Interaction between the Octamer-Binding Protein Nuclear Factor III and the Adenovirus Origin of DNA Replication

GER J. M. PRUIJN, ROB T. VAN MILTENBURG, JOHNNY A. J. CLAESSENS, AND PETER C. VAN DER VLIET*

Laboratory for Physiological Chemistry, State University of Utrecht, Vondellaan 24a, 3521 GG Utrecht, The Netherlands

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Nuclear factor III (NFIII) is a HeLa sequence-specific DNA-binding protein that stimulates initiation of adenovirus DNA replication in vitro and may be involved in regulation of transcription of several cellular and viral genes. We have studied the interaction between NFIII and the binding site in the adenovirus type 2 (Ad2) origin in detail by methidiumpropyl-EDTA · iron(II) and hydroxyl radical footprinting and by alkylation interference experiments. Our results indicate that (i) the core of the recognition sequence is 5'-TATGATAAT-3'; (ii) both major and minor groove base contacts are detected, and all base pairs in the core are involved in binding; (iii) many backbone contacts are observed divided into a large domain coinciding with the core and a small domain; (iv) contact points are not confined to one side of the DNA helix in contrast to the nuclear factor I (NFI)-binding site; (v) the binding site overlaps the NFI-binding site for at least one nucleotide. A number of Ad2 mutants as well as related binding sites in the origins of other adenovirus serotypes were systematically compared for binding with NFIII. The results are in good agreement with the contact point studies and show that at least one AT base pair is commonly required by NFI and NFIII for optimal binding. The strongest binding site, which contains the octamer/decanucleotide motif (ATGCAAAT[NA]), was found in the Ad4 origin, which lacks an NFI-binding site. Stimulation of in vitro DNA replication of Ad2, Ad4, and Ad12 by NFIII showed that the maximal level of stimulation is dependent on the affinity of NFIII for the origin.

Besides virus-encoded proteins, optimal replication of adenovirus DNA in vitro requires several proteins isolated from uninfected HeLa cell nuclei. Two of these, nuclear factors I and III (NFI and NFIII), are sequence-specific DNA-binding proteins that stimulate the initiation of adenovirus DNA replication several times (27, 30, 36). A third, NFII, a type I topoisomerase, is required for elongation of full-length adenovirus DNA chains (28). NFI and NFIII recognize specific sequences in the origin of DNA replication, which is located in the inverted terminal repeat (ITR) of the adenovirus genome. Binding of NFI and NFIII to their recognition sequences is essential for the stimulation of replication in vitro (9, 14, 25, 30, 32, 36). The NFI-binding site (consensus sequence TGGA/CN₅GCCAA) has been studied in great detail by mutagenesis of the recognition sequence, DNase I footprinting, and contact point analysis (9, 10, 13, 25, 32, 40, 53).

NFIII binds to a sequence adjacent to the NFI recognition sequence encompassing 5'-TATGATAATGA-3' in the adenovirus type 2 (Ad2) origin (30, 36). Sequences highly homologous to both the NFI- and the NFIII-binding sites within the Ad2 origin have been detected in a number of transcription regulatory elements (5, 17, 31, 42). Recently, we demonstrated that NFIII interacts with these promoter/enhancer modules which share the conserved octamer/decanucleotide ATGCAAAT(NA) (31).

The binding of NFI and NFIII to these (cellular) sequences suggests that these proteins might function as *trans*-acting factors in the regulation of transcription. This notion is supported by the observation that purified NFI is indistinguishable from a purified CCAAT box-binding transcription factor and that a protein with binding properties very similar to those of NFIII is required for transcription of histone H2B genes (22, 44).

A previous contact point analysis of the NFI recognition

sequence revealed symmetrical binding to one side of the DNA helix, presumably as a dimer, and NFI displays only major groove contacts (10) which suggest a helix-turn-helix binding mode (1). To understand the interaction of NFIII with its binding site in more detail, we determined contacts with NFIII within the Ad2 binding site by using methidiumpropyl-EDTA · iron(II) [MPE · Fe(II)] footprinting, hydroxyl radical footprinting, and alkylation interference assays. The results show that, in contrast to NFI, NFIII contacts are present all around the DNA helix and that both major and minor groove contacts are involved in binding.

Furthermore, we compared the NFIII binding sites of various adenovirus serotypes and Ad2 mutants, using a gel retardation competition assay. The results provide additional information on the borders of the Ad2-binding site and reveal differences in affinity for the binding site in different adenovirus serotypes. The level of stimulation of in vitro DNA replication by NFIII appeared to be related to the affinity of the protein for the origin.

