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 (Ad5) origin of replication and recruits the adenovirus 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 base pairs by G/C base pairs 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.

Replication of adenovirus type 5 (Ad5) DNA is well characterized and can be fully reconstituted in vitro with purified proteins. There are three viral proteins involved in this process: precursor terminal protein (pTP), which serves as a primer; adenovirus DNA polymerase; and DNA-binding protein. 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 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. The core origin at positions 9 to 18 is a conserved region bound by pTP and DNA polymerase, which forms a stable heterodimer in solution and binds DNA as a complex. The core origin is followed by the auxiliary origin recognized by transcription factors NFI and Oct-1 (nucleotides 25 to 38 and 39 to 47, respectively). The core origin and the auxiliary origin are separated by a 6-bp-long A/T-rich region (Fig. 1).

NFI is a transcription factor that was originally discovered based on its ability to stimulate adenovirus 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 to 220) is required for DNA binding and protein dimerization. The C-terminal domain (residues 220 to 499) is involved in transcription activation (19, 30). The DNA-binding domain is necessary and sufficient for the stimulation of ade-

novirus 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)(N₅)GCCAA located at the adenovirus type 5 (Ad5) origin of replication between nucleotides 25 and 38 (28, 34) (Fig. 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 modeling 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 increases not 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-polymerase 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-polymerase 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 by Mul et al. show that NFI can stabilize binding of the pTP-polymerase complex to the origin (31). Furthermore, binding of NFI to the origin

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FIG. 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-polymerase binding site and the auxiliary origin containing the consensus sequences recognized 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 to 23 with CGCG and the 6G/C origin mutant by replacing the TTATTT sequence at positions 19 to 24 with GCGCGC. The mutated nucleotides are shown in bold. The arrow indicates the middle of the NFI consensus sequence at position 31.

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, such as packaging DNA into nucleosomes, regulation of gene expression, DNA replication, repair, and recombination. DNA bending facilitates the assembly of multiprotein 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 with 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'-CAATAATATACCTCGCGTTGGAT TGAAGC-3', 5'-GCTTCAATCCAACGCGAGGTATATTG-3') and 6G/C (5'-CAATAATATACCGCGCGCTGGATTGAAGC-3', 5'-GCTTCAATCCA GCGCGCGGTATATTATTG-3') 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 A₁ (NFI-BD; position 5 to 242) was used. Of the 499-amino-acid-long NFI protein, the N-terminal 238 amino acids contain the DNA binding domain. The NFI-BD will be further referred to in the text as NFI. NFI, adenovirus DNA polymerase, pTP, and DNA-binding protein were expressed with 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 dithiothreitol and 0.5 mg of bovine serum albumin per ml.

Scanning force microscopy. For the SFM studies of NFI-DNA complexes a 711-bp *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 with 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 electrophoretic mobility shift assays (EMSAs) (data not shown).

The SFM depositions were performed as follows. NFI (4.5 pmol) was incu-

bated with the DNA fragment (0.19 pmol) for 15 min on ice in replication buffer (see below). The final mixture was diluted 20 to 30-fold in deposition buffer (5 mM HEPES-KOH, pH 7.8, 5 mM MgCl₂) and deposited onto freshly cleaved mica. After 1 min the surface of the mica was washed with 3 ml of high-pressure liquid chromatography-grade water and dried in a stream of air. The complexes were imaged in the tapping mode with a Nanoscope IIIa (Digital Instruments, Santa Barbara, Calif.). The DNA contour length and the DNA bend angle were measured with the Image SXM version 1.69 software, an NIH Image version modified for use with SXM images by Steve Barrett, Surface Science Research Centre, University of Liverpool, United Kingdom. For each measurement we analyzed 100 to 150 molecules. The data obtained were analyzed 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 the displaced strand of the Ad5 origin, respectively: T50, 5'-CTCATTATCATATTGGCTTCAATCCAAA ATAAGGTATATTATGATGATG-3'; D50, 5'-CATCATCAATAAATATACC TTATTTTGGATTGAAGCCAATATGATAATGAG-3'. 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-labeled with T4 polynucleotide kinase and [γ -³²P]ATP (4,500 Ci/mmol) in a standard kinase buffer. D50 was subsequently hybridized with T50, and the labeled TD50 was purified on a 10% polyacrylamide gel.

