

## Two Regions within the DNA Binding Domain of Nuclear Factor I Interact with DNA and Stimulate Adenovirus DNA Replication Independently

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**The cellular transcription factor nuclear factor I (NFI) stimulates adenovirus DNA replication by up to 50-fold. The NFI DNA binding domain (NFI-BD) is sufficient for stimulation and interacts with the viral DNA polymerase, thereby recruiting the precursor terminal protein-DNA polymerase complex (pTP-pol) to the origin of replication. The mechanism of DNA binding by NFI is unknown. To examine DNA binding and stimulation of adenovirus DNA replication by NFI-BD in more detail, we generated a series of deletion mutants and show that the DNA binding domain of NFI consists of two subdomains: a highly basic N-terminal domain that binds nonspecifically to DNA and a C-terminal domain that binds specifically but with very low affinity to the NFI recognition site. Both of these subdomains stimulate DNA replication, although not to the same extent as the intact DNA binding domain. The N-terminal domain has an  $\alpha$ -helical structure, as shown by circular dichroism spectroscopy. The C-terminal domain interacts with the pTP-pol complex and is able to recruit the pTP-pol complex to DNA, which leads to pTP-pol-dependent stimulation of replication. The N-terminal domain also stimulates replication in a pTP-pol-dependent manner and enhances binding of pTP-pol to DNA. Since we could not detect a direct protein-protein interaction between pTP-pol and the N-terminal domain, we suggest that this domain stimulates replication by inducing structural changes in the DNA.**

The involvement of transcription factors in the initiation of DNA replication has been described for several viral systems (for a review, see reference 13a). In most cases, the transcription factors interact with components of the initiation complex and thereby enhance the activity or the assembly of the complex. The role of transcription factors in adenovirus DNA replication has been studied extensively (for reviews, see references 21, 46, and 47). Initiation of adenovirus DNA replication occurs via a protein-priming mechanism in which a dCMP residue is coupled to a precursor terminal protein and subsequent elongation occurs via a strand displacement mechanism. The initiation reaction is stimulated by two cellular transcription factors: nuclear factor I (NFI) and Oct-1, both of which bind to the auxiliary region of the origin. The DNA binding domains of these factors (NFI-BD and the POU domain, respectively) are sufficient for stimulation (17, 32, 48), and both proteins directly interact with the precursor terminal protein-DNA polymerase (pTP-pol) complex (5, 7, 12, 35). By template commitment studies, it was shown that NFI enhances the stability of the preinitiation complex (34). Deletion analysis showed that the region of NFI-BD that interacts with the pTP-pol complex is located between amino acids 68 and 150 of human NFI (7). This was confirmed by analysis of point mutations in this region (2). Changing the position of the NFI binding site in the origin abolishes stimulation (9, 50). These results demonstrate that NFI-BD stimulates adenovirus DNA replication by recruiting and correctly positioning of the pTP-pol complex. Binding of NFI to the origin induces structural changes in the DNA, as shown by hydroxyl radical footprinting and DNA bending assays (43, 53). Whether these structural

changes contribute to the stimulatory effect of NFI is currently unknown.

The NFI family of transcription factors bind as dimers to a palindromic binding site with the consensus sequence TGG(C/A)<sub>N</sub>GCCAA (15, 18, 28). NFI family members have been shown to activate a number of promoters (6, 13, 33, 42). All NFI proteins share a large and highly conserved DNA binding domain. This domain does not contain any known DNA binding motif, which suggests that NFI employs a new mechanism for DNA binding. The N-terminal 80 amino acids are very basic and have a predicted  $\alpha$ -helical structure which may indicate that this region interacts with DNA. NFI-BD contains three conserved cysteine residues at positions 95, 148, and 154 of the human NFI (corresponding to residues 99, 152, and 158 in the rat protein) that are essential for DNA binding (36, 37). Another cysteine, at position 115 of the rat protein, is sensitive to oxidation which leads to greatly reduced DNA binding (3). Oxidation-sensitive cysteine residues have been found in a number of DNA binding domains, and in all cases this cysteine is located in the loop or helix that directly binds DNA. Interestingly, this region (amino acids 105 to 148) is the most conserved part of NFI-BD between the vertebrate NFI proteins and a putative *Caenorhabditis elegans* NFI protein. Together, these data suggest that the region around position 115 may contact DNA. Indeed Armentero et al. (2) found mutations in this region that abolish DNA binding without affecting dimerization. To study the function of this region and the basic N terminus in DNA binding and stimulation of adenovirus DNA replication, we generated a series of deletion mutants of rat NFI-BD. We conclude that the DNA binding domain of NFI can be split in two functional subdomains that both bind DNA and stimulate adenovirus DNA replication independently.

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### MATERIALS AND METHODS

**Construction of His-NFI-BD and deletion mutants.** The DNA binding domain of rat NFI-BD containing amino acids 1 to 236 was cloned in the *Bam*HI site of

pET-15b<sup>+</sup>, a derivative of pET-15b (Novagen). The resulting plasmid, pET-BD, encodes NFI-BD and 28 extra residues, including a histidine tag and a thrombin cleavage site at the N terminus and 6 additional residues at the C terminus. NFI 1-78 (numbers refer to residues present in the mutant protein) was obtained by cloning the *NcoI-EcoRV* fragment from pET-BD in pTrxFus-NB, a derivative of pTrxFus obtained from Invitrogen BV, digested with *Bam*HI, blunted with the Klenow fragment of DNA polymerase I, and then digested with *Nco*I. The protein encoded by this construct contains thioredoxin (amino acids 1 to 109), an enterokinase cleavage site (amino acids 110 to 127), a histidine tag, and a thrombin cleavage site (amino acids 128 to 155) and amino acids 1 to 78 of NFI (amino acids 156 to 233). At the C terminus, 12 additional residues encoded by the vector are present. To generate N-terminal deletion mutants, pET-BD was linearized with *Nde*I and the ends were digested with *Bal* 31 exonuclease. The ends were blunted with T4 DNA polymerase and the Klenow fragment. The DNA was then cut with *Bam*HI and NFI-BD containing fragments were ligated in pET-15b which was cut with *Xho*I, blunted with the Klenow fragment, and then cut with *Bam*HI. The resulting plasmids were sequenced to determine the size of the deletion and to check the reading frame. Deletions up to amino acid 74 were obtained. These proteins contained 23 extra amino acids at the N terminus, including the histidine tag and 6 additional amino acids at the C terminus. NFI 1-182 was obtained by PCR with pET-BD as the template and the following oligonucleotides: 5' GCTAGAGTACATATGCTCTGTCTCACCCA GGAT 3' (JD9401) and 5' GGAAGCTGGATCCTCATGCTGCATGTACA AAGTA3' (JD9403). NFI 75-182 was generated by PCR amplification with the following oligonucleotides: CGGCGAGTTACATATGTTACGGAAAGATATC CGA (JD9402) and JD9401. PCR products were digested with *Nde*I and *Bam*HI and were ligated in pET-15b digested with *Nde*I and *Bam*HI. The resulting plasmids encoded NFI 1-182 and NFI 75-182. Both proteins contained 20 additional amino acids, including a histidine tag and a thrombin cleavage site, at the N terminus.

