stimulation of replication by NFI. In this study we demonstrate that, contrary to NFI, the 4G/C substitutions do not change the DNA bend induced by Oct-1. Also the ability of Oct-1 to stimulate replication is not affected by the 4G/C mutation. This demonstrates that the A/T-rich region does not directly contribute to the Oct-1-induced DNA bend and it is also not involved in the stimulatory function of Oct-1.

In Chapter 2 we show that G/C substitutions in the A/T-rich region of the Ad5 origin of replication reduce the NFI-induced DNA bend from 60° to 33° or 37° for the 4G/C and 6G/C mutation, respectively. This decrease of the DNA bend angle has functional consequences, since it abolishes the ability of NFI to stimulate the replication. In Chapter 3 we additionally demonstrate that Oct-1 induces a 42° DNA bend angle upon binding to the origin. Since the Oct-1 binding site in the Ad5 origin is located next to the NFI recognition sequence and only 14 bp downstream from the A/T-rich region (see Fig. 1 in Chapters 2 or 3), we studied if mutation of this A/T-rich region also affects the stimulatory function of Oct-1 in replication. In view of the fact that a replacement of four AT pairs with GC pairs is already sufficient to reduce the DNA bend angle induced by NFI, we analysed the effect of the 4G/C mutation on Oct-1 function.

The A/T-rich region of the Ad5 origin is not involved in DNA bending induced by Oct-1

First, we analysed if the 4G/C mutation affects the DNA bend angle induced by Oct-1 in the Ad5 origin. For the SFM analysis a 711 bp double stranded DNA fragment containing the 4G/C origin mutation was used as described in Chapter 2. In this fragment the Oct-1 binding site is located at 37% of the fragment from one side. Oct-1 (GST-Oct-1) was incubated with DNA fragment under conditions similar to the ones used for the functional replication assays. A 50-fold molar excess of Oct-1 over DNA was used. 63 molecules were analysed and the protein position on DNA was on average 0.36 ± 0.07 (theoretical value is $r = 0.37$), confirming that Oct-1 was specifically bound to the origin (Fig. 1C, Table 1). Binding of Oct-1 to non-specific sites on the DNA was limited to very few molecules. Moreover, similarly to theresults of the wild type origin studies (Chapter 2), we did not observe shortening of a contour length of the 4G/C DNA fragment caused by Oct-1 binding (Fig. 1B, Table 1). As presented in Fig. 1A, the average DNA bendangle induced by Oct-1 in the 4G/C origin was 39° ± 15. This value is comparable with a DNA bend angle induced upon Oct-1 binding to the wild type origin (42°, Chapter 3), demonstrating that the A/T-rich region in the Ad5 origin is not involved in the DNA bend that is induced by Oct-1 binding to the origin.

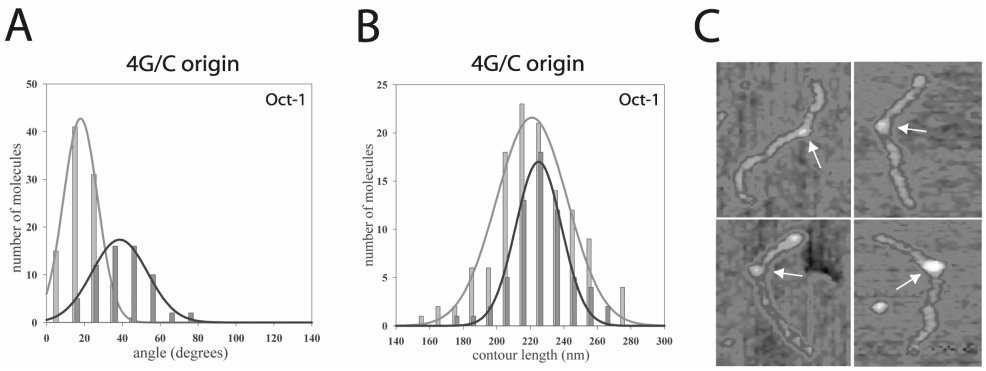

Figure 1. Binding of Oct-1 to the 4G/C origin DNA induces a 39° angle
The histograms represent distributions of the DNA bend angle (A) and contour length (B) of the protein-free DNA molecules (light grey bars, data taken from Chapter 2) and the Oct-1-DNA complexes (dark grey bars) analysed by SFM. The grey and black lines represent the Gaussian fitting of the distribution as defined in equation 1 of Schulz *et al* (6). A 711 bp DNA fragment containing the Ad5 origin of replication with 4G/C mutation was used. The Oct-1 recognition sequence is located in 37% of the DNA fragment from one site. The mean values with standard deviations are presented in able 1. (C) Zoomed SFM images of the representative Oct-1-DNA complexes. An arrow indicates Oct-1 bound to DNA.

The 4G/C mutation in the A/T-rich region does not influence the stimulation of replication by Oct-1

In order to analyse if the 4G/C mutation affected the ability of Oct-1 to stimulate replication we performed *in vitro* replication assays using wild type and 4G/C PHRI plasmid DNA linearized by EcoRI digestion as described in Chapter 2. When a concentration range of Oct-1 (his-Oct-1) was added to the reaction, a maximal stimulatory effect was observed at 120 ng of Oct-1 for wild type and 4G/C origin (Fig. 2, lanes 3-6, 9-12). This corresponded well with the optimal conditions for Oct-1 stimulation of replication using the natural XhoI-digested TP-DNA template isolated from Ad5 virions (Chapter 3), validating the use of the plasmid DNA in our study. Comparison of the maximal levels of stimulation by Oct-1 showed no significant differences between the wild type (8-fold) and the 4G/C origin (7-fold)(Fig.2). Also when 1.5-fold lower or up to 2-fold higher concentrations of Oct-1 were used, similar level of stimulation was detected for both origins. This demonstrates that the 4G/C mutation in the A/T-rich region does not affect the stimulatory function of Oct-1 in replication. In this experiment we again observed that the basal level of the 4G/C origin replication in the absence of Oct-1 was lower and showed 61% of the wild type level (Fig. 2, lanes 1-2, 7-8). It is consistent with our previous results described and discussed in Chapter 2.

Table 1. Summary of the SFM data

Origin DNA	Protein position *r*	Angle (degrees)	Contour length (nm)
Wild type			
DNA[a]	-	17 ± 7	226 ± 19
DNA-Oct-1[b]	0.35 ± 0.05	42 ± 12	223 ± 13
4G/C			
DNA[a]	-	18 ± 9	221 ± 21
DNA-Oct-1	0.36 ± 0.07	39 ± 15	225 ± 14

[a] Data from Mysiak *et al* (Chapter 2)
[b] Data from Mysiak *et al* (Chapter 3)

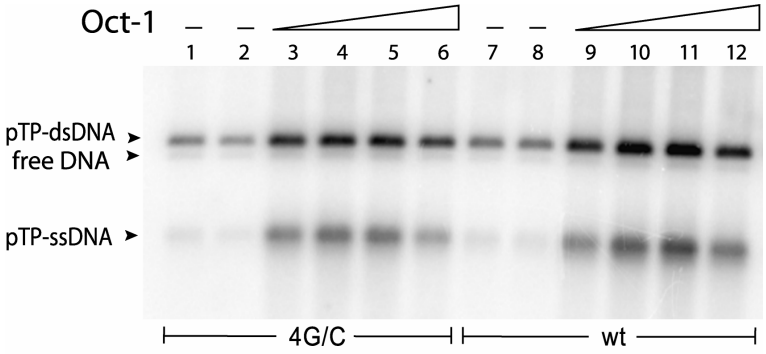

Figure 2. Stimulation of Ad5 DNA replication by Oct-1 on the templates containing 4G/C mutation is not affected
In vitro DNA replication assay using 50 ng of EcoRI linearized wt-PHRI (lanes 7-12) and 4G/C-PHRI (lanes 1-6) plasmid DNA as a template and 9 ng of Ad5 polymerase, 9 ng of Ad5 pTP and 1µg of Ad5 DBP. Replication generates a pTP containing 2.9 kb ds DNA fragment (pTP-dsDNA) which runs slightly slower than the naked DNA (free DNA). Subsequent rounds of replication lead to pTP coupled to displaced single strands (pTP-ssDNA). Lanes 1, 2, 7 and 8 represent the basal level of replication in the absence of Oct-1. To stimulate the replication, a range of Oct-1 concentrations was used: 53 ng (lanes 3, 9), 80 ng (lanes 4, 10), 120 ng (lanes 5, 11) and 180 ng (lanes 6, 12). The level of stimulation was determined by comparison of the replication signal in the presence of Oct-1 with the average basal signal.

In contrast to our observations made for NFI in Chapter 2, these data demonstrate that the A/T rich region in the Ad5 origin of replication is not involved in the Oct-1-induced DNA bending and stimulation of replication. Previous footprinting studies already ruled out a direct interaction of Oct-1 with the A/T-rich region (3, 4, 7). However, it was also proposed that the A/T-stretch with its low energy requirements for melting, could be indirectly involved in protein-induced DNA bending, in a way that the energy invested in DNA bending could facilitate base pair opening which would in turn help to bend DNA (5). We now demonstrate clearly that this is not the case for Oct-1. A possible explanation for this could be that the distance of 14 bp between the Oct-1 recognition site and the A/T-rich region in the origin is too large to functionally link these two DNA regions. It was previously demonstrated that the activity of the transcriptional elements in DNA replication depends on their close proximity to the A/T-rich stretches (1, 2). The NFI binding site is located next to the A/T-rich region in the Ad5 origin (Fig. 1, Chapters 2 and 3). Summarizing, these results add important knowledge to the description of the preinitiation complex assembly during initiation of Ad5 DNA replication.

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Chapter

4

The adenovirus priming protein pTP contributes to the kinetics of initiation of DNA replication

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The adenovirus priming protein pTP contributes to the kinetics of initiation of DNA replication

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Adenovirus (Ad) precursor terminal protein (pTP) in a complex with Ad DNA polymerase (pol) serves as a primer for Ad DNA replication. During initiation, pol covalently couples the first dCTP to Ser580 of pTP. Using an *in vitro* reconstituted replication system composed of purified proteins, we demonstrate that the conserved Asp578 and Asp582 residues of pTP, located close to Ser580, are important for the initiation activity of the pTP/pol complex. In particular the negative charge of Asp578 is essential for this process. The introduced pTP mutations do not alter the binding capacity to DNA or polymerase, suggesting that the priming mechanism is affected. The Asp578 or Asp582 mutations increase the Km for dCTP incorporation and higher dCTP concentrations or Mn^{2+} replacing Mg^{2+} partially relieve the initiation defect. Moreover the kcat/Km values are reduced as a consequence of the pTP mutations. These observations demonstrate that pTP influences the catalytic activity of pol in initiation. Since both Asp residues are situated close to the pol active site during initiation, they may contribute to correct positioning of the Ser580 hydroxyl group. Our results indicate that specific amino acids of the protein primer influence the ability of Ad5 DNA polymerase to initiate DNA replication.