MATERIALS AND METHODS

DNA preparations. Plasmid pHRI was a generous gift of R. Hay (15) and contains the Ad2 ITR. For footprinting and contact point analyses, the 331-base-pair (bp) *NdeI-XbaI* fragment of pHRI, ³²P-labeled at either the 5'-end of the *XbaI* site by polynucleotide kinase or the 3'-end of the *XbaI* site by DNA polymerase I (Klenow fragment), was used. Plasmids p4A85Δ (containing the terminal 78 bp of the Ad4 ITR), pHRΔ40, pHRΔ41, and pHRΔ43 (containing nucleotides 40 to 106, 41 to 106, and 43 to 106 of Ad2, respectively) were also obtained from R. Hay (15, 16). For competition experiments, *PvuII-PvuII* fragments of pHRI, p4A85Δ, pHRΔ40, pHRΔ41, and pHRΔ43 were isolated. These fragments consisted of 409, 384, 370, 369, and 367 bp, respectively. The simian adenovirus type 7 (SA7) origin-containing 428-bp competitor fragment was isolated from plasmid pSA7-1-565 (kindly provided by H. van Ormondt),

* Corresponding author.

which contains the terminal 565-bp *EcoRI* fragment of SA7P (cloned in pAT153; 8), by digestion with *BglII* and *SspI*. The Ad12 400-bp *EcoRI-PvuII* fragment was isolated from plasmid pAd12-RIC1 (kindly provided by J. L. Bos; 6, 50). The Ad40 and Ad41 competitors consisted of the 396-bp *SalI-DraI* fragment and the 362-bp *SalI-BamHI* fragment from pAd40ClaB and pAd41ClaD (52), respectively.

Footprinting reactions. Each footprinting reaction contained about 1 ng of the end-labeled DNA fragment, which was incubated with NFIII in the presence of 1 μ g of poly(dI-dC) · poly(dI-dC)–1 μ g of bovine serum albumin in a total volume of 50 μ l containing 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.025% Nonidet P-40, 50 mM NaCl for 30 min at room temperature. For MPE · Fe(II) footprinting, 5 μ l of a pKB67-88 DNA-cellulose fraction (see NFIII purification) was used. After binding occurred, dithiothreitol was added to the reaction mixture to a final concentration of 5 mM followed by 6 μ l of a 100 μ M MPE · 200 μ M ferrous ammonium sulfate solution (18, 37). The mixture was incubated for 2 min at 30°C, and cleavage was stopped by the addition of a mixture of EDTA (50 mM final concentration) and ammonium acetate (0.5 M final concentration).

For hydroxyl radical footprinting (49), NFIII was incubated with end-labeled DNA as described above, after which 9 μ l of a mixture of 0.13 mM EDTA, 0.07 mM ferrous ammonium sulfate, 2% H₂O₂, 6.7 mM sodium ascorbate was added, and the mixture was incubated at room temperature for 4 min. The cleavage reaction was stopped by adding thiourea to a final concentration of 67 mM.

After phenol-chloroform extraction and ethanol precipitation, samples were analyzed on an 8% denaturing polyacrylamide gel.

Alkylation interference experiments. For ethylation interference experiments, labeled DNA fragments were incubated with 1-ethyl-3-(3-dimethylammonio)propanyl carbodiimide by the method of Siebenlist and Gilbert (41). After ethanol precipitation, the probe was purified on a 5% polyacrylamide gel. The ethylated probe was eluted from the gel and ethanol precipitated. Approximately 30,000 cpm (Cerenkov) (about 2 ng) of ethylated DNA was used per incubation. For methylation interference experiments, labeled DNA fragments were methylated with dimethylsulfate as described previously (41). The methylated DNA was ethanol precipitated twice. A total of 15,000 cpm (Cerenkov) of methylated DNA was used per incubation.

Binding reactions were performed under the same conditions as those described for the footprinting experiments, using the pKB67-88 DNA-cellulose NFIII fraction. Protein-bound DNA was separated from free DNA by filtration over a nitrocellulose filter (HAWP; Millipore). DNA retained on the filter was eluted in 0.5 M ammonium acetate–0.1% sodium dodecyl sulfate (SDS)–1 mM EDTA. Both the filter-bound and free DNA fractions were phenol-chloroform extracted, ethanol precipitated, and analyzed on an 8% denaturing polyacrylamide gel.

Gel retardation analysis. Competition experiments for binding of NFIII to the Ad4 origin were performed as previously described by using the gel retardation assay (31).

DNA replication in vitro. For DNA replication assays, we used purified Ad5 DNA-binding protein and an Ad5 precursor terminal protein-DNA polymerase complex isolated from recombinant vaccinia virus-infected cells (46) and purified by phosphocellulose and denatured calf thymus DNA-cellulose chromatography followed by glycerol gradient centrifugation (33). These proteins were preincubated

with either 20 ng of an *EcoRI-AvaII* digest of pHRI or p4A85 Δ or 32 ng of an *EcoRI-AvaI* digest of pAd12-RIC1 in a total volume of 7 μ l containing 200 μ M aphidicolin, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH (pH 7.5), 4 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mg of bovine serum albumin per ml for 30 min at 30°C. After preincubation, ATP, deoxynucleoside triphosphates, and various amounts of NFIII were added and DNA replication was allowed to proceed for 90 min at 30°C in a total reaction volume of 15 μ l containing 1.7 mM ATP, 5 mM creatine phosphate, 5 μ g of creatine kinase per ml, 40 μ M each of dATP, dGTP, and dTTP, 2.5 μ M [α -³²P]dCTP (0.3 μ Ci), 25 mM HEPES–KOH (pH 7.5), 4 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, 100 μ M aphidicolin, and the indicated amounts of NFIII (pKB67-88 DNA-cellulose fraction; 0.05 mg of protein per ml). Reaction products were analyzed by electrophoresis in a 1% agarose gel in the presence of 0.1% SDS and by autoradiography of the dried gel.