**Purification of NFI derivatives.** His-tagged NFI-BD, NFI 75-236, NFI 1-182, and NFI 75-182 were expressed in *Escherichia coli* BL21(DE3)/pLysS (44). These proteins were highly insoluble; however, when induction was performed at low cell densities, reasonable amounts of protein could be purified from the soluble fraction. Overnight cultures grown at 37°C were diluted 1:200 in Luria-Bertani medium in the presence of 0.4% glucose and 50 µg of ampicillin and 30 µg of chloramphenicol per ml. Cells were grown for 2 h, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and cells were grown for another 3.5 h. Cells were harvested by centrifugation and were resuspended in lysis buffer (50 mM Tris-Cl [pH 8.0], 250 mM NaCl, 5 mM dithiothreitol (DTT), 1 mM EDTA, 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 1 mM phenylmethylsulfonyl fluoride, 0.7 mg of lysozyme per ml). Cells were freeze-thawed and sonicated (three times for 20 s, on ice). DNase I (20 µg/ml) was added, and the mixture was then incubated at room temperature for 45 min. Insoluble material was removed by centrifugation at 20,000 × g for 20 min. The supernatant was dialyzed against buffer A (50 mM Tris-Cl [pH 8.0], 250 mM NaCl, 3 mM β-mercaptoethanol, 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 0.5 mM phenylmethylsulfonyl fluoride) to remove EDTA, and DTT and was subsequently loaded on a 1-ml Ni-nitrilotriacetic acid (NTA) column (Qiagen) equilibrated in the same buffer. The column was washed with buffer A (pH 6.0) containing 10% glycerol and then with buffer A (pH 8.0) containing 20 mM imidazole and 10% glycerol. The column was eluted with a linear gradient of 20 to 500 mM imidazole in buffer A (pH 8.0) containing 10% glycerol. The His-NFI proteins eluted at 300 mM imidazole. His-NFI-BD and NFI 1-182 were further purified on an S-Sepharose column equilibrated in buffer B (50 mM Tris-Cl [pH 8.5], 1 mM DTT, 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 0.01% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) containing 200 mM NaCl. Protein was eluted with a linear gradient of 200 to 600 mM NaCl in buffer B. His-NFI-BD and NFI 1-182 eluted at 450 mM NaCl. Since NFI 75-236 and NFI 75-182 did not bind S-Sepharose or Q-Sepharose, these mutants were purified on Ni-NTA twice, exactly as described for His-NFI-BD. The purity of these mutants was approximately 80% as estimated by silver staining. The thioredoxin-His-NFI 1-78 fusion protein was expressed in *E. coli* Gl698 cells obtained from Invitrogen. Growth and induction of the cells were performed as described by the manufacturer. The cells were harvested, lysed, and purified on a 5-ml Ni-NTA column as described above, except that additional protease inhibitors were added (2 µg of aprotinin, 2 µg of Leu-peptide, 1 µg of L-1-p-tosylamino-2-phenylethyl chloromethyl ketone per ml). Thioredoxin-His-NFI 1-78-containing fractions, which eluted at approximately 200 mM imidazole, were pooled and applied to a 5-ml S-Sepharose column equilibrated in buffer C (50 mM Tris-Cl [pH 8.0], 1 mM DTT, 0.01% Nonidet P-40, 10% glycerol) containing 100 mM NaCl. Protein was eluted with a linear gradient of 100 to 1,000 mM NaCl in buffer C. Thioredoxin-His-NFI 1-78 eluted at approximately 350 mM NaCl. To remove the thioredoxin moiety, including the enterokinase cleavage site and the histidine tag, the fusion protein was treated with thrombin (1 U of protein per mg; incubation was for 1 h at room temperature, which was followed by overnight incubation at 4°C). The cleavage products were applied to a 5-ml Ni-NTA column equilibrated in buffer A containing 10% glycerol. NFI 1-78 did not bind to the column in the presence of 20 mM imidazole, while the thioredoxin-His fragment and uncleaved protein bound tightly and eluted at 200 mM imidazole. NFI 1-78-containing fractions were purified to homogeneity and concentrated on an S-Sepharose column as described above.

**Gel retardation.** The following double-stranded oligonucleotides containing intact or mutated NFI sites were used: intact NFI site, GCACGTTTTGGATT GAAGCCAATATGACGCA; one half-site mutated, GCACGTTTTTCGATTG AAGCCAATATGACGC; and both NFI half-sites mutated, GCACGTTTTTCG ATTGAAGCGAATATGACGC. These probes and the TD15 probe (24) were labelled at the 5' end by T4 polynucleotide kinase in the presence of [<sup>γ</sup>-<sup>32</sup>P]ATP. A 332-bp *Nde*I-*Xba*I fragment from pHRI containing the adenovirus type 2 origin was labelled at the *Xba*I site by the Klenow fragment of DNA polymerase I in the presence of [α-<sup>32</sup>P]dCTP. Probes (0.05 ng), and proteins were incubated in 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5)-4 mM MgCl<sub>2</sub>-0.4 mM DTT-4% Ficoll in a total volume of 20 µl for 1 h at 4°C. Concentrations of poly(dI-dC) · poly(dI-dC) were as described in the figure legends. Retardation experiments were performed in the presence of 100 mM NaCl. Bound and free DNAs were separated on a 6% polyacrylamide gel at 4°C in a running buffer containing 0.5× Tris-borate EDTA and 0.01% Nonidet P-40.