Introduction

The genome of adenovirus serotype 5 (Ad5) is a 35.935 bp double-stranded linear DNA with a terminal protein covalently attached to each DNA end. The origins of DNA replication are located at both ends of the genome in 103 bp long inverted terminal repeats. Ad employs a unique protein priming mechanism to initiate its DNA replication (1,2). First the preinitiation complex is assembled on the origin of replication. It consists of three viral proteins, precursor terminal protein (pTP) that serves as a primer, Ad5 DNA polymerase (pol), DNA binding protein (DBP), and two cellular transcription factors NFI and Oct-1, which are able to greatly enhance the replication process. During initiation pTP presents its Ser580 residue to the pol active site. After binding an incoming dCTP nucleotide, pol covalently couples dCTP to the hydroxyl group of Ser580. This reaction depends on the affinity of pol for dCTP and pTP as well as template DNA binding, and the catalysis itself that can be kinetically described by an apparent Km (Km_{app}) (3,4). After coupling of the first dCTP, two additional nucleotides are added, forming a pTP-CAT intermediate using an internal GTA triplet at positions 4-6 as a template. Subsequently, the newly synthesized CAT trinucleotide jumps back to positions 1-3 at the beginning of the template strand (2,5). Next, pTP dissociates from pol and pol elongates DNA processively (6). Late in viral infection, pTP is cleaved by a viral protease at three specific cleavage sites (Fig. 1)(7,8) resulting in the formation of a smaller terminal protein that stays attached to each DNA end during the rest of the viral life cycle.

The priming pTP is a 75 kDa protein that can form a tight heterodimer in solution with the 140 kDa pol (pTP/pol complex)(9-12). Protease cleavage experiments and mutational studies show that a large portion of the pTP surface is involved in the interaction with pol (13-16). Moreover, studies on the molecular architecture of Ad5 pol demonstrate that pTP binds at the entrance of the primer binding groove of pol (17). Although, the sequence specificity of DNA binding by pTP alone is very

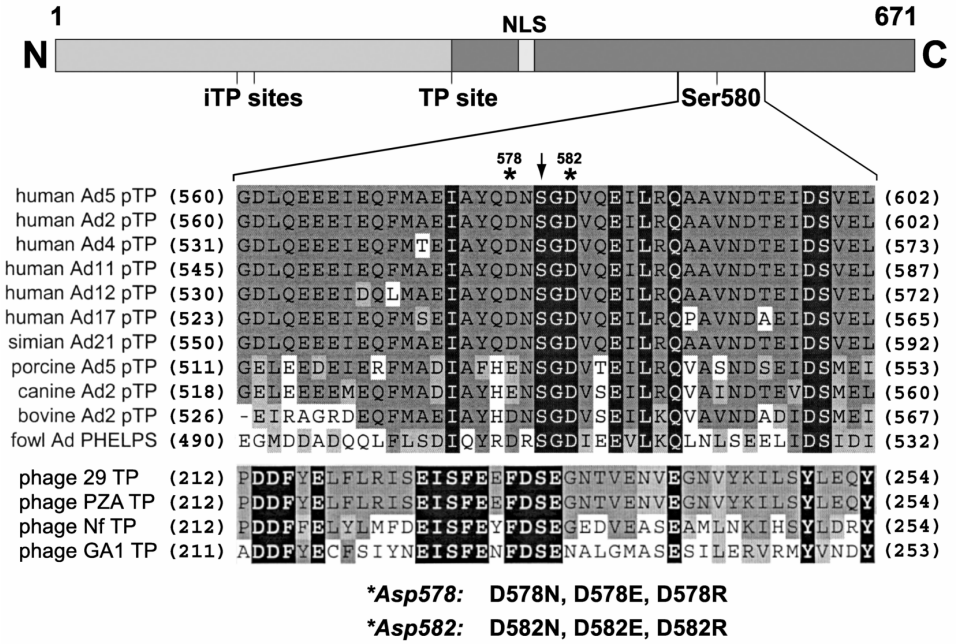

Figure 1. Alignment of terminal protein sequences of the region containing the priming Ser residue
The upper panel shows a schematic overview of the primary Ad5 pTP sequence (671 aa). Ser580 of pTP is coupled to the first nucleotide during initiation of Ad5 DNA replication. pTP contains a nuclear localization signal (NLS) and three protease cleavage sites (two iTP sites and one TP site) used by adenoviral protease in a later infection stage (7,8). The lower panel shows the aligned sequences from adenoviruses infecting human and other species. The sequences of the TP proteins from *B. subtilis* phages are also shown. Numbers in parentheses indicate the amino acid position relative to the N-terminus. Completely conserved residues are shown in black squares and similar residues are shown in grey squares. The priming Ser is marked with an arrowhead. The Asp578 and Asp582 of Ad5 pTP are indicated with asterisks and the introduced mutations are listed below.

limited (18,19), the pTP/pol heterodimer binds with increased specificity to a conserved region located between basepairs 9 and 18 in the Ad5 origin of replication (19,20). The pTP/pol complex can be recruited to the origin by the transcription factors Oct-1 and NFI, interacting with pTP and pol, respectively, resulting in stimulation of replication (21-23). Furthermore, based on the dimerization properties of pTP, the proposed interaction between pTP and the terminal protein covalently bound to the viral DNA likely stabilizes the formation of a preinitiation complex (18).

Ad5 pol can use both DNA and protein as a primer (4,24). Based on the results obtained from the study of the protein priming system in *B. subtilis* phage 29 (ϕ29) (25-28) it is likely that the same Ad5 pol catalytic site is used for interaction with both primers. We do not know the exact arrangement of pTP interacting in the pol active site, but it is remarkable that close to Ser580 several conserved negatively charged amino acids are located that might mimic DNA (Fig. 1). In this study we asked what the importance is of these negatively charged residues for the pTP-pol interaction and for the kinetics of polymerization. We demonstrated that the highly conserved Asp582 and Asp578 of Ad5 pTP are important for the optimal initiation of the Ad5 DNA replication. Furthermore, we showed that mutation of both residues influences the kinetics of initiation. Taking into consideration that Asp578 and Asp582 together with Ser580 are positioned close to the pol active site we propose that both Asp residues contribute to the arrangement of an optimally functioning active site of pol that leads to an efficient initiation.

Materials and Methods

Construction of pTP mutants

Point mutations were introduced in the pTP gene by site directed mutagenesis using the Quickchange method from Stratagene. As a template for the PCR based mutagenesis a pETpTP1S2-671 construct carrying full-length Ad5 pTP cDNA encoding amino acids 1 to 671 was used (22). Oligonucleotides used for the PCR-based mutagenesis were:
D578N 5'-GATCGCCTATCAA**AAC**AACTCAGGAGAC-3',
5'-GTCTCCTGAGTT**GTTT**TGATAGGCGATC-3',
D578R 5'-GATCGCCTATCAA**AGA**AACTCAGGAGAC-3',
5'-GTCTCCTGAGTT**TCT**TGATAGGCGATC-3',
D578E 5'-GATCGCCTATCAA**GAA**AACTCAGGAGAC-3',
5'-GTCTCCTGAGTT**TTC**TTGATAGGCGATC-3',
D582N: 5'-GACAACTCAGGA**AAC**GTGCAGGAGATT-3',
5'-AATCTCCTGCAC**GTT**TCCTGAGTTGTC-3',
D582R 5'-GACAACTCAGGA**AGA**GTGCAGGAGATT-3',
5'-AATCTCCTGCAC**TCT**TCCTGAGTTGTC-3',
D582E 5'-GACAACTCAGGA**GAA**GTGCAGGAGATT-3',
5'-AATCTCCTGCAC**TTC**TCCTGAGTTGTC-3',
with the mutations marked in bold. The presence of the mutations was confirmed by DNA sequence analysis.

Expression and purification of pTP mutants

All pTP mutants and wild type pTP were expressed as full-length proteins with an additional C-terminal His-tag (6×His). 1 liter of each BL21 expression culture was induced with 1mM IPTG at an OD_{600} of 0.6 at 30°C. After 3 hours of induction cells were harvested and 9 ml of lysis buffer was added (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol, 10 mM $Na_2S_2O_5$, 0.5 mM PMSF, 1 mg/ml lysozyme and complete protease inhibitor cocktail (Roche)). The cells were left on ice for 10 min and freeze/thawn once. Then the lysate was cleared by ultracentrifugation (2.74×10^5 g, 45 min, 4°C) and the supernatant was diluted with an equal volume of purification buffer B (20 mM HEPES-KOH pH 8.0, 5 mM β-mercaptoethanol, 10 mM $Na_2S_2O_5$, 0.5 mM PMSF and 10% glycerol). The lysate was loaded on a SP-sepharose column (1.8 ml) equilibrated with buffer B containing 150 mM NaCl (B150), the column was washed with 7 ml of B150 and proteins were eluted with buffer B containing 450 mM NaCl and 20 mM imidazole (B450/20). Eluates from SP-columns were directly loaded on NiNTA-agarose (1 ml) columns equilibrated with B450/20 and the columns were washed with 4 ml B450/20 followed by additional wash with 2 ml B150/20 in order to lower the NaCl concentration. Proteins were eluted with 500 mM imidazole in 2.5 ml of B150. Next, to stabilize purified proteins, BSA was added to a final concentration of 0.5 µg/µl and the eluates were dialyzed against buffer B150 to remove the imidazole. The purity of the proteins was estimated by coomassie staining and was approximately 90%.

Proteins and DNA templates

Adenovirus DNA polymerase was expressed using the baculovirus expression system and purified to near homogeneity as previously described (4). The protein dilution buffer contained 25 mM HEPES-KOH pH 7.5, 150 mM NaCl, 15% glycerol, 5 mM DTT and 0.5 µg/µl BSA.
As DNA templates, T30 or TD50 DNA derived from the Ad5 origin of replication was used. T30 is a single-stranded DNA representing the first 30 nucleotides of the template strand of the Ad5 origin: T30: 5'-AATCCAAAATAAGGTATATT-ATTGATGATG-3'. TD50 is a double-stranded DNA consisting of T50 and D50 that represent the first 50 nucleotides of the template and displaced strand of the Ad5 origin, respectively. T50: 5'-CTCATTATCATATTGGCTT-CAATCCAAAATAAGGTATATTATTGATGATG-3', D50: 5'-CATCATCAATAATATACCTTATTTTGGATTGAAG-CCAATATGATAATGAG-3'. TD50 was obtained by boiling equimolar amounts of T50 and D50 oligonucleotides for 5 min in 25 mM Tris-HCl pH 7.5, 100 mM NaCl and slow cooling to room temperature.

Protein-DNA interactions (EMSA)

In protein-DNA binding studies T50 was used. For the preparation of the T50 probe, the T50 oligonucleotide was end-labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP (4500 Ci/mmol) in a standard kinase buffer and the labelled T50 was purified on a 10 % polyacrylamide gel. In the DNA binding assay pTP mutants and wild type pTP were incubated with DNA for 30 min on ice in binding buffer (25 mM HEPES KOH pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 4 mM DTT, 0.2 µg/µl BSA, 3.2 % Ficoll). Protein-DNA complexes were separated on a 7 % polyacrylamide gel in TBE buffer at 4°C and the intensity of the bands was quantified using a Storm 820 phosphorimager. Where indicated, α-pTP polyclonal antibody (Ab) (29) at a 1:100 dilution was added to the binding reaction in order to identify the pTP-DNA complexes.