NFIII purification. NFIII was purified from a HeLa cell nuclear extract. Extraction of the nuclei and the first purification steps (DEAE-cellulose and phosphocellulose column chromatography) were performed as previously described (30). NFIII-containing fractions were further purified by sequence-specific DNA affinity chromatography on pKB67-88 DNA-cellulose as described by Rosenfeld and Kelly (35). After the column was washed with buffer B (30) containing 0.2 M NaCl, NFIII was eluted with 0.5 M NaCl in buffer B. Finally, the active fractions were chromatographed on a denatured calf thymus DNA-cellulose column. This column was washed with 0.1 M NaCl followed by step elutions at 0.35 M NaCl and 1.0 M NaCl. NFIII eluted with the 0.35 M NaCl step. During the entire purification procedure, NFIII was monitored by its stimulatory activity of adenovirus DNA replication in vitro. A 95-kilodalton (kDa) protein co-eluted with the replication-stimulatory and DNA-binding activities and was shown, by renaturation after SDS-polyacrylamide gel electrophoresis, to be responsible for DNA binding (G. J. M. Pruijn and P. C. van der Vliet, manuscript in preparation).

Densitometry and computer graphics. The results of protection and interference experiments were quantitated by densitometric scanning of the autoradiograms with a chromatogram spectrophotometer (model KM-3; Zeiss). Levels of protection were determined by using the formula (band intensity in the presence of NFIII)/(band intensity in the absence of NFIII) normalized for regions outside the protected area.

Ethylation interference levels were calculated with the formula $100\% \times [(\% \text{ unbound experimental} - \% \text{ unbound average}) / (100\% - \% \text{ unbound average})]$. Methylation interference levels were calculated by taking the average of $(100\% - \% \text{ bound})$ and $\% \text{ unbound}$.

Three-dimensional representations of the NFIII- and NFII-binding sites of Ad2 were generated on a picture system (PS300; Evans & Sutherland) by using the MOGLI program. The bp 31 to 54 or 20 to 54 of Ad2 were visualized according to the coordinates of idealized B-DNA. Van der Waals surfaces of phosphate and base contact points were displayed as dotted spheres.

RESULTS

Borders of NFIII-binding site in Ad2 origin. The DNase I footprint of NFIII on the Ad2 origin partially overlaps the NFII footprint (30, 36). In order to determine the borders of

FIG. 1. MPE · Fe(II) footprints of NFIII on the Ad2 origin. The 331-bp *NdeI-XbaI* fragment of pHRI, labeled at either the 3' (top) or the 5' (bottom) end of the *XbaI* site, was briefly digested with MPE · Fe(II) in the absence (–) or presence (+) of NFIII (pKB67-88 DNA-cellulose fraction). Products were electrophoresed on an 8% polyacrylamide gel in parallel with an A+G sequencing reaction (M). Numbers indicate position in the Ad2 ITR.

the NFIII-binding site more accurately, we performed MPE · Fe(II) footprinting with NFIII. The use of MPE · Fe(II) instead of DNase I as cleaving agent has two major advantages. (i) In contrast to DNase I, cleavage by MPE · Fe(II) occurs much more randomly, and (ii) MPE · Fe(II) is smaller than DNase I and consequently artifacts caused by steric hindrance are less likely to occur. Thus, MPE · Fe(II) footprinting delimits the binding site more accurately than DNase I footprinting (18, 37).

The end-labeled 331-bp Ad2 origin-containing *NdeI-XbaI* fragment of pHRI was incubated with NFIII, cleaved with MPE · Fe(II), and analyzed on a denaturing polyacrylamide gel (Fig. 1). Densitometer scanning of the autoradiogram revealed a gradual increase of protection at the borders of the binding site. NFIII protects 11 to 13 nucleotides in both strands more than 50% against cleavage by MPE · Fe(II) (see Fig. 5A), while 20 phosphate bonds were protected against cleavage by DNase I (30). If we define the binding site as the region protected more than 50% against MPE · Fe(II) cleavage, the NFIII-binding site extends from position 37 to position 51. This agrees well with results obtained by the analysis of deletion mutants spanning the left border (see Table 1).