**DNase I footprinting.** DNase I footprinting was performed on a 332-bp *Nde*I-*Xba*I fragment of pHRI, which was labelled as described above. Incubations of 1 ng of DNA with various amounts of NFI-BD or deletion mutants were performed in a total volume of 50 µl in a buffer containing 20 mM HEPES-KOH (pH 7.5), 1 mM MgCl<sub>2</sub>, 0.018% Nonidet P-40, 100 mM NaCl, and 1 µg of bovine serum albumin. Protein was allowed to bind for 30 min on ice, and the reaction mixtures were incubated with DNase I (0.1 U per reaction mixture) for 90 s at 25°C. Reactions were stopped by the addition of 20 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), and 1 µg of calf thymus DNA and then by phenol-chloroform extraction and isopropanol precipitation. DNA was analyzed on an 8% denaturing polyacrylamide gel.

**CD spectroscopy.** Circular dichroism (CD) spectra were recorded at room temperature with a Jasco-600 spectropolarimeter. The protein concentration, as determined by the Bio-Rad protein assay, was 0.6 mg/ml in a buffer containing 10 mM sodium phosphate (pH 7.5) and 50 mM NaCl. Spectra were recorded from 310 to 190 nm in a cuvette with a 0.1-mm path length. The data from 10 spectra were averaged.

**Adenovirus DNA replication in vitro.** Replication reactions were performed in a total volume of 25 µl containing 25 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 40 µM dATP, dTTP, dGTP, 500 nM [α-<sup>32</sup>P]dCTP, 0.5 µg of Adenovirus DNA-binding protein (DBP), and various amounts of pTP-pol and NFI-BD or NFI deletion mutants. DBP, pTP-pol, and TP-DNA were purified as described previously (11). Incubation was for 45 min at 37°C. The reactions were stopped by the addition of 2.5 µl of stop mix (40% sucrose, 1% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol). The products were analyzed on a 1% agarose gel run for 16 h (50 V) in 0.5× Tris-borate-EDTA containing 1% SDS. The gels were dried and quantified by densitometric analysis with a PhosphorImager.

## RESULTS

**Bacterial expression of NFI-BD and deletion mutants.** To study the function of NFI-BD by deletion mutagenesis employing purified proteins, an efficient expression system was required. NFI-BD produced with the vaccinia virus expression system is fully active, but the yield is low (2, 35). NFI-BD expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein is insoluble, and although the protein can be purified by denaturation and renaturation protocols, the specific DNA binding activity is approximately five times less than that for vaccinia virus-expressed NFI-BD (10). We expressed His-tagged rat NFI-BD in *E. coli*. Although the bulk of the protein was insoluble, as much as 5% (1 mg/liter) was soluble when protein expression was induced at low cell densities. The His-tagged NFI-BD was purified on Ni-NTA and S-Sepharose columns and was as active in DNA binding and stimulation of adenovirus DNA replication as vaccinia virus-produced NFI-BD (data not shown).

To study the role in DNA binding of the basic N-terminal region and the region around C-115, His-tagged NFI 75-236 and NFI 1-78 were constructed (Fig. 1A). In addition, two C-terminal deletion mutants, NFI 1-182 and NFI 75-182, were made. Although all of the deletion mutants except NFI 1-78 were highly insoluble, employing growth and induction conditions identical to those used for the intact DNA binding domain, we obtained reasonable amounts of soluble protein. NFI 1-78 fused to thioredoxin was soluble, and large quantities

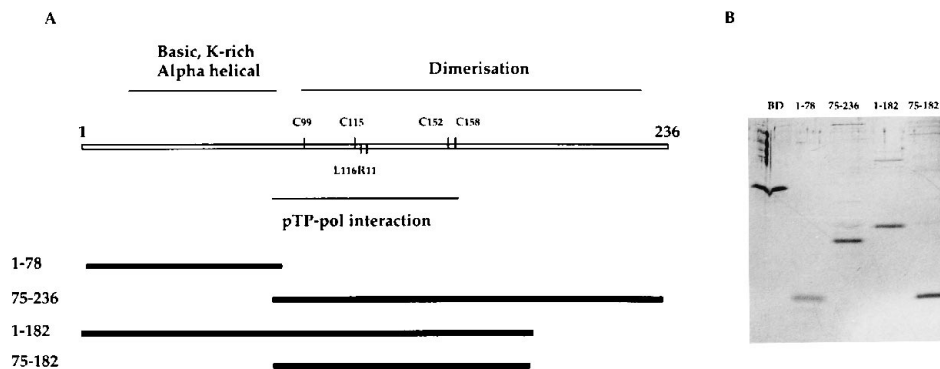


FIG. 1. (A) Schematic representation of the DNA binding domain of rat NFI. The amino-terminal 80 amino acids are very basic and fold into an  $\alpha$ -helical structure (see Fig. 5). The cysteine residues at positions 99, 152, and 158 are essential for DNA binding (36, 37). The cysteine residue at position 115 is oxidation sensitive (3). Mutations at positions 116 and 117 abolish DNA binding without affecting dimerization (2). Regions implicated in dimerization and interaction with pTP-pol are indicated. All of these data were obtained by mutational analysis of the DNA binding domain of human NFI, which is more than 95% identical to the binding domain of rat NFI. Residue numbers indicated here refer to the rat protein. (B) Purified NFI-BD and deletion mutants analyzed by SDS-polyacrylamide gel electrophoresis and silver staining.

could be obtained. The His-tagged deletion mutants were purified in a way similar to that for His-NFI-BD (Fig. 1B).