Co-immunoprecipitation assay of pTP with pol

30 µl of pre-swollen 50% protein A-agarose beads were washed twice with IP buffer (25 mM HEPES KOH pH 8.0, 0.5 mM β-mercaptoethanol, 1 mM $Na_2S_2O_5$, 0.5 mM PMSF, 0.1% NP-40 and 10% glycerol) containing 500 mM NaCl and 2% BSA (IP500/2%). Next, 10 µl of α-pol Ab (rabbit, polyclonal) (4,29) was added to the beads in 400 µl of IP500/2%. The beads were tumbled for 30 min at 4°C and washed twice with IP500/0.02% and twice with IP300/0.02%. In a separate tube, 600 ng Ad5 pol and 800 ng wild type pTP or pTP mutants (a two-fold molar excess of pTP over pol) were preincubated on ice for 15 min in 70 µl of IP300/0.02%. Then, the protein mixtures were added to the beads and incubation continued for 1 hour at 4°C in 400 µl of IP300/0.02%. Non-bound pTP was removed by washing three times with IP300 without BSA. After the last wash the supernatant was carefully removed, 17 µl of Laemmli sample buffer was added, the samples were boiled for 10 min and loaded on a 7.5 % SDS-PAGE gel. Proteins were detected by Western blotting using α-His Ab (mouse, monoclonal) (Qiagen) and α-pol Ab (rabbit, polyclonal) for pTP and pol detection, respectively.

In vitro initiation and partial elongation assays

In vitro initiation and partial DNA elongation was assayed using 60 ng Ad5 DNA polymerase and 50 ng wild type pTP or pTP mutants in a 25 µl reaction mixture containing initiation buffer (20 mM HEPES-KOH pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 40 ng/µl BSA) and 4 µCi of [α-^{32}P] dCTP (3000 Ci/mmol) at the final

concentration of 53 nM. When indicated, 3 mM $MnCl_2$ was used in the initiation buffer instead of $MgCl_2$. As a template 10 ng of TD50 was used for the initiation reaction and 100 ng of T30 for the partial elongation reaction. In addition, for partial elongation assays extra 0.7 μM dCTP, 40 μM dTTP and 40 μM dATP was added to allow DNA elongation. Reactions were incubated for 45 min at 37°C and stopped by the addition of EDTA to a final concentration of 80 mM. Reaction products were precipitated with 20% trichloroacetic acid (TCA), washed with 5% TCA, dissolved in sample buffer and analysed on a 7.5% SDS-PAGE gel. The intensity of the initiation and partial elongation bands was quantified using a Storm 820 phosphorimager at the linear range of the signal.

The kinetic analysis of dCTP incorporation by pol for wild type and pTP mutants was determined by performing initiation assays as described above with the addition of increasing concentrations of unlabeled dCTP (0.6-20 μM). Km_{app} and $Vmax_{app}$ were determined from Lineweaver-Burk plots. $Kcat_{app}$ was calculated from $kcat = Vmax_{app} /[E_t]$, where $[E_t]$ is a total amount of enzyme (pol). The average values were calculated from at least two independent experiments.

Results

Conservation of negatively charged residues in the priming region of Ad5 pTP

The crucial amino acid of Ad5 pTP is Ser580, which is used to prime Ad5 DNA replication (30). There are several negatively charged amino acids located in the neighbourhood of Ser580 in adenoviruses and also in other protein priming systems (Fig. 1). In this study we analysed Asp578 and Asp582 of Ad5 pTP located near Ser580, (Fig. 1). The amino acid residue at position 578 exists as Asp or Glu in different serotypes and Asp582 is completely conserved in all adenovirus serotypes known to date. To analyse the role of these two residues in initiation they were mutated to Asn (D to N) in order to remove a negative charge, or mutated to Arg (D to R) to replace a negative charge by a positive charge. In addition, to verify the significance of the negative charge, Asp was changed to Glu (D to E), keeping the negative charge unchanged. All mutants were expressed and purified as his-tagged full-length pTP proteins as described in Materials and Methods.

Mutation of Asp578 or Asp582 of pTP affects the initiation activity

During initiation covalent coupling of the first C-residue to the Ser580 hydroxyl group of the pTP-dCMP product (pTP-C) which can be detected by SDS-PAGE. The ability of the pTP mutants to serve as a primer was tested in an initiation assay (Fig. 2, Table II). The origin-dependent formation of pTP-C was studied using a dsDNA fragment (TD50) consisting of the first 50 nucleotides of the Ad5 core origin. Substitution of Asp578 to Asn or Arg decreased pTP initiation activity to 29% and 7% of wild type activity for D578N and D578R, respectively. Changing Asp578 to Glu (D578E) did not affect the initiation activity at all. Mutation of Asp582 resulted in more severe effects on initiation. D582N showed only 15% activity, whereas the activity of D582R was completely abolished. Furthermore, the initiation activity of D582E decreased to 35%. When higher amounts of pTP up to 2-fold were used, the relative initiation activity of the pTP mutants compared with wild type did not change (data not shown). These results demonstrate the importance of Asp578 and Asp582 in initiation.

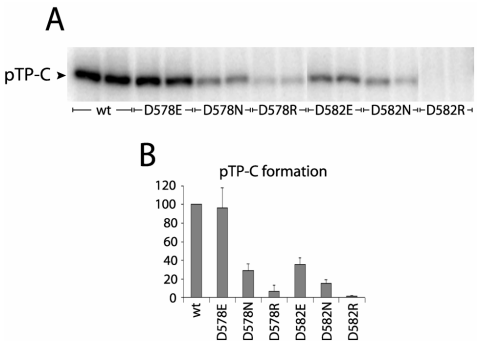

Figure 2. Initiation activity of the pTP mutants is affected
(A) The initiation activity of Asp578 and Asp582 mutants was measured by the formation of the [α-^{32}P]-dCMP-pTP product (pTP-C). 60 ng of polymerase and 50 ng of wild type pTP or the pTP mutants were incubated with 10 ng of TD50 template. (B) Percentage of initiation activity of the pTP mutants compared with the wild type activity (set at 100%). The error bars represent the standard deviation from three independent experiments.

Mutation of Asp578 or Asp582 of pTP does not decrease the DNA elongation

When all nucleotides are present the pTP-C complex can be elongated leading first to a pTP-CAT initiation intermediate, which jumps back and is subsequently extended generating longer products. In the absence of dGTP elongation proceeds only to position 25, since at position 26 the first G residue should be incorporated. This leads to the formation of a pTP-25 product

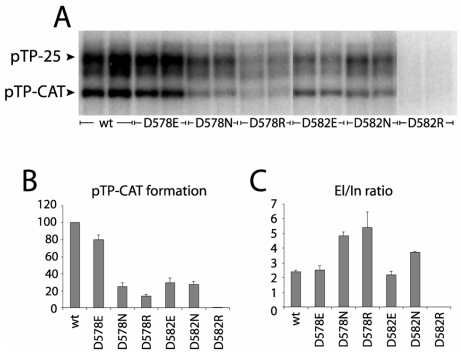

Figure 3. Elongation activity of the pTP mutants is not decreased

(A) Partial elongation was measured in the absence of dGTP resulting in DNA elongation only up to 25 nucleotides (pTP-25). At low dCTP concentration only part of the pTP-CAT intermediate jumps back and elongates, allowing simultaneous analysis of both products. 60 ng of polymerase and 50 ng of wild type pTP or the pTP mutants were incubated with 100 ng of T30 template. The positions of the pTP-CAT and pTP-25 product are indicated with arrowheads. The band directly below pTP-25 represents a faster migrating pTP-25 product due to secondary structure formation of the 25 nucleotide-long ssDNA. For our measurements of DNA elongation we have added these two signals. (B) Amount of pTP-CAT formation for the pTP mutants compared with the wild type. Wild type pTP activity was set to 100%. (C) Elongation/Initiation (El/In) values calculated as the ratio between the amount of pTP-25 and pTP-C product. The error bars in both graphs represent the standard deviation from three independent experiments.

Table II. Analysis of the initiation activity of the pTP mutants

pTP	Initiation activity (%) [a]		
	1 mM Mg^{2+}	1 mM Mg^{2+} 20 μM dCTP [b]	3 mM Mn^{2+} [c]
wild type	100	100	100 [c]
D578E	97	118	145
D578N	29	48	90
D578R	7	26	65
D582E	35	65	69
D582N	15	46	45
D582R	<1	<1	<1

[a] Numbers represent the percentage of initiation activity of the pTP mutants compared with wild type pTP. Wild type activity was set to 100% for each condition. [α-^{32}P] dCTP was present at the standard 53 nM concentration.

[b] The values represent the activity in the presence of an additional 20 μM of unlabeled dCTP.

[c] The initiation activity of wild type pTP in the presence of 3 mM Mn^{2+} was 8-fold higher than in the presence of 1 mM Mg^{2+}.

that migrates around 90 kDa in SDS-PAGE (Fig. 3A). At a low dCTP concentration only part of the pTP-CAT intermediate elongates, enabling the study of the pTP-CAT formation and DNA elongation simultaneously. As shown in Fig. 3AB, the formation of pTP-CAT decreased to 25% and 14% of wild type activity for D578N and D578R, respectively, whereas D578E showed 80% activity. The activity of D582N decreased to 27% and D582R showed no detectable activity. D582E showed 30% activity compared with wild type pTP. The ability of the pTP mutants to form pTP-CAT (Fig. 3AB) correlated with their ability to form the initiation pTP-C product (Fig. 2).

The level of DNA elongation was calculated from the pTP-25/pTP-CAT ratio (elongation/initiation ratio, El/In, Fig. 3C). Under the experimental conditions used the El/In ratio for wild type pTP was 2.4 ± 0.12. Mutants D578E and D582E also showed a wild type El/In ratio of 2.5 ± 0.26 and 2.2 ± 0.21, respectively. This indicates that substitution of Asp578 and Asp582 with Glu does not disturb the jumping back process or the subsequent DNA elongation. For the D578N, D578R and D582N the El/In ratio was even higher than that of wild type pTP, 4.9 ± 0.25, 5.4 ± 1.07 and 3.7 ± 0.06, respectively, showing that more elongating product (pTP-25) was formed after initiation. No elongation was observed for the D582R mutant.

The DNA binding capacity of pTP mutants is not affected

In order to verify that the mutations introduced in pTP did not affect DNA binding we performed electrophoretic mobility shift assays (EMSA) using ssDNA representing the first 50 nucleotides of the Ad5 origin template strand (T50). Addition of pTP to the DNA gave rise to multiple protein-DNA complexes (Fig. 4A). Two of these were specific pTP-DNA complexes containing one (pTP1) or two pTP molecules (pTP2), which is consistent with a previous study of DNA binding properties of pTP (18). Two different concentrations of pTP were tested for each mutant (Fig. 4A).

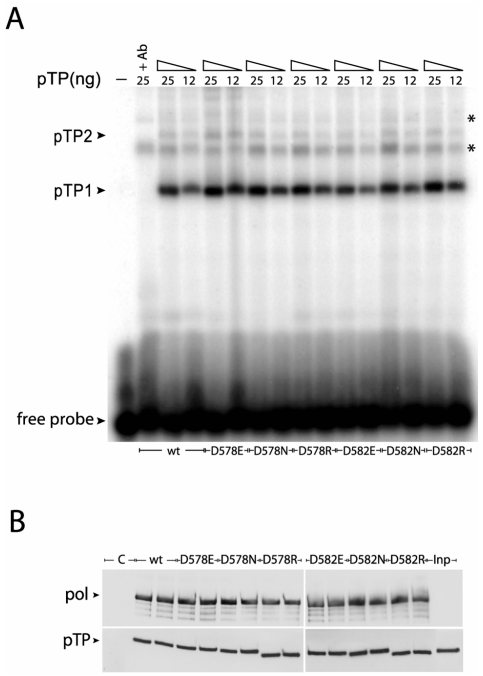

Figure 4. Binding of the Ad5 pTP mutants to DNA or to pol is not altered

(A) EMSA experiments with single-stranded T50 probes representing the first 50 nucleotides of the Ad5 template strand. The positions of the pTP-DNA complexes (pTP1 monomer and pTP2 dimer) and free DNA are marked with arrowheads. The DNA probe was incubated with 12 ng or 25 ng of wild type pTP or the pTP mutants. In the second lane (+Ab) α-pTP polyclonal antibody was added to the binding reaction with wild type pTP at a 1:100 dilution in order to identify the protein-DNA complexes containing pTP molecules. Two asterisks point to non-specific protein-DNA complexes that do not contain pTP, possibly as a result of some impurity in the pTP samples. The first lane represents free probe.