NFIII contact points within Ad2 origin-binding site. To identify contact points of NFIII with its recognition sequence, we employed hydroxyl radical footprinting and chemical modification of DNA by alkylating reagents.

FIG. 2. Hydroxyl radical footprints of NFIII on the Ad2 origin. Labeled DNA fragments, as described in the legend to Fig. 1, were digested by hydroxyl radicals for 4 min after incubation with 4 or 8 μ l or without (–) NFIII (pKB67-88 DNA-cellulose fraction). Products were analyzed on an 8% polyacrylamide gel in parallel with an A+G sequencing reaction (M). Numbers indicate position in the Ad2 ITR.

Hydroxyl radical footprinting. MPE · Fe(II) strand scission is probably mediated by the production of hydroxyl radicals which induce oxidative degradation of the deoxyribose ring. Recently, another footprinting technique has been developed on the basis of the same reaction among hydroxyl radicals and DNA, designated hydroxyl radical footprinting (49). With this technique, hydroxyl radicals generated by the reduction of hydrogen peroxide by iron(II) cleave the DNA strand unless the protein is in close contact with the sugar ring in the DNA backbone. Figure 2 shows the hydroxyl radical footprint of NFIII on the Ad2 origin. Densitometric scanning revealed that two regions on both strands were partially protected by NFIII. The most strongly protected region coincided with the center of the MPE · Fe(II) footprint (see Fig. 5A), whereas a smaller, weakly protected region was detected around position 38.

Ethylation interference. To detect phosphate contacts between NFIII and the DNA backbone, an end-labeled Ad2

origin-containing DNA fragment was partially ethylated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide under conditions in which less than one phosphate group per 200 bp is modified. After binding to NFIII and separation of protein-bound and free DNA by filtration over nitrocellulose, the DNA was cleaved at the sites of ethylation and analyzed on a denaturing polyacrylamide gel (Fig. 3). Bands that were relatively weak in the filter-bound DNA pattern and predominant in the filtrate DNA pattern represent positions of ethylated phosphate groups that interfere with binding of NFIII (Fig. 3A). The percentage of interference is plotted in a histogram (Fig. 3B) which illustrates that ethylation of some phosphates almost completely prevents binding of NFIII while ethylation at other positions only partially interferes. More than 50% interference was observed after ethylation of the phosphate 5' from position 39, 44, 45, 46, or 47 in the top strand and 5' from position 48 or 49 in the bottom strand. A 25 to 50% level of interference was observed after ethylation of the phosphate 5' from position 41, 44, 45, or 47 in the bottom strand. The positions of phosphate contacts are shown in Fig. 5A.

The differences in interference might be explained by differential effects on NFIII binding of ethylation at either of the two free oxygen atoms of the phosphate or by variable binding modes between the phosphate group and the amino acid side chains involved in backbone binding.

Methylation interference. Dimethyl sulfate methylates guanine residues at the N7 position in the major groove and adenine residues at the N3 position in the minor groove of the B-DNA helix. The DNA can be specifically cleaved at methylated residues by chemical treatment. This procedure provides an approach to identify G and A contacts of a (sequence-specific) DNA-binding protein since methylation of certain residues might interfere with binding. Partially methylated Ad2 origin-containing DNA fragments were incubated with NFIII, and protein-bound DNA fragments were separated from free DNA by filtration over nitrocellulose filters. After cleavage at the modified residues, filter-bound (protein-bound) and filtrate (free) DNA were analyzed on a sequencing gel (Fig. 4). Methylation of the G at position 42 and of the A's at positions 43, 45, and 46 in the top strand and at positions 44 and 47 in the bottom strand shows relatively strong interference (30 to 60%) with binding of NFIII. The histogram displaying the percentages of interference shows that about 20% interference is observed for methylation of the A residues at positions 39, 40, and 41. Methylation of all other purines did not significantly interfere with NFIII binding. Since the probe was not completely saturated with protein, a small percentage of control DNA bands is present in the f lanes. These methylation-interference experiments indicate that both major and minor groove contacts are involved in the interaction between NFIII and the Ad2 origin in contrast to the NFII-Ad2 interaction for which only major groove contacts were observed (10).

NFIII-binding site in Ad2 origin. The results of all protection and interference experiments are summarized in Fig. 5A. For the top strand, hydroxyl radical footprinting detected contacts at all positions in which phosphate contacts were found, while in the bottom strand additional phosphate contacts were observed at positions 48 and 49.

The spatial pattern of the contacts identified in the NFIII-binding site on the Ad2 origin is shown in computer-generated stereographics (Fig. 5B). Contact points are indicated as orange (N7 of G residue, N3 of A residue) and green (O of phosphate) spheres and as purple deoxyribose rings. This pattern illustrates that NFIII contact points are scattered

FIG. 3. Ethylation interference analysis of the NFIII-binding site. (A) Partially ethylated DNA fragments (described in the legend to Fig. 1) were incubated with NFIII and separated in a protein-bound (lane b) and a free (lane f) fraction by filtration over nitrocellulose. After cleavage at ethylated positions, both fractions were analyzed on an 8% polyacrylamide gel in parallel with the cleaved input DNA fragment (lane c) and an A+G sequencing marker (M). Positions interfering for more than 25% when ethylated are indicated. Numbers correspond to position in the Ad2 ITR. (B) Quantitation of ethylation interference. Levels of interference were determined after densitometric scanning of the autoradiogram and are plotted in a histogram for the Ad2 sequence between positions 31 and 56.