The DNA binding assays were performed in EMSA experiments as follows. NFI or Ad5 DNA polymerase was incubated with DNA for 30 min on ice in binding buffer (25 mM HEPES-KOH, pH 7.5, 10 mM MgCl₂, 4 mM dithiothreitol, 1 mM EDTA, 0.1 mg of bovine serum albumin per ml, 4% Ficoll). For the NFI binding studies, 80 mM NaCl and 1 μ g of poly(dI-dC)-(dI-dC) was used to exclude nonspecific DNA-protein interactions. The Ad5 polymerase binding studies were performed at 50 mM NaCl in the absence of competitor DNA to optimize binding to the core origin (41). TD50-protein complexes were separated on a 10% polyacrylamide gel in Tris-borate-EDTA buffer at 4°C, and the intensity of the bands was quantified with a Storm 820 phosphorimager. In the case of the 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 with the indicated amounts of Ad5 DNA polymerase, Ad5 pTP, Ad5 DNA-binding protein, and NFI-BD in a 20- μ l reaction mixture containing replication buffer (25 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 1.5 mM MgCl₂, 1 mM dithiothreitol), 40 μ M each dATP, dTTP, dGTP, 0.7 μ M dCTP, and 4 μ Ci of [α -³²P]dCTP (3,000 Ci/mmol). As a template, 100 ng of pHRI plasmid linearized with *EcoRI* or 30 ng of *XhoI*-digested viral terminal protein-DNA were used. TP-DNA is a 36-kb linear Ad5 genome with the terminal protein 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 μ l of stop mix (40% sucrose, 1% sodium dodecyl sulfate, 0.1% bromophenol blue and 0.1% xylene cyanol). The replication products were separated on a 1% agarose gel containing 0.1% sodium dodecyl sulfate in 0.5 $\times$ Tris-borate-EDTA-0.1% sodium dodecyl sulfate buffer. The replicated bands were quantified with a Storm 820 phosphorimager.

In vitro initiation was assayed with the indicated amounts of Ad5 DNA polymerase and Ad5 pTP, in a 20- μ l reaction mixture containing initiation buffer (20 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 40 μ g of bovine serum albumin per ml) and 4 μ Ci of [α - ^{32}P]dCTP (3,000 Ci/mmol). As a template, 10 ng of 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, washed with 5% trichloroacetic acid, dissolved in a sample buffer, and analyzed on a 7.5% polyacrylamide-sodium dodecyl sulfate gel. The intensity of the initiation bands was quantified with 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 double-stranded DNA fragment containing the first 103 bp of the Ad5 inverted terminal repeat 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 recognized by NFI (Fig. 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, amino acids 5 to 242), which will be referred to as NFI. The DNA binding domain of NFI is sufficient for dimerization 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-fold molar excess of NFI over DNA, for 15 min 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 center of the protein in a protein-DNA complex, to each end of the DNA (Fig. 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 0.36 ± 0.09 , which confirms that NFI is bound to the origin (Table 1). Very few NFI molecules (3 of 150) were bound to nonspecific 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 180°. 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 nonorigin 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 equation 1 in Schulz et al. (39). From the fitting defined in equation 2, which cor-

rects for different directions of bending, we obtained similar results with at most a 3° 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 (Fig. 2A; Table 1). The average bend induced by NFI at the Ad5 origin, was $60^\circ \pm 19^\circ$. A DNA bend of $17^\circ \pm 7^\circ$ 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 analyzed 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 (Fig. 2A; Table 1). 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.

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 (Fig. 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 to 23 with CGCG and the 6G/C mutant by replacing the TTATTT sequence at positions 19 to 24 with GCGCGC (Fig. 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-polymerase binding sequences in the origin, and moreover, the correct spacing between the core origin and the NFI binding site is maintained.