(B) Analysis of pTP/pol complex formation in co-immunoprecipitations. A mixture of 800 ng of wild type pTP or the pTP mutants with 600 ng of pol was incubated with protA-αpolAb beads. The ability of pTP to bind pol was analysed by western blotting using specific antibodies against pTP or pol. Positions of pol and pTP are marked with arrowheads. The first lane is a control lane (C) where pol was omitted in the incubation mix and the last lane represents 10 % of the pTP added to the beads (Inp). The smaller size of D578R and D582R pTP is most likely caused by the fact that an Arg substitution affects the protein migration in the gel. Careful restriction analysis and sequencing of the expression vectors carrying the D578R and D582R pTP mutants did not show any sequence deletions. The weaker signals below the actual pol bands are caused by pol degradation.

The binding affinity of the pTP mutants was not significantly changed compared to wild type binding, demonstrating that mutations of Asp578 and Asp582 do not affect the pTP-DNA interaction.

The interaction of pTP mutants with pol is not disturbed

It is likely that during initiation the priming region of pTP interacts with the pol active site. Therefore it is possible that the defects in initiation are caused by a reduced interaction between pTP and pol. The contribution of the Asp578 and Asp582 residues to pTP-pol interaction was analysed in a co-immunoprecipitation assay. The control experiment without pol showed no non-specific pTP binding to the protA beads or to the α-pol antibody (Fig. 4B). When pol was added, approximately 10% of pTP was co-immunoprecipitated at the conditions used. All mutants showed unaffected pol binding (Fig. 4B). We also performed experiments testing the stability of the pTP-pol interaction at 37°C and up to 60 min, but no differences in half-life were observed for the pTP/pol complexes containing pTP mutants compared to the wild type (data not shown). Based on these results we conclude that binding of the pTP mutants to pol is not disturbed.

The kinetics of the initiation reaction is affected by Asp578 or Asp582 mutations of pTP

Since a decreased activity of the pTP mutants was noticeable already in the protein priming step, we performed a kinetic analysis of the initiation reaction. Unlabeled dCTP ranging from 0.6 to 20 μM was added to the reaction mixture containing wild type or mutated pTP and wild type polymerase. The reactions were incubated for 45 min, which is still in the linear time range. Based on the Lineweaver-Burk plots the apparent Km_{app} for dCTP incorporation was determined (Table I). Interestingly the Km_{app} values increased for the D578N, D578R, D582E and D582N pTP mutants. When initiation was performed in the presence of 20 μM dCTP, the D578N, D578R, D582E

and D582N mutants gained activities up to 48 %, 26%, 65% and 46% of wild type activity, respectively (Table II). However, the severe initiation defect of D582R could not be rescued. The increased values of the Km_{app} of the pTP/pol complexes containing pTP mutants and their increased activities at higher dCTP concentrations suggest a reduced affinity of pol for dCTP when it is in complex with the pTP mutants.

In addition to the Km_{app}, the $kcat_{app}/Km_{app}$ ratios of pTP/pol complexes in initiation were determined (Table I). The $kcat_{app}/Km_{app}$ ratios were seven-fold lower for D578R, and two-fold lower for D578N, D582E and D582N pTP, demonstrating that the catalytic efficiency of the initiation reaction is reduced by these pTP mutations. These results correlate with the decreased initiation activity of the mutants (Fig. 2). The pTP/pol complex containing D578E showed a wild type Km_{app} and $kcat_{app}/Km_{app}$ (Table I), which is in agreement with the wild type activity found in initiation. The initiation activity of D582R was below the detection limit, so the specific kinetic properties could not be determined.

Table I. Analysis of the kinetics of the initiation of Ad5 DNA replication [a]

pTP	Km_{app} (μM dCTP)	$kcat_{app}/Km_{app}$ [b] ($M^{-1}s^{-1}$)
wild type	2.06 ± 0.33	2.20
D578E	2.12 ± 0.11	2.01
D578N	4.16 ± 0.16	1.16
D578R	5.68 ± 1.48	0.31
D582E	3.45 ± 0.62	1.13
D582N	4.12 ± 0.30	0.98
D582R	/	/

[a] The Km_{app} and $Vmax_{app}$ values of pTP deoxycytidylation by polymerase in the initiation reaction were calculated from Lineweaver-Burk plots (0.6-20 μM dCTP) based on at least two independent experiments for each pTP. The Km_{app} and $Vmax_{app}$ of D582R pTP could not be determined since the initiation activity of this mutant was below the detection limit.

[b] $kcat_{app}/Km_{app}$ ratio describes the catalytic efficiency of initiation. $kcat_{app}$ was calculated from $kcat_{app} = Vmax_{app}/[E_t]$, where $[E_t]$ is the total amount of enzyme (pol).

The presence of Mn^{2+} partially relieves the initiation defect of the pTP mutants

Ad5 pol uses two Mg^{2+} ions in the active site for efficient catalysis. However, it can also make use of other divalent cations with different efficiencies depending on the cation (31). Mn^{2+} increases the activity of DNA polymerase not only of Ad5 pol but also of other polymerases (31,32). This suggests that Mn^{2+} induces subtle alterations in the active site of pol that might result in a more efficient catalysis. In order to analyse the influence of Mn^{2+} on the activity of the pTP mutants, initiation assays were performed in the presence of 3 mM Mn^{2+}, the optimal conditions for Mn^{2+}, instead of the standard 1 mM Mg^{2+} (Table II). Indeed, Mn^{2+} increased the initiation activity of the pTP mutants resulting in 145%, 90%, 65%, 69% and 45% of the wild type activity for D578E, D578N, D578R, D582E and D582N, respectively. The abolished initiation activity of the D582R pTP mutant could not be rescued by Mn^{2+} confirming that the D582R mutation affects pTP/pol function severely. These data demonstrate that in the presence of Mn^{2+} pol tolerates the mutations present in pTP and that Mn^{2+} can lead to a more efficient initiation for the pTP mutants compared with the initiation in the presence of Mg^{2+} (Fig. 2, Table II).

Discussion

Functional importance of Asp578 and Asp582 of pTP for Ad5 DNA replication

We show that two conserved amino acids, Asp578 and Asp582, of Ad5 pTP are essential for the initiation of replication. Substitution of these residues with Asn or Arg greatly reduced the ability of pTP to form the pTP-C and pTP-CAT products (Fig. 2, Fig. 3AB). The strongest effect was observed when Asp was changed into a positively charged residue. We demonstrate that specifically the negative charge of Asp578 is essential for the initiation, since its change to Glu did not affect the initiation. Some Ad serotypes have a Glu at this position (Fig. 1) confirming that this residue is already functional when a negative charge is

present. On the other hand Asp582 is completely conserved, and cannot be substituted by a Glu residue without loss of initiation activity. Since Glu has a larger side chain than Asp, it is likely that such a mutation is not tolerated by a functionally optimal protein structure.

During initiation, Ser580 of pTP is located in the active site of pol. Asp578 and Asp582 must be in or near the active site as well, and may contact pol or even template DNA. However, with the assays used in this study we can not find any effect on the interaction with DNA or pol (Fig. 4). Since multiple regions of pTP are involved in pol binding (14-16) it is likely that single point mutations do not significantly disrupt this interaction. Our assay conditions do not rule out that the mutations locally affect the pTP/pol interaction within the active site. This could affect the optimal positioning of Ser580 of pTP in the pol active site leading to an impaired initiation.

After initiation, formation of pTP-CAT and jumping back is followed by dissociation of TP from pol, which is almost complete by the time seven nucleotides are synthesized (5,6). The elongation/initiation ratio (El/In) ratio (Fig. 3C) showed that substitution of Asp578 and Asp582 with Glu does not impair the jumping back event and the subsequent DNA elongation. Interestingly, in case of the D578N, D578R and D582N mutants the elongation was more efficient than wild type pTP. It is possible that these mutations locally weaken the interaction of pTP with pol, thereby facilitating the dissociation of pol from pTP after jumping back, leading to an increased amount of pTP-25. An alternative explanation might be that the D578N, D578R and D582N mutants need a lower dNTP concentration to elongate efficiently and that the increased El/In ratio is a result of changed kinetics of the DNA elongation step. Kinetic studies also showed that a higher dCTP concentration is required for optimal activity of pol during elongation compared with initiation (3,6).

Contribution of pTP to the kinetics of the initiation of Ad5 DNA replication

We show that mutations of Asp578 and Asp582 change the kinetics of the initiation reaction. The Km_{app} for incorporation of dCTP was increased for the pTP/pol complexes containing pTP mutants shown to be defective in initiation. Moreover, at high dCTP concentrations the mutants gained some initiation activity suggesting a reduced affinity of the pTP/pol complex for dCTP. These observations demonstrate that pTP contributes to the affinity of pol for dCTP in the initiating pTP/pol complex. We also observed that the presence of Mn^{2+} instead of Mg^{2+} partially relieved the initiation defect of the pTP mutants. Reduced $kcat_{app}/Km_{app}$ values show that also the overall catalytic efficiency of the reaction is decreased. These results demonstrate that the decrease in initiation activity caused by the Asp578 and Asp582 mutations is a direct consequence of changed kinetics of initiation. So far, polymerase mutations were only reported to affect the kinetics of initiation for Ad5 or ϕ29 (4,32,33). In this study we show that the protein primer pTP, interacting with polymerase directly influences the kinetics of the initiation. Such an effect was already suggested by our previous study that revealed a change in the active site of pol after dissociation from pTP upon transition from initiation to elongation (6). Also, the elongation and exonuclease activities of pol are inhibited by pTP (24), showing that pTP indeed influences the pol active site. The kinetic properties of the active site of pol are described by Km_{app} for dCTP incorporation and might depend on several parameters like affinity of pol for dCTP, pTP and template DNA binding and positioning of the hydroxyl group of Ser580. The increase of the Km_{app} caused by the Asp578 and Asp582 mutations indicates that pTP contributes to the correct arrangement of the active site of pol resulting in the optimal activity of pol.

A structural model of a protein priming

Studies on Ad5 pol and *B. subtilis* ϕ29 polymerase demonstrated that the protein primer as well as the DNA primer bind to a common site of polymerase (17,25,26). Therefore, it is likely that protein primers mimic DNA in order to interact with pol in the primer-binding groove. Interestingly, the priming region of pTP surrounding Ser580 contains 13 out of 43 conserved acidic residues (Fig. 1). They cluster at one side of two predicted α-helices. We observed that two of these residues (Glu585 and Asp594) are also important for the initiation of replication (data not shown). Similarly, the priming region of ϕ29 TP is also rich with negatively charged residues, conserved among other *B. subtilis* phages (11 aa out of 43 aa, Fig. 1). The secondary structure prediction of the Ad5 pTP and the ϕ29 TP priming region revealed that in both proteins the priming Ser residue is located in a β-turn surrounded from both sides by α-helices (34). Asp578 and Asp582 of Ad5 pTP could similarly be positioned in a β-turn and surface exposed. Such a location could facilitate the optimal charge-charge interactions between pTP and the active site of pol leading to the optimal positioning of the Ser580 hydroxyl group. Similarly, TP of ϕ29 also contains conserved acidic residues located next to the priming Ser232 (Asp231 and Glu233, Fig. 1), but their role in initiation has not been studied so far.