FIG. 4. Methylation interference analysis of the NFIII-binding site. (A) Partially methylated DNA fragments (described in the legend to Fig. 1) were incubated with NFIII and separated in a protein-bound (lane b) and a free (lane f) fraction by filtration over nitrocellulose. After cleavage at methylated positions, these fractions were analyzed on an 8% polyacrylamide gel in parallel with the cleaved input DNA fragment (lane c). Residues that interfered more than 10% when methylated are indicated. Numbers correspond to position in the Ad2 ITR. (B) Quantitation of methylation interference. Levels of interference were determined by densitometric scanning of the autoradiogram and are plotted in a histogram for the purines between positions 31 and 56 in the Ad2 sequence.

over the recognition sequence and not confined to one side of the helix, which is even more evident from a view down the axis of the helix (Fig. 5C). Previously, the NF1-binding site on the Ad2 origin was analyzed in a similar way (10). Since the binding sites for NF1 and NFIII on the Ad2 origin are so closely spaced, we also generated a stereograph in which all known contact points for both NF1 (phosphate contacts are purple, base contacts are yellow) and NFIII (phosphate contacts are green, base contacts are orange) were highlighted (Fig. 5D). This picture emphasizes the slight overlap of the binding sites for these two proteins on the Ad2 origin. Remarkably, the two bases of bp 39 and the two phosphate groups in each strand next to bp 39 contact both proteins. Also (weak) deoxyribose contacts for NFIII were detected very close to NF1 phosphate contacts at positions 36 and 37. It should be stressed that these contact point analyses were performed for both proteins independently and thus do not exclude the possibility that changes occur when both proteins are bound simultaneously.

Interaction of NFIII with origin of other adenovirus serotypes. We compared the NFIII-binding sites of a number of different adenovirus serotypes containing small differences within the NFIII-binding region. Gel retardation competition experiments were carried out with an end-labeled 85-bp *EcoRI-XbaI* fragment of p4A85Δ containing the Ad4 origin as probe. Without specific competitor DNA, a single retarded band is observed after incubation with NFIII, which represents a specific NFIII-DNA complex (31) (Fig. 6). Origin-containing fragments of about 400 bp, either from

the human serotypes Ad2, Ad4, Ad12, Ad40, or Ad41 or from the simian serotype SA7, were used as competitors. Figure 6 shows the results for the Ad2, Ad4, and Ad12 competitors. Relative binding affinities were calculated from the molar concentration of competitor DNA that resulted in 50% competition (Table 1). In agreement with previous results (31), the affinity for the Ad4 origin is higher than that for the Ad2 origin. NFIII also binds stronger to the SA7-binding site, which contains only one point mutation compared with the Ad2-binding site (23), whereas the affinity for Ad12 is lower. The NFIII-binding sequence of Ad40 and Ad41 deviates from Ad2 only at the right border of the binding site. Similar binding affinities were observed for these origins. The relative binding affinities for two control DNA (pUC) fragments were less than 0.2% (31).

Interaction of NFIII with mutated Ad2-binding sites. The very similar affinities of NFIII for Ad2, Ad40, and Ad41 suggests that nucleotides beyond position 50 of Ad2 are not important for NFIII binding, thereby defining a right border to the binding site. The presence of several deletion mutants deleting the NFIII-binding sequence from the left enabled us to obtain information about the left border. Fragments were isolated from deletion mutants pHRΔ40, pHRΔ41, and pHRΔ43, which contain the Ad2 ITR starting at position 40, 41, and 43, respectively. The results in Table 1 show that mutation of nucleotides before position 40 of Ad2 reduced the binding affinity more than threefold. Mutation of one additional nucleotide of the recognition sequence (pHRΔ41) resulted in a further twofold decrease in affinity. Surprisingly

FIG. 5. (A) Summary of footprinting and alkylation interference assays of the NFIII-binding site in the Ad2 ITR. Results for top-strand analyses are shown above the sequence; results for bottom-strand analyses are shown below. DNase I footprints (30) are indicated by large brackets. Levels of protection (0 to 100%) by bound NFIII from cleavage by MPE · Fe(II) are indicated by the histogram silhouette. Bases whose deoxyribose is protected from attack by hydroxyl radicals are indicated by arrows; the length of the arrow indicates the level of protection by NFIII. Arrowheads indicate phosphate groups whose ethylation interferes more than 25% with NFIII binding. Bases whose methylation interferes more than 10% with NFIII binding are in bold type. (B) Computer-generated stereographic side view of the NFIII-binding site. The Van der Waals radii of G-N7, A-N3 (both orange) and phosphate-free oxygen (green) atoms in contact with NFIII are shown as spheres; protected deoxyribose rings (hydroxyl radical footprinting) are shown as purple pentagons. In panel C, the helix is tilted 90° to obtain a view down the axis of the helix. (D) Stereographic side view of both the NFII- (phosphate contacts, purple; base contacts, yellow) and the NFIII (phosphate contacts, green; base contacts, orange)-binding sites. In panels B and D, bp 54 of Ad2 is the top base pair, and in panel C, bp 31 is the front base pair.