To analyze the effect of the 4G/C and 6G/C mutants on DNA-NFI complex formation with SFM, we again used the 711-bp DNA fragment, but with the mutated origins. The experiments were performed in the absence and in the presence of NFI (Fig. 2B and C; Table 1). Binding of NFI to the 4G/C origin caused clear bending of DNA, but the angle was smaller ($33^\circ \pm 14^\circ$) compared with the bend induced in the wild-type origin (Fig. 2B). A similar effect was seen with the origin containing the 6G/C mutation, where NFI induced a bend of $37^\circ \pm 17^\circ$ (Fig. 2B and C). For both mutants the position of NFI on the DNA corresponded with correct origin binding (Table 1). 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 $18^\circ \pm 9^\circ$ angle, and the 6G/C origin is bent by $18^\circ \pm 10^\circ$ (Fig. 2B and C; Table 1). 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 (Fig. C; Table 1). Similar results as for the wild-type DNA were found. Again a slight but consistent short-

A wt **B 4G/C** **C 6G/C**

FIG. 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 is represented 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 103 bp of the Ad5 origin of replication with the middle of the NFI consensus sequence at position 249 of the fragment. For each experiment 100 to 150 molecules were analyzed. The mean values with standard deviations are presented in Table 1. 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 (<i>r</i>)	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

ening of the protein-bound DNA molecules was observed, which was ≈ 8 nm (≈ 26 bp) and ≈ 10 nm (≈ 33 bp) for the 4G/C and 6G/C origins, 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 (Fig. 1). Therefore, we performed electrophoretic mobility shift assays (EMSAs) with the first 50 bp 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 (Fig. 3). 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 was 100% and 105%, respectively, compared to the wild-type affinity (100%).

Stimulation of Ad5 replication by NFI on mutated origins is impaired. To analyze 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 *Xho*I-digested TP-DNA templates from Ad5 virions. TP-DNA is the 36-kbp-long linear viral genome including ter-

минаl protein covalently attached to each 5' end. When a range of NFI concentrations was used, optimal stimulation of TP-DNA replication up to sevenfold was observed (Fig. 4A). This indicates that the NFI used in the SFM experiments was functionally active.

Since it is not technically possible to create terminal protein-containing mutated templates, we investigated the NFI stimulation ability on mutated terminal protein-free DNA origins. Terminal protein-free origin DNA is a less effective template but still supports replication (40, 43). Digestion of the pHRI plasmid with *Eco*RI generated a linear 2.9-kb DNA fragment with the origin of replication at the end. Under conditions similar to those for TP-DNA, replication of the pHRI DNA fragment containing the wild-type origin was stimulated by NFI up to fivefold (Fig. 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 at just 63 ng of NFI, whereas pHRI template replication was still stimulated at higher concentrations of NFI (up to 250 ng).

Next, we used *Eco*RI-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 was severely affected (Fig. 4B). Since the stimulation by NFI is maximized at low pTP-polymerase levels (31, 32) we also tested the mutant templates at a lower pTP-polymerase concentration. The stimulation was 32-fold under these conditions with the 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 that 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 was lower than for the wild-type origin (65% and 20% of the wild-type level 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.

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-polymerase binding site (Fig. 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. Polymerase-DNA interactions were checked in EMSAs, with TD50 wild-type and the mutant probes (Fig. 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, nonspecific 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 con-

FIG. 3. DNA binding of NFI is not significantly affected by the mutations. The ability of NFI to bind to origins containing the 4G/C and 6G/C mutations was studied in EMSA experiments with wild-type and mutated double-stranded TD50 probes containing the first 50 nucleotides of the Ad5 origin of replication. The positions of the NFI-DNA complexes and free probe are marked with arrowheads. Radiolabeled TD50, wild type (lanes 1 to 4), 4G/C (lanes 5 to 8), and 6G/C (lanes 9 to 12) were incubated with 30 ng (lanes 2, 6, and 10), 60 ng (lanes 3, 7, and 11) and 120 ng (lanes 4, 8, and 12) of NFI. Lanes 1, 5, and 9 represent free probe.