**Amino acids 75 to 182 of NFI-BD are sufficient for specific DNA binding, but high-affinity binding requires amino acids 1 to 74.** To study the DNA binding activities of the NFI-BD deletion mutants, three different probes were used (Fig. 2); probe 1 contains the NFI binding site present in the adenovirus type 2 origin, probe 2 has a mutation in one half-site, and probe 3 has both half-sites mutated. NFI-BD bound the intact NFI site; however, at this concentration no binding to the mutated probes was observed. When more NFI-BD was added, binding to the half-site was observed (data not shown), and we estimate that the affinity for the half-site was approximately 10 times lower, in agreement with earlier results (17). NFI 75-236 also bound specifically to DNA, since no binding to probes 2 and 3 was observed. However, we estimate that the amount of protein required for 50% binding of the intact NFI site was 200 times higher than that for NFI-BD. Deletion of amino acids 183 to 236 resulted in a fivefold reduction in affinity for the intact site, but NFI 1-182 still bound specifically. These results indicate that amino acids 75 to 182 are sufficient for specific DNA binding. However, NFI 75-182 itself had undetectable DNA binding activity (Fig. 2, lane 19). Therefore, residues outside amino acids 75 to 182, especially amino acids 1 to 74,

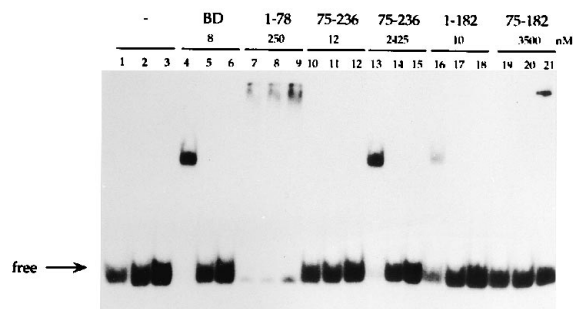


FIG. 2. DNA binding properties of NFI-BD deletion mutants. DNA binding was studied with short double-stranded oligonucleotides (30 bp) containing the wild-type NFI binding site (lanes 1, 4, 7, 10, 13, 16, and 19), containing one mutated half-site (lanes 2, 5, 8, 11, 14, 17, and 20), or containing both half-sites mutated (lanes 3, 6, 9, 12, 15, 18, and 21). The amounts of protein added are indicated. The spot in lane 21 is due to some aggregates in the slot and was not observed in other experiments.

seem to be required for high-affinity binding. The protein-DNA complexes containing the various deletion mutants migrate at identical positions in the gel. This indicates that the mass of the protein does not significantly influence the electrophoretic migration of the protein-DNA complex. We purified a number of mutants with smaller N-terminal deletions, and all of these mutants retarded the probe to identical positions in the gel (data not shown). A similar observation was made with deletion mutants of p53 (49). The basic N terminus of NFI-BD, NFI 1-78, bound to all three probes. It formed large protein-DNA complexes with very low mobility. This suggested nonspecific DNA binding by this fragment.

DNase I footprinting was performed to test the specificity of DNA binding and to compare the extents of the region protected by the various deletion mutants (Fig. 3). The footprint obtained with His-tagged NFI-BD was similar to the footprints obtained with intact NFI purified from HeLa cells (28) and vaccinia virus-expressed NFI-BD (35). NFI-BD, NFI 75-236, and NFI 1-182 all have identical footprints, indicating that these mutants bind specifically to an NFI binding site as a dimer. This confirms that amino acids 75 to 182 are responsible for specific binding. The affinity of NFI 75-182 was too low to obtain a footprint. NFI-BD 1-78 did not show a footprint, although complete binding of the DNA was detected under these conditions in a bandshift assay (see also Fig. 4, lanes 18 to 23). At high concentrations of NFI 1-78, the intensities of all bands decreased somewhat (Fig. 3, lanes 17 to 21), but no distinct footprint was observed. This indicates that amino acids 1 to 78 interact with DNA in a nonspecific way. These results suggest that NFI contains two DNA binding activities: specific binding via amino acids 75 to 182 and nonspecific binding via amino acids 1 to 78. To show that the intact DNA binding domain indeed contains specific and nonspecific DNA binding properties, a gel retardation assay was performed with a long probe containing only one intact NFI binding site (Fig. 4). In the presence of low concentrations of competitor DNA [10 ng of poly(dI-dC) · poly(dI-dC); Fig. 4, lanes 2 to 7] NFI-BD formed at least six complexes. In the presence of 500 and 1,000 ng of poly(dI-dC) · poly(dI-dC) (Fig. 4, lanes 8 and 9), only one major complex was detected. The weak second complex is probably due to the binding to an extra half-site present on the probe. NFI 75-236 formed only one major complex on this probe, and no additional complexes were observed. Since the affinity of this protein is very low, we could not add the amount

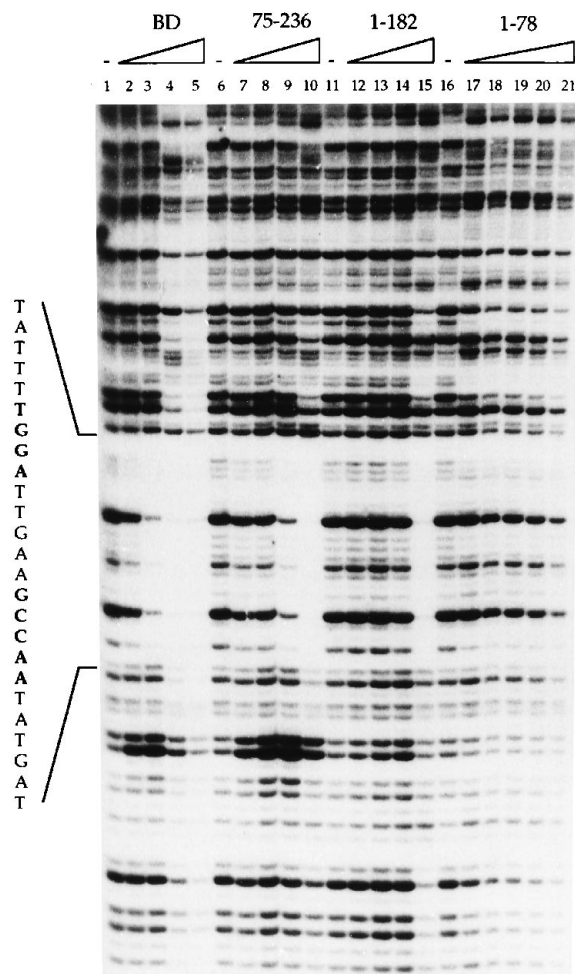


FIG. 3. DNase I footprint analysis of NFI-BD and deletion mutants on the top strand of the adenovirus type 2 origin. Lanes: 1, 6, 11, and 16, no protein; 2 to 5, respectively, 4, 8, 17, and 33 nM NFI-BD; 7 to 10, respectively, 150, 300, 600, and 1200 nM NFI 75-236; 12 to 15, respectively, 21, 43, 85, and 170 nM NFI 1-182; 17 to 21, respectively, 1, 2, 4, 8, and 16  $\mu$ M NFI 1-78.

of protein required to achieve the same degree of saturation as with NFI-BD in lane 7. Therefore, it cannot be excluded that NFI 75-236 can also interact nonspecifically with DNA. NFI 1-78 formed large DNA-protein complexes. Binding of this fragment was completely abolished by addition of 500 ng of poly(dI-dC)  $\cdot$  poly(dI-dC) (lane 24), confirming that the binding is nonspecific. It is clear from these data that NFI-BD can bind specifically and nonspecifically to DNA. It is likely that amino acids 1 to 78 and 75 to 182 are responsible for the nonspecific binding and for the specific binding of NFI, respectively.