FIG. 6. Gel retardation competition assay for Ad2, Ad4, and Ad12. An end-labeled 85-bp *EcoRI-XbaI* fragment containing the Ad4 origin was incubated with NFIII in the presence of increasing amounts of unlabeled competitor DNA, and the resulting complexes were analyzed on a nondenaturing polyacrylamide gel. Lanes: 1, no competitor; 2 to 5, 16, 32, 64, and 128 ng of a 409-bp fragment containing the Ad2 origin; 6 to 9, 4, 8, 16, and 32 ng of a 384-bp fragment containing the Ad4 origin; 10 to 13, 32, 64, 128, and 256 ng of a 400-bp fragment containing the Ad12 origin. B and F indicate the positions of the DNA-protein complex and of the free DNA fragment in the gel, respectively. The arrow marks the start point of migration.

however, pHRΔ43 binds somewhat stronger than pHRΔ41. This might be explained by the restoration of the original sequence from position 37 to 39 by the ligated *EcoRI* linker. In this region, backbone contact points with the protein were detected (Fig. 5A). Moreover, the presence of two consecutive A residues in front of T-39 coincided with high binding affinities when cellular NFIII-binding sites were compared (31).

Finally, the relative binding affinity for the point mutant Xpm46G was 10-fold reduced. This A→G transversion was previously shown to prevent stimulation of DNA replication by NFIII (30).

Stimulation of DNA replication of Ad2, Ad4, and Ad12 by NFIII. The previously reported differences in the level of

stimulation of DNA replication of Ad2 and Ad4 by NFIII (31) and the results obtained for the point mutant Xpm46G (30) suggested that stimulation of adenovirus DNA replication by a fixed amount of NFIII is directly related to the affinity of NFIII for the adenovirus origin. In order to investigate this more systematically, we determined the levels of stimulation of Ad2, Ad4, and Ad12 DNA replication in vitro by increasing amounts of NFIII. Using terminal protein-free plasmid DNA as template, initiation in vitro only takes place when the origin is exposed at the end of a DNA molecule (48, 50). Therefore, plasmids pHRI and p4A85Δ were digested with *EcoRI* and *AvaII* and pAd12-RIC1 was digested with *EcoRI* and *AvaI* to yield origin-containing fragments of 1,528, 1,503, and 1,642 bp, respectively. Equimolar amounts of origin-containing fragments were incubated with the purified viral replication factors (Ad5 DNA-binding protein, Ad5 precursor terminal protein-polymerase complex) in the presence of deoxynucleoside triphosphates. NFI was left out of the reaction mixtures since the Ad4 origin lacks an NFI-binding site. Previous results showed that NFIII alone can stimulate Ad2 DNA replication (31). Replication products were visualized by electrophoresis of the reaction mixture in an SDS-containing agarose gel followed by autoradiography. Due to the reduced mobility of primary replication products which are covalently attached to the primer pTP, a separation from input DNA fragments was obtained. The results in Fig. 7 show that the maximal level of stimulation by NFIII is dependent on the affinity of NFIII for the adenovirus origin. A saturating amount of NFIII stimulated Ad2 replication 5-fold, Ad4 replication 10-fold, and Ad12 replication 2-fold. With the Ad4 template, a faster-migrating band was observed, which presumably represents single-stranded DNA originating from multiple rounds of replication, thus indicating a high level of replication (19, 51).

DISCUSSION

Our detailed study of the NFIII-binding site on the Ad2 genome has defined the binding borders as well as many of the possible contacts that NFIII makes with its recognition sequence. MPE · Fe(II) footprinting delimited the binding site between positions 37 and 51 in the Ad2 ITR. The previously derived consensus sequence for optimal NFIII binding, 5'-TATGCAAAT-3' (31), corresponds to the center of the MPE · Fe(II) footprint, taking into account the two

TABLE 1. Adenovirus NFIII-binding site sequences and their relative binding affinities