FIG. 4. Stimulation of Ad5 replication by NFI on templates containing the 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 1 μ g of DNA-binding protein were incubated with 60 ng of *Xho*I-digested TP-DNA, resulting in the synthesis of the two labeled double-stranded origin fragments (dsDNA B and C) and labeled displaced single strands originated from the second and subsequent rounds of replication (ssDNA B and C). The D fragment (dsDNA D) is not replicated, since it does not have the origin but is nonspecifically labeled 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 linearized terminal protein-free origin DNA; 100 ng of *Eco*RI-linearized wild-type-pHRI (lanes 1 to 5), 4G/C-pHRI (lanes 6 to 10), and 6G/C-pHRI (lanes 11 to 15) plasmid were incubated with 15 ng of polymerase, 12 ng of pTP, and 1 μ g of DNA-binding protein. Replication leads to a pTP containing a 2.9-kb double-stranded 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 replication, a range of NFI concentrations were used: 250 ng (lanes 3, 8, and 13), 125 ng (lanes 4, 9, and 14), and 63 ng (lanes 5, 10, and 15).

taining 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-polymerase complex is not affected by AT to GC mutations. During initiation, Ad5 polymerase catalyzes 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 analyzed the effect of mutations introduced in the A/T-rich region on the ability of polymerase to initiate the replication (Fig. 6). The initiation activity of the pTP-polymerase complex on the 4G/C and 6G/C origins was not significantly affected and resulted in $98\% \pm 9\%$ and $82\% \pm 15\%$ 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-polymerase 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-polymerase the amount of initiation product was dependent on the pTP-polymerase concentration, demonstrating that the pTP-polymerase concentration was not saturating for the experimental conditions (data not shown). However, the initiation activity of the pTP-polymerase complex on the mutated origins was still comparable to that of 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 60° 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, also by applying circular permutation analysis (46).

We demonstrate that the 6-bp-long A/T-rich region located between the pTP-polymerase binding site and the NFI binding site in the Ad5 origin (Fig. 1) contributes to DNA bending induced by NFI, since substitution of four or six AT pairs with GC pairs decreases the bend angle from 60° to 33° to 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.

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 destabilization 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 $17^\circ \pm 7^\circ$ bend of the Ad5 origin (Table 1). 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-contain-

FIG. 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 with double-stranded TD50 probes. The positions of polymerase-DNA complexes and free probe are marked with arrowheads. Radiolabeled TD50, wild type (lanes 1 to 4), 4G/C (lanes 5 to 8), and 6G/C (lanes 9 to 12) were incubated with 50 ng (lanes 2, 6, and 10), 100 ng (lanes 3, 7, and 11) and 200 ng (lanes 4, 8, and 12) of Ad5 polymerase. Lanes 1, 5, and 9 represent free probe.

ing 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 *Saccharomyces cerevisiae* profilin promoter generates a 25° natural bend (3). A DNA bend of 8° to 11° is also present in the phage T7 promoter (16 A/T's out of 21 bases) (42). The Ad5 origin

FIG. 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 radiolabeled [α -³²P]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 with the wild type (lanes 1 and 2), 4G/C (lanes 3 and 4), and 6G/C (lanes 5 and 6).

contains 20 A/T's out of the first 25 bases (Fig. 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 four or six AT pairs with GC pairs in the A/T-rich region abolishes the ability of NFI to stimulate Ad5 DNA replication (Fig. 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 (Fig. 3 and 5) or that it is due to impaired initiation activity of pTP-polymerase (Fig. 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 pairwise 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 dimerization of pTP, an interaction between pTP and the terminal protein was postulated (12). Formation of such a multiprotein 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 positions 40 to 43, is induced by Oct-1. Based on the available data we cannot 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 cooperative 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 relationship between an A/T-rich stretch and DNA bending is suggested for the regulation of the *S. cerevisiae* 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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