**Amino acids 1 to 78 fold into a stable, highly  $\alpha$ -helical structure.** From the bandshift and footprint experiments, we conclude that NFI 75-236 binds DNA as a dimer, indicating that amino acids 75 to 236 are still correctly folded. To determine whether the N-terminal domain, amino acids 1 to 78, could adopt a stable structure, CD measurements were performed. The result is shown in Fig. 5. The spectrum contains double minima at 208 to 210 and 222 nm and a maximum at 191 to 193 nm, which indicates that NFI 1-78 is  $\alpha$ -helical. By comparison with standard spectra of Lys<sub>n</sub> (51), the  $\alpha$ -helical content was estimated to be around 80% with little if any

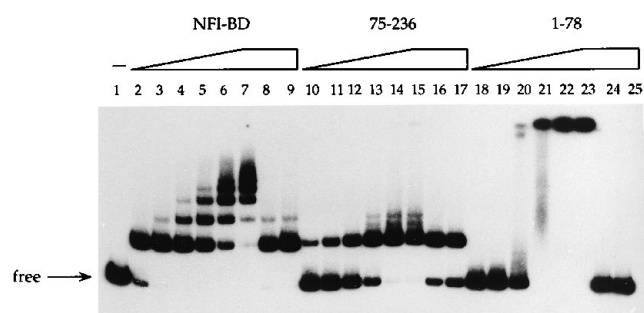


FIG. 4. DNA binding of NFI-BD, NFI 75-236, and NFI 1-78 on a probe of 332 bp containing one intact NFI binding site. Lanes: 1 to 7, respectively, 0, 2.5, 5, 10, 21, 42, and 83 nM NFI-BD in the presence of 10 ng of poly(dI-dC)  $\cdot$  poly(dI-dC); 8 and 9, 83 nM NFI-BD in the presence of 500 and 1,000, respectively, ng of poly(dI-dC)  $\cdot$  poly(dI-dC); 10 to 15, 30, 60, 121, 242, 484, and 970 nM NFI 75-236, respectively, in the presence of 10 ng of poly(dI-dC)  $\cdot$  poly(dI-dC); 16 and 17, 970 nM NFI 75-236 in the presence of 500 and 1,000 ng, respectively, of poly(dI-dC)  $\cdot$  poly(dI-dC); 18 to 23, respectively, 78, 156, 312, 625, 1,250, and 2,500 nM NFI 1-78 and 10 ng of poly(dI-dC)  $\cdot$  poly(dI-dC); 24 and 25, 2,500 nM NFI 1-78 and 500 and 1,000 ng, respectively, of poly(dI-dC)  $\cdot$  poly(dI-dC).

$\beta$ -sheet. Apparently, the N-terminal and C-terminal parts of NFI-BD can fold independently.

**Both NFI-BD subdomains stimulate adenovirus DNA replication.** NFI-BD stimulates adenovirus DNA replication by recruiting the pTP-pol complex to the origin of replication (2, 5, 7, 34). The level of stimulation strongly depends on the pTP-pol concentration (35). NFI binds the pTP-pol complex, and the region of NFI-BD that interacts with the polymerase has been mapped between amino acids 68 and 150 of human NFI (2, 7), corresponding to amino acids 71 to 153 of the rat protein (39). NFI 75-236, NFI 1-182, and NFI 75-182 all contain this region and the domain required for specific DNA

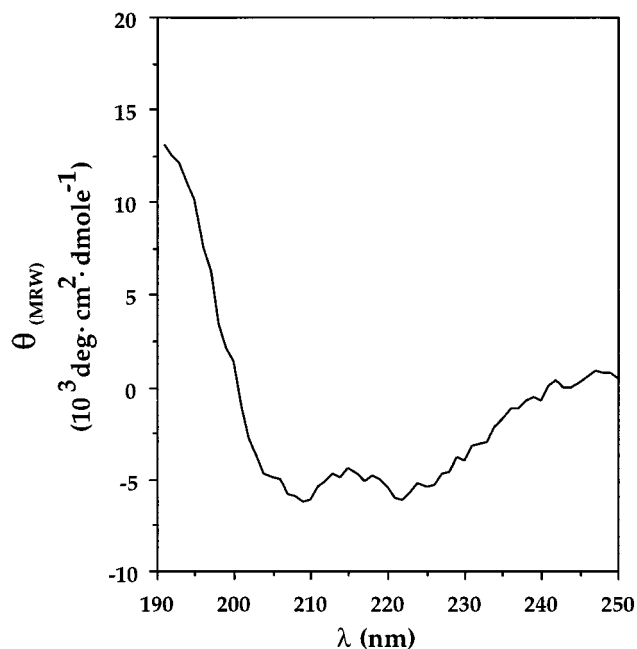


FIG. 5. CD spectrum shows that NFI 1-78 is  $\alpha$ -helical. Mean residue ellipticity,  $\theta$  (mean residue weight [MRW]), is plotted against wavelength,  $\lambda$  (in nanometers).