Virus or mutant		NFIII recognition sequence ^a																Relative binding affinity (%) ^b
		<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> 40° ↓ </div> <div style="text-align: center;"> 50° ↓ </div> </div>																
Ad2	C	C	A	A	T	A	T	G	A	T	A	A	T	G	A	G	G	100
Ad4	T	T	C	A	T	A	T	G	c	A	A	A	T	A	A	G	G	250
SA7	C	C	A	A	T	A	T	G	c	T	A	A	T	G	A	G	T	200
Ad12	C	C	A	A	T	A	T	T	A	A	A	A	T	G	A	A	G	55
Ad40	C	C	A	A	T	A	T	G	A	T	A	A	T	G	A	G	G	83
Ad41	C	C	A	A	T	A	T	G	A	T	A	A	T	G	A	G	T	83
pHRΔ40	A	T	T	C	C	A	T	G	A	T	A	A	T	G	A	G	G	30
pHRΔ41	A	A	T	T	C	C	T	G	A	T	A	A	T	G	A	G	G	15
pHRΔ43	G	G	A	A	T	T	C	C	A	T	A	A	T	G	A	G	G	25
Xpm46G	C	C	A	A	T	A	T	G	A	T	A	G	T	G	A	G	G	10

^a Nucleotides differing from the Ad2 sequence are indicated in small capitals.

^b Calculated from the molar ratio of competitor DNA to probe at 50% competition.

^c Numbers correspond to position in the Ad2 ITR.

FIG. 7. Stimulation of Ad2, Ad4, and Ad12 DNA replication in vitro by NFIII. Ad2, Ad4, and Ad12 origin-containing plasmids were digested with restriction enzymes to yield origin-containing fragments (o) of 1,528, 1,503, and 1,642 bp, respectively, which were incubated in a reconstituted DNA replication system (see Materials and Methods) in the absence (lanes 1, 7, and 13) or presence of 31 nl (lanes 2, 8, and 14), 62 nl (lanes 3, 9, and 15), 125 nl (lanes 4, 10, and 16), 250 nl (lanes 5, 11, and 17), or 500 nl (lanes 6, 12, and 18) of NFIII (pKB67-88 DNA-cellulose fraction). Due to the protein-priming mechanism of adenovirus DNA replication, replication products are covalently attached to pTP, leading to a reduced electrophoretic mobility (arrows). The faster-migrating replication product with Ad4 (s) presumably represents single-stranded DNA originating from multiple rounds of replication.

mutations (CA $\rightarrow$ AT) that have occurred in the Ad2 sequence compared with the optimal binding sequence present in Ad4.

Both at the left- and right-hand side of this consensus sequence, base pairs modulate the relative binding affinity as observed from the mutant studies described here and elsewhere (31). At the left-hand side, two consecutive A residues improve binding, as indicated by the affinity for immunoglobulin heavy and light chain promoters (31) as well as for deletion mutant pHR Δ 43. At the right-hand side, 4 additional bp are strongly protected against MPE $\cdot$ Fe(II) cleavage. It should be noted that one of these is an AT base pair (position 49), which appears to be highly conserved among immunoglobulin promoters, a reason why the conserved box in this case was preferably indicated as a decanucleotide (11) instead of an octanucleotide. Recent competition experiments with synthetic oligonucleotides indicated that a mutation of this particular base pair slightly lowers the binding affinity of NFIII (26a). The affinity of Ad41 suggests that position 51 can be mutated without alteration of the affinity.

Contact point analysis revealed many base contacts. Interference with NFIII binding in alkylation interference experiments is most likely due to steric hindrance by the alkyl groups or to alteration of the charge distribution on the alkylated part of the DNA. Therefore, a close interaction of the protein with the site of alkylation is required for interference; consequently, these sites are denoted contact points. Strikingly, all A and G residues corresponding to the previously derived consensus sequence (positions 39 to 47) appeared to interfere to some extent with NFIII binding when methylated, albeit to various extents. Some of these contacts had been detected previously by methylation protection, i.e., the G residue at position 42 in the top strand and the A residues at positions 44 and 47 in the bottom strand (30, 31). The results of the contact point analysis summa-

rized in the computer-generated stereographic representations shown in Fig. 5 clearly demonstrate that contact points were identified at all sides of the DNA helix and indicate that NFIII penetrates both the major and minor groove of the DNA helix.

The hydroxyl radical footprinting and ethylation interference experiments confirm that the right part of the consensus sequence (positions 42 to 47) represents the core of the binding site. Nevertheless, additional contacts outside the core were identified. Around position 49, contacted phosphates whose ethylation strongly interfered with the binding of NFIII were observed. Around position 38, a second area of backbone contacts was observed which confirm the importance of this region.