Binding of pTP to pol might structurally resemble interaction of the regulatory factor GreB to bacterial RNA polymerase (RNAP) (35,36). The globular C-terminal domain of GreB binds at the edge of the RNAP active site channel, while the N-terminal coiled-coil domain extends into a channel leading to the RNAP active site with its tip making contacts in the active site. Two conserved acidic residues in the coiled-coil tip (Asp 41 and Glu 44) are located very close to the RNAP active site and are proposed to influence its function. Mutations of these residues cause severe functional defects without affecting the interaction with RNAP, while substitution of Asp 41 with Glu had little or no effect on GreB function (36). Similar roles are proposed for Asp 290 and Glu 291 of the eukaryotic elongation factor TFIIS (37).

Our results demonstrate that the negatively charged residues Asp578 and Asp582 of Ad5 pTP play an important role in the arrangement of an optimally functioning polymerase active site, leading to an efficient initiation of Ad5 DNA replication. This shows that pTP is an integral component of the pol active site contributing to the catalytic activity of pol. It is likely that the contribution of the acidic residues to protein priming is a general mechanism among other organisms that use protein as a primer to start the DNA replication.

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Addendum

The negative charges of Glu585 and Asp594 of Ad5 pTP are important for initiation of replication

Addendum

The negative charges of Glu585 and Asp594 of Ad5 pTP are important for initiation of replication

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During initiation of Ad5 DNA replication, Ser580 of pTP is positioned in the active site of polymerase (pol). Interestingly, the pTP region around Ser580 contains 13 out of 43 amino acids that are negatively charged and conserved among different serotypes. Two residues, Asp578 and Asp582, which are located close to Ser580 are involved in initiation. In this study we demonstrate that also residues located a bit further from Ser580, Glu585 and Asp594, are important for initiation. We propose a model in which the optimal positioning of the Ser580 in the pol active site is facilitated by charge-charge interactions within the pTP/pol complex.

In Chapter 4 we demonstrated the importance of the conserved Asp578 and Asp582 residues of the Ad5 pTP for the optimal initiation of Ad5 DNA replication. These amino acids are located close to Ser580, which is coupled to the first nucleotide during the initiation process (Fig. 1). Moreover, we show that specifically the negative charge of Asp578 is essential for this process. It is remarkable that within 43 amino acids of the pTP priming region around Ser580, 13 residues are negatively charged and also conserved.

In this study we raised the question if other acidic residues located in this region are also important for Ad5 initiation. Therefore, we constructed additional pTP mutants of Glu566, Glu573, Glu585 and Asp594, respectively (Fig. 1). To verify the significance of the negative charge a set of two mutants for each residue was constructed. Substitution of Glu with Asp or Asp with Glu kept the charge unchanged (E to D or D to E substitution) and substitution of Glu with Gln and Asp with Asn removed the negative charge (E to Q or D to N). The ability of the mutants to serve as a primer was analysed in an initiation assay that was performed as described in Chapter 4. Replacement of Glu585 with Gln (E585Q) and Asp594 with Asn (D594N) decreased the initiation activity to 71% of wild type activity for E585Q and 24% for D594N pTP, whereas mutations of Glu585 to Asp (E585D) and Asp594 to Glu (D594E) did not significantly affect the initiation activity (Fig. 2). This demonstrates that the negative charge of Glu585 or Asp594 is important for initiation of Ad5 DNA replication. When the initiation reaction was performed in the presence of 20 μM dCTP, the activity of E585Q was rescued to a wild type level (Fig 2B). The activity of the D594N mutant increased more significantly, up to 61% of the wild type activity compared with 24% at 53 nM dCTP. This indicates that the affinity of pol for dCTP when it is in a complex with the E585Q and D594N pTP mutants is reduced, and it might be the result of changed kinetics of initiation.

Mutations of Glu566 or Glu573 residues did not affect the initiation activity of the pTP mutants, showing that these residues are not involved in the initiation process. The simple explanation for that would be that not all acidic residues from the pTP priming region are essential for initiation. Alternatively, single point mutations are not sufficient to affect the initiation activity of pTP.

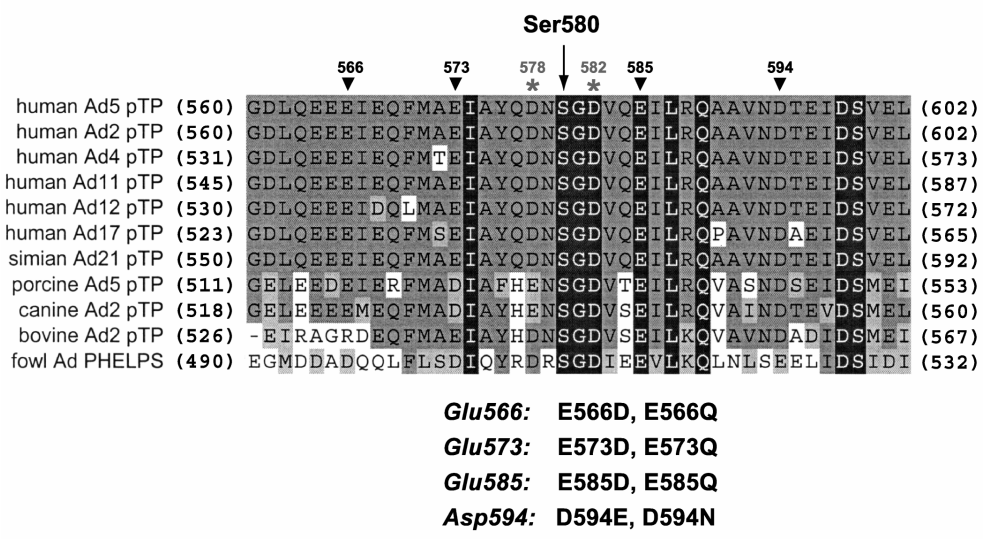

Figure 1. Conservation of the Ad pTP priming region containing the Ser residue
Sequence alignment of the priming pTP proteins from adenoviruses infecting human and other species. Numbers in parentheses indicate the amino acid position relative to the N-terminus. Residues identical among the listed viruses are shown in black squares, conserved residues are shown in dark grey squares and similar residues are shown in light grey squares. The priming Ser is marked with an arrowhead. The Glu566, Glu573, Glu585 and Asp594 of Ad5 pTP are marked with black triangles and the introduced mutations are listed below. The Asp578 and Asp582 analysed in Chapter 4 are indicated with grey asterisks

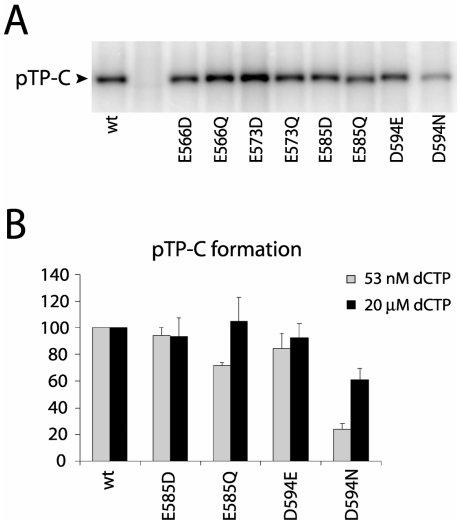

Figure 2. Analysis of the initiation activity of the pTP mutants
(A) The initiation activity of the pTP mutants was analysed in the initiation assay representing formation of the [α-^{32}P]-dCMP-pTP product (pTP-C). 60 ng of polymerase and 50 ng of wild type pTP or the pTP mutants were incubated with 10 ng of TD50 template in the presence of 53 nM dCTP.
(B) Percentage of initiation activity of the pTP mutants at 53 nM dCTP (grey bars) and 20 µM dCTP (black bars), compared with the wild type activity (set at 100%). The error bars represent the standard deviation from at least two independent experiments

These data demonstrate that Glu585 and Asp594 located in the vicinity of Ser580 play an important role in the initiation of Ad5 DNA replication. Secondary structure prediction studies of the Ad5 pTP showed that the priming region consists of two α-helices that surround a β-turn containing the Ser580 residue (2). Fig. 3 shows a helical wheel presentation of the two predicted α-helices from the priming region of pTP. Interestingly, for both α-helices, the

negatively charged residues cluster at one side of the α-helix, whereas the other side of the α-helix consists of hydrophobic residues. We propose that the two predicted α-helices can form a coiled-coil domain through hydrophobic interactions, with the negatively charged residues exposed on its surface. Ser580 would be then positioned in a β-turn on the tip of this domain. The negatively charged residues of pTP, like the Glu585 analysed in this study, could mimic DNA in order to interact with pol in the primer-binding groove. The fact that the pTP primer or the DNA primer bind to a common site of pol supports this hypothesis (1). In other processes such as regulation of eukaryotic or bacterial transcription a coiled-coil domain was already shown to be involved in the interaction of the regulatory proteins with RNA polymerase (3, 4).

In our study we only mutated single selected acidic residues from the pTP priming region. In order to strengthen our hypothesis, mutation of other acidic residues in the priming region of pTP, followed by functional analysis of the mutants is required. Moreover, substitution of hydrophobic amino acids of that region with hydrophilic residues would validate the possibility of coiled-coil domain formation. Such mutation would eliminate the hydrophobic surfaces of the two predicted α-helices and consequently affect or even prohibit formation of the coiled-coil domain.

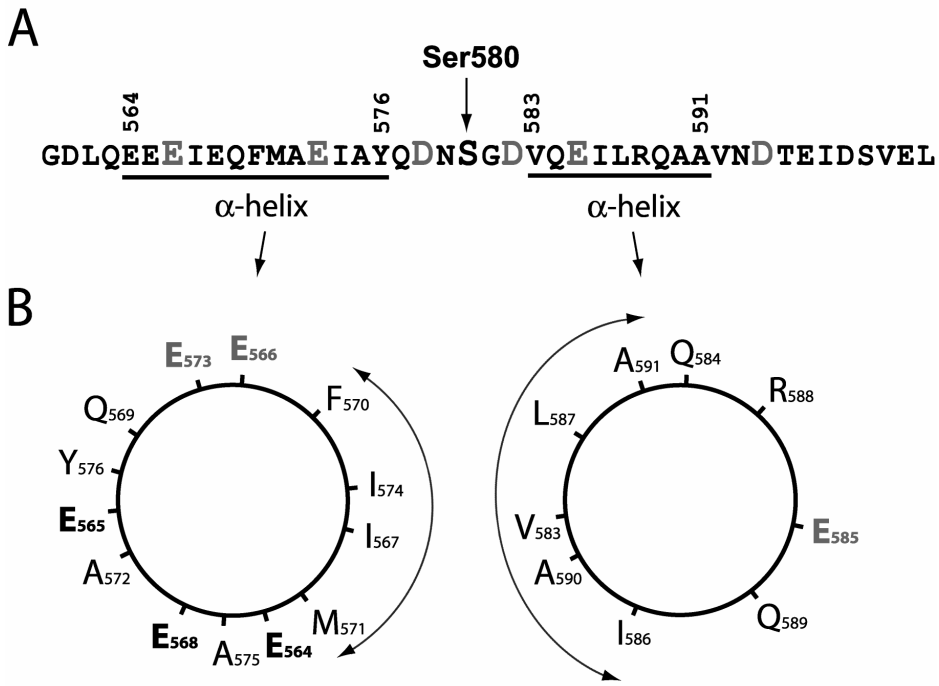

Figure 3. Helical wheel presentation of the Ad5 pTP priming region containing Ser580

(A) Sequence of the Ad5 pTP priming region containing Ser580. The amino acids forming two α-helices according to the secondary structure prediction are underlined (2). Ser580 is marked with an arrowhead.