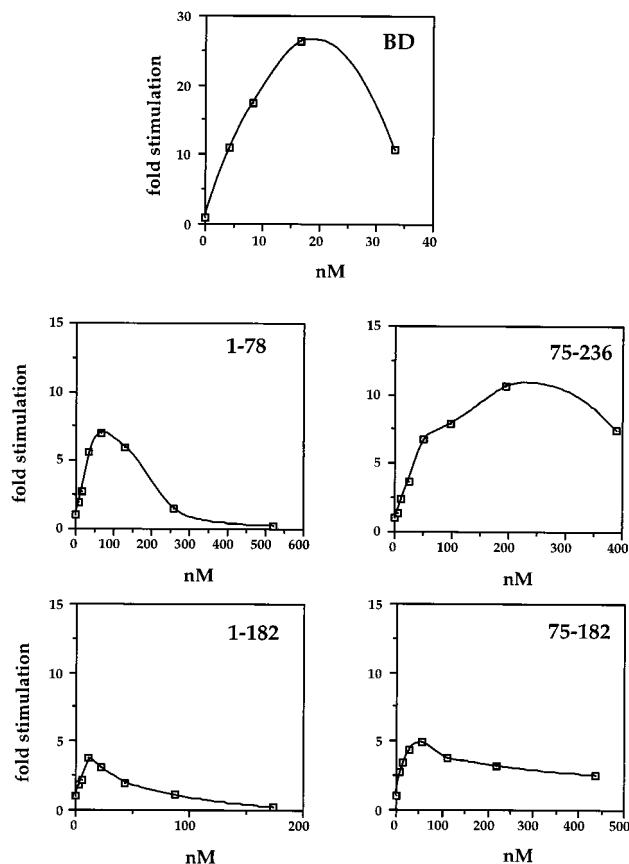


FIG. 6. Stimulation of adenovirus DNA replication in vitro. The replication activities of NFI-BD and NFI deletion mutants were tested at various protein concentrations. Note that the scales of the axes are not the same in every plot.

binding. The abilities of the NFI deletion mutants to stimulate adenovirus DNA replication in vitro were tested. The results are shown in Fig. 6. Low amounts of pTP-pol (0.5 nM) were used to obtain optimal levels of stimulation. Under these conditions, NFI-BD stimulated by as much as 27-fold. At high concentrations of NFI-BD, the level of stimulation decreased. This may be explained by nonspecific DNA binding at these high concentrations of NFI-BD (Fig. 4, lanes 2 to 7) which results in tethering of pTP-pol to other positions in the genome, thereby reducing the efficient pTP-pol concentration at the origin. The deletion mutants all stimulated replication, although to a level lower than that for NFI-BD, whereas an unrelated protein (the DNA binding domain of P15 [26], amino acids 63 to 127) did not stimulate. NFI 1-78 stimulated replication by as much as 7-fold. This was unexpected, since this protein binds nonspecifically to DNA and lacks the reported pTP-pol interaction domain. NFI 75-236 stimulated replication by 10-fold. Maximal stimulation by NFI 1-182 was 4-fold, and NFI 75-182 stimulated replication 5-fold. When only pTP-dCMP formation was tested, similar results were obtained, which shows that the mutants, like NFI-BD, act during initiation. Optimal stimulation by the NFI deletion mutants required protein concentrations higher than those in the case of NFI-BD. From these data, we conclude that the DNA-binding domain of NFI contains two regions, amino acids 1 to 78 and 75 to 182, that can both stimulate replication. When subsaturating amounts of NFI 1-78 and NFI 75-236 were mixed, their effects were additive and the high levels of stim-

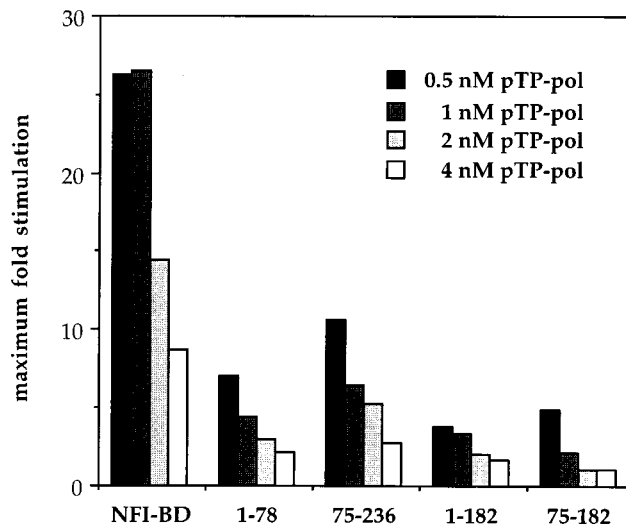


FIG. 7. NFI-BD and the deletion mutants stimulate DNA replication in a pTP-pol concentration-dependent manner. Maximum levels of stimulation are presented. Protein concentrations were as follows: NFI-BD, 16 nM; NFI 1-78, 50 nM; NFI 75-236, 190 nM; NFI 1-182, 17 nM; and NFI 75-182, 140 nM.

ulation that were obtained with the intact NFI-BD were not observed. Probably covalent linkage is required for the two subdomains to cooperate effectively.

**Both subdomains of NFI enhance the binding of the pTP-pol complex to the origin.** To test if the stimulation of replication by the NFI deletion mutants was dependent on the pTP-pol concentration, replication was performed at various concentrations of pTP-pol. Figure 7 shows the maximal levels of stimulation obtained in the presence of four different pTP-pol concentrations. Like NFI-BD, all of the deletion mutants stimulated replication in a pTP-pol concentration-dependent manner. This indicates that the mutants exert their stimulatory effect via the pTP-pol complex. Therefore, we assumed that these mutants would still be able to recruit pTP-pol to the origin in a manner similar to that for the intact DNA binding domain. This was tested in a bandshift assay with the TD15 probe (Fig. 8), which has been used before to study pTP-pol recruitment by NFI (2). This probe contains the first 50 nucleotides of the adenovirus type 2 origin of replication, but the first 14 nucleotides of the template strand are single stranded, leaving the NFI binding site double stranded (24). We used this probe because pTP-pol forms discrete complexes on this probe, in contrast to a fully double-stranded origin. NFI and pTP-pol concentrations required for optimal stimulation of DNA replication were used. At the low concentration used here, the pTP-pol complex binds very weakly to the TD15 probe (Fig. 8, lane 2), but in the presence of NFI-BD pTP-pol binding is enhanced and a pTP-pol-NFI-DNA complex is formed (lane 4). NFI 75-236, NFI 1-182, and NFI 75-182 also enhance binding of pTP-pol to the DNA (lanes 7 to 12). As a negative control, the DNA binding domain of P15 was tested. This protein bound to the TD15 probe but did not stimulate the binding of pTP-pol (data not shown). In the cases of NFI 75-236 and NFI 75-182, increased pTP-pol binding is observed; however, hardly any pTP-pol-NFI-DNA complex is formed. This suggests that NFI 75-236 and NFI 75-182 are no longer stably bound to the pTP-pol complex after recruitment of the complex to the DNA. At the concentration used here, no binding of NFI 1-78 to the TD15 probe was detected (Fig. 8, lane 5). NFI 1-78 does not contain the region implicated in