Closely spaced binding sites for NFI and NFIII. The summary of NFI and NFIII contacts (Fig. 5D) indicates, in addition to the pronounced difference between the two binding modes, that NFI and NFIII bind very close to each other. In particular, at position 39, a T-base contact (C5, major groove) with NFI is observed (10), while at this position not only an A-base contact (complementary strand; N3, minor groove) but also a phosphate backbone contact with NFIII was found. The importance of this AT-base pair both for NFI and NFIII binding is also apparent from deletion studies. A mutant containing only the first 38 bp is defective for NFI binding (25), whereas bp 39 is also important for optimal NFIII binding as shown by analysis of pHR Δ 40 and pHR Δ 41 (Table 1). Despite this close spacing, NFI and NFIII are able to bind simultaneously to the same DNA molecule and do not exclude each other (30). The close spacing raises the possibility of protein-protein interactions which could improve the binding by a cooperative effect. Thus far we have obtained no indications for strong cooperativity by comparing the binding affinity of NFI in the presence and absence of NFIII and vice versa, but more

stringent analyses with higher concentrations of purified proteins may be required to establish this point. Presently, we are investigating whether alteration of the position and orientation of the NFIII-binding site will lead to any change in binding or, more importantly, to the functioning of NFIII in DNA replication. It should be stressed that in transcription regulatory elements, NFIII sites are present in both orientations and at various positions relative to the transcription initiation site.

Comparison with other octamer-binding proteins. Several reports describe the identification of octamer/decanucleotide-binding activities in crude nuclear extracts of various cell types (2-4, 7, 20, 21, 24, 26, 34, 39, 43-45). Recently, the purification of four octamer/decanucleotide-binding transcription factors has been described (12, 29, 38, 47). OTF-1 and OBP100 were purified from HeLa nuclear extracts, whereas the B-cell-specific factor OTF-2 was purified from Namalwa cell nuclear extracts. OTF-1, which stimulates transcription of a human histone H2B gene, was identified as a 90-kDa protein; OBP100 was identified as a 100-kDa protein. The similar molecular sizes (NFIII was identified as a 92-kDa protein (29) or as a 95-kDa protein; Pruijn and van der Vliet, in preparation) and DNA-binding specificities raise the possibility that NFIII, OTF-1, and OBP100 are identical proteins. A strong indication that at the least they share identical DNA-binding domains comes from a comparison of methylation interference experiments and effects of mutations within binding sites. The methylation interference results for NF-A1, NF-A2, oct-B1, oct-B2, OTF-1, OTF-2, and OBP100 mostly coincide with the methylation interference/protection results for NFIII (30, 31, 34, 38, 47). Furthermore, the reduction of binding affinity by a number of mutations within the binding sites of these proteins reflects the observed effects of similar mutations on NFIII binding (3, 4, 34, 44, 45). For instance, a double point mutant (oct - a) of an H2B promoter octamer, containing two transversions comparable to those of Xpm46G and Ad12 together, lost the ability to compete for binding of OTF-1 (crude extract) to the wild-type H2B promoter (44).

The results of competition experiments performed by Rosales et al. (34) for binding of HeLa oct-B1 to the octamer motif of the SV40 enhancer, however, only very marginally correspond to our results (31). The high binding affinity for the Ad2 NFIII-binding site compared with sequences containing a complete match with our consensus sequence is especially surprising. At present, the only plausible explanation for these discrepancies is the assumption that crude extracts and (partially) purified proteins behave differently in competition assays. Recent competition experiments comparing the binding to the octamer/decanucleotide- and ACCC-containing motifs of a κ light chain promoter of factors present in a crude Raji nuclear extract with the binding of a partially purified NFIII preparation and a highly purified NFIII preparation strengthen the existence of such a phenomenon. In these studies, an ACCC-containing oligonucleotide competed for octamer/decanucleotide binding of a partially purified NFIII preparation which was shown to contain a protein that interacts with the ACCC-containing sequence by footprinting techniques. The same oligonucleotide, however, did not compete for octamer/decanucleotide binding of highly purified NFIII (26a).

Stimulation of adenovirus DNA replication by NFIII. The replication assays for Ad2, Ad4, and Ad12 showed that the level of stimulation by NFIII was dependent on the affinity of NFIII for the origin. Even with high concentrations of NFIII, stimulation of Ad2 and Ad12 replication did not reach

the level of Ad4, suggesting that NFIII stimulation is not merely related to occupation of the binding site but also to the strength of the interaction with the binding site. Alternatively, sequence differences outside the NFIII-binding site might affect the activity of NFIII or give rise to qualitatively different interactions among NFIII and other replication proteins. In agreement with the former hypothesis, the lack of stimulation of replication of the Ad2 point mutant Xpm46G (30) coincides with a low relative binding affinity. The results obtained with the in vitro DNA replication system do not necessarily mean that similar differences exist in vivo. However, since the origins of adenovirus serotypes containing a strong NFIII-binding site lack an NF1-binding site (e.g., Ad4), it is very attractive to assume that these adenoviruses require a strong NFIII-binding site and consequently that their DNA replication is stimulated strongly by NFIII.

At present, the mechanism of action of NFIII in DNA replication is unclear. The protein might change the DNA structure either at its binding site or at the replication initiation site in a manner that favors the initiation reaction. Alternatively, NFIII might interact (when bound to the DNA) with other replication factors by direct protein-protein interaction, thus promoting the formation of an initiation complex.

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