(B) The two predicted α-helices are presented in the form of a helical wheel. Since one turn in an α-helix is 3.6 residues long, the distance between two amino acids is 100° of the circle, and the consequent residues can be plotted along the circle. This allows analysis of the position of the amino acids in the α-helix. Negatively charged residues are marked in bold and the hydrophobic surfaces of α-helices are indicated with curved lines. pTP residues analysed in this study and in Chapter 4 are marked in grey in both panels.

Studies on the pTP/pol architecture revealed that the distance from the active site of pol to the primer binding groove is 9-10 nucleotides, which is about 30Å (1). To present the Ser580 to the active site of pol, pTP needs to insert one of its domains 30Å deep into the primer binding channel of pol. Theoretically, two predicted α-helices of pTP would form a coiled-coil structure that has a length of only 19Å (9 or 13 aa). Therefore, it is likely that pTP residues positioned outside the α-helices of the priming region are also involved in correct initiation. Indeed, we showed that the negative charge of Asp594, located 14 aa from Ser580 is important for initiation activity of pTP. Previously it was also demonstrated that insertion of four amino acids between Val592 and Asn593 of pTP abolished the initiation activity (5). This could be explained by the fact that such mutation would cause a change in the positioning of Asp594. Consequently, possible Asp594 interactions that are important for the optimal initiation would be lost. It needs to be mentioned that in order to fully demonstrate the importance of the Glu585 and Asp592 residues, the activity of the pTP mutants in pTP/pol interaction, DNA binding and elongation as well as kinetics of initiation should be additionally analysed.

Materials and Methods

Construction of pTP mutants

Point mutation were introduced in the pTP gene as described in Chapter 4 using the following oligonucleotides:
E566D 5'-CTCCAGGAGGAA**GAT**ATCGAGCAG-TTC-3',
5'-GAACTGCTCGAT**ATC**TTCCTCCTG-GAG-3',
E566Q 5'-CTCCAGGAGGAA**CAG**ATC-GAGCAGTTC-3',
5'-GAACTGCTCGAT**CTG**TTC-CTCCTGGAG-3',
E573D 5'-CAGTTCATGGCC**G-AT**ATCGCCTATCAAG-3',
5'-CTTGATAGGCG-AT**ATC**GGCCATGAACTG-3',
E573Q 5'-CAGT-TCATGGCC**CAG**ATCGCCTATCAA-3',
5'-TTG-ATAGGCGAT**CTG**GGCCATGAACTG-3',
E585D 5'-GGAGACGTGCAG**GAT**ATTTTGCGCCAG-3',
5'-CTGGCGCAAAAT**ATC**CTGCACGTCTCC-3',
E585Q 5'-GGAGACGTGCAG**CAG**ATTTTGCGC-CAG-3',
5'-CTGGCGCAAAAT**CTG**CTGCACGTC-TCC-3',
D594E 5'-GCCGCCGTCAAC**GAA**ACC-GAAATTGAT-3',
5'-ATCAATTTCGGT**TTC**GTT-GACGGCGGC-3',
D594N 5'- GCCGCCGTCAA-C**AAC**ACCGAAATTGAT-3',
5'-TCAATTTCGGT-**GTT**GTTGACGGCGGC-3',
with the mutations marked in bold.

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Chapter

5

Discussion

DNA replication is a crucial step in the Ad life cycle and after infection of a host cell Ad rapidly enhances this process, utilizing two cellular transcription factors NFI and Oct-1. The replication of Ad DNA starts with the formation of a preinitiation complex (PIC), which consists of pTP, pol, DBP, NFI and Oct-1. All proteins bind to the 50 bp-long origin DNA, which contains TP protein covalently attached to the 5' terminus. The next important step of replication is initiation. Ad employs a unique protein priming mechanism, in which pol covalently couples the first nucleotide (C) to the Ser580 hydroxyl group of pTP. The initiation is followed by the addition of two more nucleotides, A and T, generating the pTP-CAT product that jumps back to the beginning of the origin. Subsequently, pol processively synthesizes a new DNA strand. The research presented in this thesis is mainly focused on the two early events of Ad DNA replication, which are tightly connected with each other, the assembly of the PIC and the subsequent initiation process.

Architecture of the preinitiation complex

In order to achieve optimal initiation, the PIC has to adopt the most favourable nucleoprotein architecture involving protein-protein and protein-DNA interactions. The data presented in Chapters 2 and 3 demonstrate that NFI and Oct-1 serve as architectural factors that facilitate formation of the nucleoprotein structure of the PIC by induction of a DNA bend in the origin. NFI and Oct-1 bend the origin DNA independently, NFI induces a 60° DNA bend and Oct-1 induces a bend of 42°. These two bends are oriented in the same direction, resulting in an 82° collective bend when NFI and Oct-1 are simultaneously bound to the origin DNA. Although the collective bend is not the sum of the two individual bends, the stimulation of replication in the presence of both proteins is synergistically enhanced. This shows that the presence of an 82° bend in the origin DNA results in a more efficient DNA replication than when the origin is bent by 60° or 42°. From these data a clear correlation emerges that the more the origin DNA is bent, the larger the enhancement of replication is. The two DNA bends induced by NFI and Oct-1 are not the only structural changes in the origin DNA induced during the PIC assembly. Data in Chapter 2 demonstrate that the origin DNA is intrinsically bent by 17°, which is likely due to the high content of A and T bases. Furthermore, TP that is covalently bound to the 5' end of the origin DNA additionally destabilizes the terminus of the origin (20). Also DBP changes the DNA structure resulting in stimulation of initiation by enhancement of NFI and the pTP/pol complex binding to the origin (4, 24, 25, 27). Moreover, during initiation the core origin has to be unwound since pol uses single-stranded DNA as a template for initiation, while the displaced strand is protected by DBP (7, 28). All these structural features contribute to an optimal geometry of the PIC and lead to the efficient initiation of Ad5 DNA replication.

The stimulation of replication by NFI and Oct-1

Over the last years there have been two models proposed that explain the mechanism of stimulation by NFI and Oct-1. The first model refers to DNA bending and suggests that structural DNA changes induced by NFI or Oct-1 upon binding to the origin, stabilize binding of other proteins and facilitate PIC formation. This model is supported by the observation that the level of stimulation of replication by NFI or Oct-1 depends on the DNA binding affinity of these proteins (8, 21). The second model, the recruitment model, is based on direct interactions between NFI and pol and between Oct-1 and pTP and proposes that each transcription factor recruits the pTP/pol complex to the origin and thus increases the number of active initiation complexes. It is likely that both mechanisms are involved in stimulation. However, up till now it is not known what is the contribution of each mechanism to the stimulatory function of NFI or Oct-1. The data presented in Chapter 2 are in favour of the first model and clearly demonstrate that the DNA bend induced by NFI is

functionally important, since mutations in the origin that reduce the bend angle to 33-37° also abolish the stimulation of replication by NFI. On the other hand, the same mutations do not affect the degree of the Oct-1-induced DNA bend nor do they affect the stimulatory function of Oct-1. In order to verify the functional importance of the Oct-1-induced DNA bend, a mutant of the Oct-1 protein itself or a mutant of the origin needs to be created that reduces this bend angle. The attempts to change the Oct-1-induced bend angle by changes in the length of the linker connecting the POUs and POUh domains were unsuccessful (30). Alternatively, since the level of Oct-1 stimulation depends on the affinity of DNA binding as analysed using different Oct-1 binding sites (21), it would be also interesting to determine the DNA bend induced by Oct-1 upon binding to these different Oct-1 binding sites. In agreement with the first model of stimulation, NFI and Oct-1 were shown to stabilize binding of the pTP/pol complex to the origin (16, 29). Therefore, it would be interesting to link the NFI-induced and Oct-1-induced DNA bend with their ability to stabilize the pTP/pol complex binding to the origin DNA. However, initial EMSA experiments using mutated Ad5 origins that reduce the NFI-induced DNA bend did not yet give conclusive results.

What is the mode of NFI binding and bending of DNA?

The structural data on the interaction of the Oct-1 POU domain with its recognition site in DNA are available from the crystal structure (11). However, the structure of NFI bound to DNA has not yet been solved and interesting questions on what interactions are involved in NFI binding to DNA and how NFI induces the DNA bend, remain unanswered. A recent alignment study using the novel COMPASS method postulated a structural relationship between the DNA binding domain of NFI and the MH1 domain of the Smad proteins, transcription factors involved mainly in TGF-β signalling (22). According to the crystal structure of the Smad3 MH1 domain bound to DNA, the functional part of this domain is a β-hairpin that binds the DNA in the major groove (23). Since NFI also binds the major groove of DNA (6, 31), it is likely that the NFI-DNA interactions involve a putative β-hairpin of the NFI DNA binding domain. Data in Chapter 3 suggest that NFI induces a DNA bend by broadening of the major groove. This change could be achieved by insertion of a putative β hairpin of NFI into the DNA structure. Such interaction was reported for phage T7 RNA polymerase that inserts a β-hairpin into the major groove of promoter DNA, thereby inducing a slight bend and melting of the DNA structure (3). It is not easy to analyse the nature of NFI-DNA interactions and the structural mechanism of NFI-induced DNA bending using biochemical techniques. Therefore, one of the remaining aims in the Ad DNA replication research is to solve the crystal structure of NFI in complex with DNA. The efforts to crystallize the intact NFI or only the DNA binding domain of NFI have been so far faced with difficulties to produce sufficient amount of soluble protein. Alternative attempts to obtain a hybrid protein consisting of the DNA binding domain of NFI flanked by the amino acid sequences from the MH1 domain of Smad3 and also the expression of NFI from *C. elegans* resulted still in an insoluble protein (Lars Meijer, data not shown).