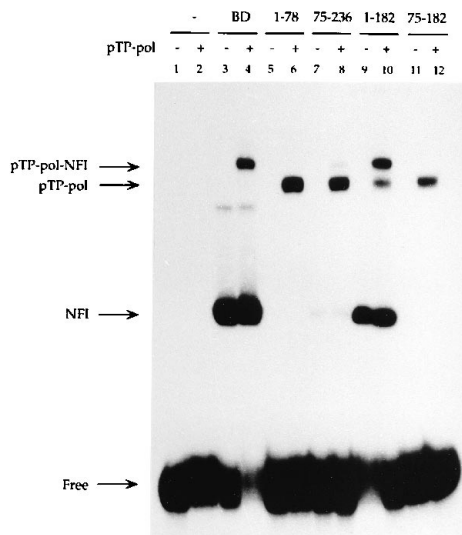


FIG. 8. DNA binding of pTP-pol in the absence and presence of NFI-BD and deletion mutants. Protein concentrations that give optimal stimulation of replication were used. Lanes 2, 4, 6, 8, 10, and 12 contain 0.5 nM pTP-pol. Concentrations of intact NFI-BD and deletion mutants were the as described in the legend to Fig. 7. The experiment was performed in the presence of 100 ng of poly(dI-dC)-poly (dI-dC).

binding to the pTP-pol complex but does stimulate binding of the complex to DNA (lane 6). In addition, in this case no supershift was detected. Since this could be due to the small size of NFI 1-78, we tested thioredoxin-NFI 1-78 (molecular mass, 23 kDa) and GST-NFI 1-78 (molecular mass, 36 kDa). Both fusion proteins stimulated replication and enhanced binding of the pTP-pol complex to DNA, but no supershift was detected (data not shown). By using GST-NFI 1-78 bound to glutathione-agarose beads, pulldown assays were performed to determine whether an interaction with pTP-pol could be detected. No such interaction was observed, whereas a mutant containing the pTP-pol binding domain (GST-NFI 1-140) could bind pTP-pol in this assay. This is consistent with the results described by Chen et al. (7), who have shown that the middle part, amino acids 68 to 150, of human NFI-BD binds the pTP-pol complex. Since NFI 1-78 seems to bind nonspecifically to DNA and does not bind the pTP-pol complex in a direct assay, we suggest that it stimulates pTP-pol binding and DNA replication by inducing structural changes in the DNA. The fact that the deletion mutants stimulate the binding of pTP-pol to the origin explains the pTP-pol concentration-dependent stimulation of replication by these proteins.

## DISCUSSION

**The DNA-binding domain of NFI contains two adjacent regions that interact with DNA.** Previous deletion analyses of NFI-BD showed that even small deletions at the N or C terminus abolished DNA binding (17, 32). We expressed a series of deletion mutant in *E. coli* and were able to purify amounts of protein sufficient to detect even very low affinities for DNA. Our data show that the DNA binding domain of NFI consists of two functional subdomains. Amino acids 75 to 236 bind specifically to DNA but with a greatly reduced affinity compared with that of NFI-BD. NFI 1-182 binds specifically, and the affinity is slightly reduced. From this we deduce that amino acids 75 to 182 are required for specific DNA binding, although we could not detect DNA binding by NFI 75-182 itself.

From the bandshift and footprint experiments, we conclude that amino acids 75 to 182 can still dimerize. Using in vitro-translated proteins, Gounari et al. (17) reported that deletion of amino acids 183 to 220 abolished dimerization and DNA binding. However, Armentero et al. (2) described a number of double point mutations that affect dimerization that are all located between amino acids 75 and 182. Our observation that amino acids 183 to 236 are not essential for dimerization is in agreement with the observation that two members of the human family of NFI proteins, CTF-1 and CTF-3, can heterodimerize (27). CTF-3 lacks amino acids 187 to 208, corresponding to amino acids 183 to 204 of rat NFI, but binds specifically to an NFI binding site as a dimer. However, it is possible that deletion of amino acids 183 to 236 results in a somewhat reduced dimerization potential, which would explain the lower DNA binding activity of NFI 1-182.

Several observations support our results that indicate that amino acids 75 to 182 are essential for specific DNA binding. Three conserved cysteines, at positions 99, 152, and 158 of the rat protein, are essential for DNA binding (36, 37). The cysteine residue at position 115 is sensitive to oxidation (3), which leads to inactivation of the protein. When NFI is bound to DNA, it is partially protected against oxidation. Several DNA binding domains, of which the structure is known, contain cysteine residues with similar properties, and in all of these cases this cysteine is very close to the DNA, e.g., NF- $\kappa$ B/rel (29), Myb (19), p53 (20, 23), c-fos and c-jun (1, 4), or even makes specific base contacts, as in the case of the bovine papillomavirus E2 protein (22, 30) and the POU homeodomain (25, 41). Furthermore, the region around this cysteine residue in NFI is the only continuous stretch of amino acids that is more than 95% identical to the *C. elegans* NFI, as predicted from the genomic sequence identified in the *C. elegans* genome sequencing project. Finally, mutation of amino acids 112 and 113 of human NFI abolishes DNA binding but does not affect dimerization (2).

The N-terminal 80 amino acids have also been implicated in contacting DNA (17, 31). This region is very basic, and CD spectroscopy showed that this region adopts an  $\alpha$ -helical structure. This domain interacts with DNA in a nonspecific way and is required for high-affinity binding, but not for specific binding by NFI. Deletion of this region, in the mutant NFI 75-236, reduced DNA binding dramatically; however, this did not result in a smaller DNase I footprint. Possibly, amino acids 1 to 78 make DNA backbone contacts within the binding sites of NFI 75-236 and NFI 1-182. Binding of NFI 75-236 and NFI 1-182 resulted in the DNase I-hypersensitive sites observed with the intact DNA binding domain, which indicates that these mutants also induce structural changes in the DNA. We conclude that the DNA binding domain of NFI contains two regions that interact with DNA: an N-terminal domain that binds nonspecifically and a C-terminal domain that binds specifically to the NFI recognition site. In addition, in the context of the intact DNA binding domain, such specific and nonspecific DNA binding properties were observed. de Vries et al. (14) obtained evidence for a sliding mechanism by which NFI finds its binding site. An attractive model would be that NFI interacts nonspecifically with DNA via amino acids 1 to 78 until it encounters an NFI binding site to which the specific domain will then bind.