Structural view on protein-priming

The main player in initiation of Ad5 DNA replication is the pTP/pol complex. The initiation can take place in the absence of DBP, NFI or Oct-1, but it fails when pTP or pol is not present. The pTP/pol complex exists as a stable heterodimer in solution and it binds the origin DNA in order to initiate replication. During initiation pol covalently couples the first nucleotide to the hydroxyl group of pTP Ser580. Analysis of the pTP/pol complex architecture showed that pTP binds the primer binding groove of pol and the distance from the active site of pol to the primer binding groove is 9-10 nucleotides (2). Structural data on the pTP-pol interaction are not available, since there is no

crystal structure of a terminal protein complexed with DNA polymerase from any protein-priming system. However, the logical model is that pTP inserts one of its putative domains into the primer binding channel of pol in order to position Ser580 in the pol active site. A good candidate of such a domain is the priming region of pTP (see Figure 1, Chapter 4). It is predicted to fold into two α-helices that surround a β-turn in which the Ser580 residue is located (9). Since the protein primer as well as the DNA primer bind to a common site of pol (2, 5, 26), it is likely that the protein primer mimics DNA in order to interact with pol in the primer-binding groove. DNA mimicry involving negatively charged amino acids plays an important role in the regulation of transcription, and DNA repair as demonstrated by the crystal structures of the protein complexes, human TBP-TAF(II)230 complex and human uracil-DNA glycosylase in complex with an inhibitor, respectively (13, 14). In the pTP priming region there are 13 out of 43 conserved acidic residues, and they cluster at one side of the two predicted α-helices, whereas the other side of the α-helices contains predominantly the hydrophobic residues (see Fig. 3 in Addendum to Chapter 4). We propose a model in which the two predicted α-helices of pTP form a coiled-coil domain through hydrophobic interactions, with the negatively charged residues exposed on the surface and Ser580 positioned in a β-turn on the tip of this domain. Such a location could facilitate the optimal charge-charge interactions between acidic residues of pTP and basic residues of the active site or the primer binding channel of pol leading to an optimal positioning of the Ser580 hydroxyl group. Chapter 4 supports this model demonstrating that pTP residues, Glu585, Asp582 and specifically the negative charge of Asp578 and Asp594 are involved in the initiation of replication. An additional experiment such as the construction of a pol mutant with a basic residue changed to an acidic residue, which would complement for the acidic to basic residue mutation in the priming region of pTP, would strengthen this model. However, there are numerous basic residues in the sequence of pol that are good candidates for mutagenesis and analysis of all of them would be a tedious task. Structural features analogous to the interaction of the proposed pTP coiled-coil domain with the active site of pol were already reported in other processes such as regulation of eukaryotic or bacterial transcription (17). The interaction of the bacterial regulatory factor GreB and the eukaryotic elongation factor TFIIS with RNA polymerase involves a coiled-coil domain with conserved acidic residues on its tip that are essential for stabilization of one of the Mg^{2+} ions in the active site of RNA pol (10, 12, 18). Also the bacterial transcription factor DksA uses negatively charged residues positioned on the tip of a coiled-coil domain to modulate the function of RNA pol (19). Moreover two recent studies demonstrated that the 21 aa-long peptide antibiotic Microcin J25 (MccJ25) adopts a coiled-coil structure and blocks nucleotide entry into the active site of RNA pol, thereby inhibiting RNA pol activity (1, 15). This observation raised the possibility to design new antimicrobial compounds. The priming region of pTP has a similar size as MccJ25. Since it is inserted into the primer binding channel of pol it is likely a separate and protruding domain of pTP. Therefore, the expression and purification of a soluble peptide representing this region might be possible. An alternative solution would be the synthesis of such a pTP peptide. Because the intact pTP/pol complex is difficult to crystallize, such a truncated pTP peptide could then be used for crystallization purposes. In a view of the fact that adenoviral infection is a major problem for patients undergoing immunosuppression treatment during transplantation or for patients with a defect of immune system (AIDS), it is also tempting to construct a pTP peptide that could serve as an antiviral drug. A good candidate might be a pTP peptide with mutation of Ser580 that would interact with pol and inhibit initiation of replication.

The data described in this thesis add to the general knowledge of the mechanism of Ad DNA replication. Novel information is presented

on two important aspects. Firstly, the determination of the individual and collective DNA bend angles induced by NFI and Oct-1 in the origin, provides a view on the architecture of the PIC. These structural features also have important functional implications in the enhancement of Ad DNA replication by NFI and Oct-1. Secondly, the evidence that negatively charged amino acids of pTP are involved in initiation reveals an interesting aspect of the mechanism of protein-priming, which is that pTP mimics DNA in order to correctly position Ser580 in the active site of pol. It is challenging to combine our structural observations together with all known protein-protein and protein-DNA interactions in one three-dimensional view of the PIC, thus our model of PIC assembly and protein-primed initiation should be additionally confirmed by crystal structure analysis. Moreover, comparison of the crystal structures of the pTP/pol complex alone and bound to the origin DNA, would give insight into the structural changes of pTP/pol upon DNA binding. Also the differences in pol structure when it is in the protein-priming or in the DNA-priming state would be revealed after comparison of the pTP/pol-DNA complex with the pol-DNA complex.

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Summary

After infection of a host cell, adenovirus (Ad) aims for generation of progeny viruses, and thus it rapidly replicates its genomic DNA. The replication process starts with the assembly of the preinitiation complex (PIC) on the origin DNA. The PIC consists of three viral proteins, DNA polymerase (pol), precursor terminal protein (pTP), DNA binding protein (DBP) and two transcription factors of the host cell, Nuclear Factor I (NFI) and Octamer binding protein (Oct-1). Both transcription factors enhance the replication process up to 80 fold. The efficiency of Ad DNA replication is an essential issue for gene therapy procedures, and because most human cell types contain the cellular transcription factors NFI and Oct-1, it is important to study how NFI and Oct-1 perform their function. One of the mechanisms by which NFI and Oct-1 could stimulate replication is by changing the structure of the origin DNA. Furthermore, NFI and Oct-1 recruit pol and pTP to the core origin through direct protein-protein interactions. Experiments in **Chapter 2** and **Chapter 3** of this thesis investigate the first mechanism. The ability of NFI and Oct-1 to induce a DNA bend in the Ad5 origin is analyzed using scanning force microscopy (SFM). **Chapter 2** demonstrates that NFI induces a 60° bend upon binding to the origin DNA, which is functionally important. Mutations of the A/T-rich region preceding the NFI recognition sequence in the origin reduce the bend angle and lead to a loss of NFI-stimulated replication. **Chapter 3** shows that Oct-1 induces a 42° bend in the origin DNA. Moreover, simultaneous binding of NFI and Oct-1 to the origin increases the collective DNA bend up to 82°, which indicates that the two DNA bends induced individually by NFI and Oct-1 are oriented towards each other. Consequently, such extensive DNA bending leads to the synergistic stimulation of replication. Furthermore, the **Addendum** presents that, in contrast to NFI, mutation of the A/T-rich region does not effect the Oct-1-induced DNA bend and its ability to stimulate replication. All these data generate a model, in which bending of the origin DNA by NFI and Oct-1 facilitates the formation of an optimal nucleoprotein structure of the PIC.

After PIC formation, pol initiates Ad DNA replication using pTP as a primer. Both proteins form a tight heterodimer, the pTP/pol complex, which plays a central role in initiation. During this process pol covalently couples the first nucleotide of a new viral genome to the hydroxyl group of the Ser580 residue of pTP that is positioned in the active site of pol. Protein priming is a unique system to start DNA replication, and therefore it is important to understand its mechanism. The priming region of pTP that surrounds Ser580 contains a number of negatively charged amino acids (13 aa out of 43), and since the protein primer and the DNA primer bind to a common site of pol, it is likely that pTP mimics DNA in order to interact with pol. **Chapter 4** demonstrates that the conserved acidic residues of pTP, Asp578 and Asp582, located close to Ser580 are important for the optimal initiation of Ad DNA replication. Moreover, they contribute to the kinetics of initiation, showing that these residues play an important role in the arrangement of an optimally functioning active site of pol that leads to an efficient initiation. This also suggests that pTP is not a simple primer but rather an integral part of the initiating pTP/pol complex. The **Addendum** demonstrates that also other conserved acidic residues from the priming region, Glu585 and Asp594, located somewhat further from Ser580 are important for the initiation. This suggests that the optimal positioning of the Ser580 hydroxyl group in the active site of pol is facilitated by charge-charge interactions between pTP and pol. In **Chapter 5**, a model of the PIC assembly and protein priming is proposed and future perspectives for the Ad5 DNA replication are discussed.

Samenvatting

Na infectie van een gastheercel, is het belangrijk voor adenovirus (Ad) om nieuwe virussen te genereren. De duplicatie van het Ad genoom wordt replicatie genoemd. Het replicatie proces begint met de vorming van het zogenaamde preinitiatie complex (PIC) op de Ad origin. Het PIC bestaat uit een complex van vijf verschillende eiwitten. Drie eiwitten worden door adenovirus zelf gemaakt, adenovirus DNA polymerase (pol), precursor terminal protein (pTP) en het DNA binding protein (DBP). De andere twee eiwitten worden door de gastheercel gemaakt en zijn de transcriptie-factoren Nuclear Factor I (NFI) en Octamer binding protein (Oct-1). Deze laatste twee factoren kunnen de adenovirus replicatie tot 80 maal stimuleren. Deze stimulering is van belang bij gentherapie voor de productie van genoeg van Ad, omdat de meeste humane cellen de transcriptie factoren NFI en Oct-1 bevatten. Dit proefschrift beschrijft een gedetailleerde studie van de functie van de diverse eiwitten van het PIC en het mechanisme van efficiënte Ad replicatie.

De cellulaire transcriptiefactoren NFI en Oct-1 binden respectievelijk aan pol en pTP en rekruteren deze eiwitten naar replicatie origins. Bovendien kunnen NFI en Oct-1 ook de structuur van het origin DNA veranderen. Experimenten in Hoofdstuk 2 en Hoofdstuk 3 onderzoeken dit tweede mechanisme. De mogelijkheid van NFI en Oct-1 om buiging van het Ad5 origin DNA te induceren is met de scanning force microscopie techniek (SFM) bestudeerd. **Hoofdstuk 2** beschrijft dat NFI een buiging van het origin DNA van 60° kan induceren. Deze buiging in de origin structuur veroorzaakt een versnelling van de replicatie reactie. Mutaties in het A/T-rijke gebied voorafgaand aan de NFI binding site in de origin, verkleinen de buigingshoek en verlagen de stimulatie door NFI. In **Hoofdstuk 3** wordt beschreven dat Oct-1 het origin DNA buigt in een hoek van 42°. Verder wordt aangetoond dat wanneer NFI en Oct-1 tegelijkertijd het origin DNA binden, de buiging vergroot wordt tot 82°. Deze experimenten tonen aan dat de DNA buigingen die door NFI of Oct-1 geïnduceerd worden, in hetzelfde vlak georiënteerd zijn. Verder wordt aangetoond dat de gecombineerde DNA buiging een synergistische stimulering van de replicatie veroorzaakt. De experimenten in het **Addendum** laten zien dat, anders dan bij NFI, de mutaties in het A/T-rijke gebied geen invloed hebben op de buigingshoek van Oct-1 en op de mogelijkheid van Oct-1 om de replicatie te stimuleren. De gevonden resultaten leiden tot een model waarin de buiging van de origin een zodanige structuurverandering in het DNA aanbrengt dat het met de eiwitten van het PIC een optimaal complex vormt.

Na vorming van het PIC begint pol met de DNA replicatie en gebruikt daarbij pTP als primer. pTP en pol hebben een hoge affiniteit voor elkaar en vormen een sterk eiwit complex, het pTP/pol complex, dat een belangrijke rol speelt bij de initiatie van de replicatie. Tijdens de initiatie word het eerste nucleotide van het nieuwe virale genoom covalent gebonden aan het pTP aminozuur serine 580, dat in het actieve centrum van pol ligt. Het gebruik van eiwit als primer is een unieke manier om de DNA replicatie te beginnen, en dus het is belangrijk om het mechanisme te begrijpen. Het priming gebied van pTP dat het aminozuur Ser580 omringt, bevat een groot aantal aminozuren met een negatieve lading (13 van de 43 aminozuren). Omdat een eiwit primer en een DNA primer aan dezelfde bindingsplaats van pol kunnen binden, lijkt het waarschijnlijk dat pTP voor z'n pol interactie negatief geladen DNA nabootst. De experimenten in **Hoofdstuk 4** laten zien dat de geconserveerde aminozuren van pTP, Asp578 en Asp582, rondom Ser580 liggend, belangrijk zijn voor de optimale initiatie van het virale DNA. Bovendien dragen zij bij aan de snelheid van initiatie. Dit suggereert dat deze aminozuren een belangrijke rol spelen in de optimale positionering van pTP in het actieve centrum van pol. Het **Addendum** beschrijf verder dat ook andere geconserveerde negatief geladen aminozuren van pTP, die iets verder van Ser580 liggen, belangrijk zijn voor initiatie.

Streszczenie

Dla rodziny i przyjaciół

Adenowirus jest patogenem, wywołującym głównie infekcje oczu i układu oddechowego. Do rozmnażania się oraz podstawowych procesów biologicznych niezbędny jest mu organizm żywy, taki jak komórka ludzka lub zwierzęca. Kompletna forma adenowirusa, wirion, przypomina z wyglądu sztucznego satelitę. Wirion zbudowany jest z jednej cząsteczki kwasu deoksyrybonukleinowego (DNA), zamkniętej w kapsydzie tworzącym swoisty płaszcz białkowy (rysunek 1 na stronie 8). Dodatkowo, z wierzchołków kapsydu wystają włókna białkowe zwane pentonami. Średnica wirionu wynosi około 100 nm, czyli można go zobaczyć dopiero pod mikroskopem elektronowym. Po wniknięciu do organizmu, wirus przyczepia się do powierzchni komórki gospodarza wykorzystując białka znajdujące się na końcu wystającego pentonu (rysunek 2 na stronie 9). Komórka gospodarza rozpoznając wirusa jako intruza, otacza go błoną komórkową i następnie wchłania w pęcherzyku do środka w celu zniszczenia. Jednakże, wirus sprytnie ucieka z pęcherzyka do cytoplazmy i po przyczepieniu się do błony jądra komórkowego, wprowadza swój materiał genetyczny (DNA) do jądra. W jądrze rozpoczyna się proces tworzenia nowych cząsteczek wirusowych, które w końcowym rezultacie powodują śmierć komórki. Ważnym krokiem w tym procesie jest powielenie cząsteczek wirusowego DNA, czyli replikacja DNA.

DNA adenowirusa jest liniową cząsteczką zbudowaną z dwóch pasujących do siebie nici. Adenowirus posiada dwa miejsca startu replikacji, tak zwane *origin* które znajdują się na obydwu końcach wirusowego DNA (rysunek 5 na stronie 16). Głównymi wykonawcami replikacji są trzy białka wirusowe, polimeraza DNA, pTP oraz DBP. Białka te specyficznie rozpoznają sekwencje DNA zlokalizowane w *origin* i wiążą się do nich. Zadaniem polimerazy DNA jest tworzenie nowej nici DNA z pojedynczych nukleotydów. Polimeraza DNA do rozpoczęcia procesu replikacji potrzebuje tak zwanego startera, czyli cząsteczki oferującej miejsce, do którego zostanie przyłączony pierwszy nukleotyd nowej nici DNA. Takim miejscem jest grupa hydroksylowa (-OH). W komórkach ludzkiego organizmu, jak też i w komórkach innych organizmów donorem grupy hydroksylowej jest cząsteczka DNA. Jednakże adenowirus posiada unikalna metodę rozpoczynania replikacji, a mianowicie używa on swojego białka pTP jako startera. Proces ten polega na tym, że pierwszy nukleotyd zostaje przyłączony do hydroksylowej grupy jednego z aminokwasów pTP. Tym aminokwasem jest seryna, która jest zlokalizowana na pozycji 580 (Ser580) w sekwencji aminokwasów budujących pTP. Trzecie białko wirusowe, DBP, ochrania DNA w trakcie procesu replikacji poprzez wiązanie się do nici DNA jedno obok drugiego generując multimetry podobne do koralików na nici. Oprócz trzech białek własnych, adenowirus wykorzystuje dwa białka obecne w każdej komórce, NFI i Oct-1, w celu zwiększenia efektywności procesu replikacji. Białka te są czynnikami

transkrypcyjnymi, które w normalnych okolicznościach zaangażowane są w zachodzącym w komórce procesie transkrypcji, czyli przepisania informacji genetycznej z DNA na cząsteczkę RNA. Ponieważ komórki posiadają dużą liczbę białek NFI i Oct-1, replikacja wirusowego DNA jest efektywnie stymulowana po wprowadzeniu wirusowego DNA do jądra komórkowego.

Replikacja adenowirusowego DNA rozpoczyna się od związania się wyżej wymienionych białek do *origin* DNA, czyli uformowania kompleksu preinicjacyjnego (rysunek 6 na stronie 17). Kolejnym krokiem jest inicjacja replikacji, czyli przyłączenie pierwszego nukleotydu do pTP przez polimerazę. Następnie polimeraza przyłącza kolejne nukleotydy, wydłużając tym samym nowo powstałą nić DNA.

Interesującym aspektem jest to w jaki sposób NFI i Oct-1 powodują wzmocnienie procesu replikacji adenowirusa. Aktualnie proponowane są dwa mechanizmy. Po pierwsze NFI wiąże się z polimerazą a Oct-1 wiąże się z pTP. Te oddziaływania miedzy białkami sugerują, ze NFI i Oct-1 przynoszą polimerazę i pTP do miejsca startu replikacji na DNA i dzięki temu zwiększają efektywność replikacji. Druga możliwość jest taka, że NFI i Oct-1 zmieniają liniową strukturę DNA poprzez wprowadzenie w nim zgięcia. Badania opisane w mojej pracy dotyczą drugiego mechanizmu stymulacji replikacji przez NFI i Oct-1. W celu zbadania czy wiązanie się NFI albo Oct-1 do DNA powoduje jego zgięcie, użyliśmy specjalnego mikroskopu, pod którym można zobaczyć białka związane z DNA (rysunek 2 na stronie 32 i 45). Okazało się, że NFI zgina DNA o 60 stopni, natomiast Oct-1 wprowadza zgięcie o wartości 42 stopni. W sytuacji kiedy obydwa białka są związane z DNA zgięcie to wynosi 82 stopnie. Strukturalne dane uzyskane z mikroskopu zostały następnie potwierdzone w funkcjonalnych eksperymentach dowodząc, że zginanie DNA wprowadzone przez NFI i Oct-1 jest faktycznie czynnikiem stymulującym replikację. Jest bardzo prawdopodobne, że zgięcie DNA ułatwia optymalne wiązanie się oraz ułożenie białek wchodzących w skład kompleksu preinicjacyjnego na *origin* DNA.

Oprócz formowania się kompleksu preinicjacyjnego, kolejnym ważnym krokiem w procesie replikacji adenowirusowego DNA jest przyłączenie pierwszego nukleotydu do pTP przez polimerazę. Rozpoczynanie replikacji DNA przy użyciu białka jako startera jest bardzo unikalnym procesem, dlatego też interesującym pytaniem jest to jaki jest jego mechanizm. Druga część mojej pracy podejmuje próbę odpowiedzi na to pytanie. Polimeraza i pTP wiążą się ze sobą i tworzą bardzo stabilny kompleks białkowy. Wśród aminokwasów budujących pTP położonych blisko Ser580 jest kilka takich, które posiadają ujemny ładunek. Rezultaty przeprowadzonych eksperymentów pokazują, iż aminokwasy te pełnią ważną role w procesie inicjacji. Ponieważ cząsteczka DNA jest ujemnie

naładowana, jest bardzo prawdopodobne, że w trakcie oddziaływania pTP z polimerazą, pTP w pewnym sensie imituje DNA, co w rezultacie prowadzi do takiego ułożenia hydroksylowej grupy Ser580, że polimeraza efektywnie przyłącza pierwszy nukleotyd.

Podsumowując, badania przedstawione w mojej pracy doktorskiej skupiają się na ważnym aspekcie replikacji wirusowego DNA, a mianowicie na zwiększeniu efektywności tego procesu. Dotyczy to zarówno etapu formowania się kompleksu preinicjacyjnego jak i etapu przyłączania pierwszego nukleotydu do pTP. Zdolność adenowirusów do wnikania do komórki ludzkiej i wprowadzenia swojego DNA do jądra komórkowego jest wykorzystywana w walce z nowotworami i chorobami genetycznymi, w tak zwanej terapii genowej. Terapia genowa polega na wprowadzaniu „terapeutycznych" genów do patologicznie zmienionych komórek. Białka kodowane przez te geny umożliwiają przywrócenie prawidłowych funkcji uszkodzonych komórek. Adenowirusy sa popularnym nośnikiem takich terapeutycznych genów. Dzięki inżynierii genetycznej możliwe jest usunięcie z DNA adenowirusa genów związanych z cyklem rozwojowym i wstawianie w ich miejsce genu terapeutycznego. Tak zmodyfikowany wirus nie ulega namnażaniu w komórce, ale pozostawia w niej wprowadzony leczniczy DNA. Jednym z ważnych procesów w terapii genowej jest produkcja zmodyfikowanych wirusów w większych ilościach. Proces ten odbywa się poza organizmem ludzkim i zależy od efektywności procesu replikacji DNA adenowirusa. Przedstawione w tej pracy badania nad zwiększeniem efektywności tego procesu stanowią ważny wkład teoretyczny, który w przyszłości mógłby być wykorzystany w rozwoju terapii genowej.

Innym aspektem, do którego mogą się odnieść rezultaty moich badań jest zwalczanie infekcji wywołanych przez adenowirusa. Infekcje te są bardzo powszechne i w zazwyczaj przebiegają bezobjawowo, dlatego też większość ludzi posiada w organizmie przeciwciała zwalczające wirusa już w wieku 15 lat. Jakkolwiek w przypadku pacjentów, których układ odpornościowy nie funkcjonuje poprawnie, infekcja adenowirusowa może być bardzo uciążliwa. Takimi pacjentami są ludzie chorzy na AIDS, których układ odpornościowy jest zniszczony przez wirusa HIV oraz pacjenci po transplantacji organów, których układ udpornościowy jest „wyłączony" odpowiednimi lekami. Dokładne poznanie mechanizmu replikacji DNA adenowirusa, może ułatwić znalezienie czułych punktów tego procesu, co w rezultacie może prowadzić do rozwoju nowych rozwiązań terapeutycznych, takich jak znalezienie nowego leku, który zwalczyłby infekcję poprzez zahamowanie replikacji DNA i tym samym zahamowanie rozwoju adenowirusa w organizmie.

Curriculum vitae

Monika Mysiak was born on the 11th of December 1976 in Lubartów in Poland. She obtained her secondary school diploma in June 1995. In October the same year she entered the Jagiellonian University in Kraków to study biotechnology. In February 1998 she got an EU Tempus scholarship and for six months she joined the group of Prof. Leslie A. Grivell in the Institute of Molecular Cell Biology (BioCentrum) at the University of Amsterdam. In October 1999 she started the Master research project under supervision of Prof. Juliusz Pryjma in the Department of Immunology at the Jagiellonian University and wrote a Master Thesis entitled "Release of soluble FasL by monocytes phagocytizing bacteria *Staphylococcus aureus*". On 16th of June 2000 she obtained her MSc degree in Biotechnology and Molecular Biology. From September 2000 till November 2004 she worked as a graduate student under the supervision of Prof. Peter C. van der Vliet in the Department of Physiological Chemistry at the University Medical Center in Utrecht. Research performed during that period was focused on adenovirus DNA replication and is described in this PhD Thesis. In January 2005 she will begin as a postdoctoral fellow in the Laboratory of Neurodegeneration headed by Prof. Jacek Kuźnicki at the International Institute of Molecular and Cell Biology in Warsaw.

List of publications

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Page 90, top: Kinderdijk
Page 90, bottom: Utrecht, Schimmelstraat 1, my own klompen
Page 91: Utrecht, Domplein 9, Pandhof, the herb garden
Page 92, top: Utrecht, Oudegracht 132, Bakkerij Mario
Page 92, bottom: Utrecht, the Saturday Market, hmmmm…lekker!
Page 93: Utrecht, Vismarkt, woman with two chickens on Koninginnedag (April 30th)