**The two subdomains of NFI-BD can stimulate replication independently, but full stimulation requires the intact DNA binding domain.** Our data show that the two subdomains of NFI-BD, amino acids 1 to 78 and 75 to 182 can both stimulate adenovirus DNA replication, although to a level lower than that of NFI-BD. NFI-BD stimulates replication by binding the

polymerase and bringing it to the origin by binding to its binding site in the auxiliary region of the origin. The region of human NFI that interacts with the polymerase has been mapped between amino acids 68 and 150 (7), corresponding to amino acids 71 to 153 of rat NFI. Mutation of amino acids 83/84 and 91/92 reduces the binding between human NFI-BD and pTP-pol and the stimulation of replication by NFI-BD (2), indicating that the interaction between this region of NFI-BD and pTP-pol is functional in replication. We show that amino acids 75 to 182 containing the specific DNA binding domain as well as the pTP-pol interaction domain are sufficient for low levels of stimulation. High-affinity binding does not seem to be absolutely required, although the deletion mutants stimulate considerably less compared to intact NFI-BD. NFI 1-182 binds specifically to DNA with a slightly reduced affinity compared with that of NFI-BD but stimulates replication by only four times. As described above, deletion of amino acids 183 to 236 may reduce dimerization and thereby specific DNA binding. Monomeric NFI 1-182 may interact with pTP-pol, which can result in mistargeting the complex because of nonspecific DNA binding by amino acids 1 to 78. This would result in reduced levels of stimulation. NFI 1-78 does not bind specifically to DNA but nevertheless stimulates replication by as much as sevenfold. These results show that two separate regions within the DNA binding domain of NFI are involved in stimulation of adenovirus DNA replication. The action of both subdomains results in the formation of a pTP-pol-DNA complex, which explains the additive effects of the two domains. NFI-BD stimulates to a higher level, possibly because the intact DNA binding domain forms a stable pTP-pol-NFI-DNA complex which may result in a better positioning of the polymerase on the origin.

**NFI may employ two mechanisms to enhance preinitiation complex formation.** Apparently, binding of NFI-BD, NFI 75-182, or NFI 75-236 to the pTP-pol complex increases the specificity of the ternary complex for binding the origin containing an NFI binding site. Possibly, the polymerase also adopts another conformation when bound to NFI that has an increased affinity for the origin. NFI 75-182 and NFI 75-236 enhance binding of the pTP-pol complex without becoming committed to the complex itself, as shown by bandshift analysis. This means that NFI 75-182 and NFI 75-236 no longer interact with pTP-pol once the complex binds DNA. Although intact NFI-BD is more stably incorporated in the preinitiation complex, nucleotide binding by polymerase is sufficient to displace NFI-BD from the DNA (10). There are more examples of proteins that stimulate binding of another protein to DNA via a direct protein-protein interaction without becoming stably committed to the complex. The HMG-1 protein can stimulate binding of progesterone receptor to its binding site without participating in the complex (38). HMG-2 interacts with the DNA binding of Oct-1 and enhances binding of Oct-1 to DNA; however, no HMG-2 could be detected in the Oct-1-DNA complex (54).

NFI 1-78 does not interact with the pTP-pol complex (7; our unpublished data) and does not bind specifically to DNA. However, it does stimulate replication in a pTP-pol-dependent way, and it enhances binding of pTP-pol to DNA in an unknown fashion. Binding of pTP-pol to the origin induces structural changes in the DNA resulting in DNase I-hypersensitive sites (34). Possibly, NFI 1-78 can induce or stabilize alterations in the DNA structure which facilitate binding of pTP-pol. If NFI 1-78 exerts its effect indeed via the DNA, the interaction between NFI 1-78 and DNA must be very transient. Stimulation of binding of proteins to DNA via transient structural changes in DNA induced by sequence-nonspecific DNA bind-

ing proteins has been described for several systems. The bacterial protein Hu stimulates binding of Lac repressor and catabolite activator protein to DNA without participating in the final complex (16). Hu has been proposed to stabilize a structural conformation of the DNA to which the sequence specific DNA binding protein preferentially binds (16), (40). Another example is the adenovirus DNA binding protein DBP, which stimulates binding of NFI (8, 45) and USF (52) to DNA, probably via structural alterations in the DNA. CD spectroscopy can be used to study changes in the structure of DNA induced by binding of a protein. We have used this technique to study the effect of binding of NFI 1-78 on the structure of DNA. However, because of aggregation of the protein-DNA complex at the high concentrations of protein and DNA required for CD spectroscopy, we have not been able to obtain structural information of the DNA in the complex. Therefore, clear experimental evidence that NFI 1-78 induces structural changes in the DNA is still lacking.

From Fig. 8, it is clear that recruitment of pTP-pol does not correlate with the binding affinity of the NFI deletion mutants. No DNA binding by NFI 75-182 is observed, but this protein is able to recruit pTP-pol only slightly less efficiently than NFI-BD. However, it should be noted that the level of pTP-pol binding to the partially single-stranded TD15 probe is not directly comparable to binding to a TP-containing double-stranded origin. Therefore, the levels of stimulation of pTP-pol binding to TD15 cannot be directly compared to stimulation of replication.

These results support a model in which NFI enhances preinitiation complex formation via two mechanisms. NFI-BD directly interacts with pTP-pol via amino acids 75 to 182 and tethers the complex to the origin by binding its recognition site located in the auxiliary region of the origin. Possibly via structural changes in the DNA induced by amino acids 1 to 78, the pTP-pol-DNA complex is stabilized. The formation of a stable pTP-pol-NFI-DNA complex that is formed by intact NFI-BD may then ensure correct positioning of the pTP-pol complex on the core origin. The combined stimulatory effects of both domains would result in high levels of stimulation.

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