JOURNAL OF VIROLOGY, Nov. 1990, p. 5510–5518
0022-538X/90/115510-09$02.00/0

Vol. 64, No. 11

Transcription Factors NFI and NFIII/oct-1 Function Independently, Employing Different Mechanisms To Enhance Adenovirus DNA Replication

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Initiation of adenovirus DNA replication is strongly enhanced by two transcription factors, nuclear factor I (NFI) and nuclear factor III (NFIII/oct-1). These proteins bind to two closely spaced recognition sequences in the origin. We produced NFI and NFIII/oct-1, as well as their biologically active, replication-competent DNA-binding domains (NFI-BD and the POU domain), in a vaccinia virus expression system and purified these polypeptides to apparent homogeneity. By DNase I footprinting and gel retardation, we show that the two proteins, as well as their purified DNA-binding domains, bind independently and without cooperative effects to their recognition sequences. By using a reconstituted system consisting of the purified viral proteins (precursor terminal protein-DNA polymerase complex (pTP-pol) and DNA-binding protein, we show that NFIII/oct-1 or the POU domain stimulates DNA replication in the absence of NFI or NFI-BD and vice versa. When added together, the enhancing effect of the two transcription factors was independent and nonsynergistic. Interestingly, stimulation by NFI or NFI-BD was strongly dependent on the concentration of the pTP-pol complex. At low pTP-pol concentrations, NFI or NFI-BD stimulated up to 50-fold, while at high concentrations, the stimulation was less than twofold, indicating that the need for NFI can be overcome by high pTP-pol concentrations. In contrast, stimulation by NFIII/oct-1 or the POU domain was much less dependent on the pTP-pol concentration. These data support a model in which NFI enhances initiation through an interaction with pTP-pol. Glutaraldehyde cross-linking experiments indicate contacts between pTP-pol and NFI but not NFIII/oct-1. The site of interaction is located in the NFI-BD domain.

Control of gene expression requires the binding of several different proteins to specific sites present in promoter and enhancer regions. These binding sites are often closely spaced or even partially overlapping, and interactions between bound proteins are supposed to play a role in transcription activation. Such transcription regulatory elements are also found in the replication origins of several eucaryotic DNA viruses (papovavirus, bovine papillomavirus, and Epstein-Barr virus), where they function as auxiliary regions enhancing initiation (5). Binding of cellular transcription factors to these elements is clearly documented for adenovirus DNA replication. This system can be studied in vitro by using purified proteins, enabling a detailed study of the effects of transcription factors on DNA replication.

The origins of adenovirus types 2 and 5 (Ad2 and Ad5) consist of a core region and an auxiliary region which enhances initiation up to 100-fold, both in vivo and in vitro (2, 15, 37). This region, encompassing nucleotides 25 to 50, binds two proteins, nuclear factor I (NFI) (25), and nuclear factor III (NFIII/oct-1) (31, 34). NFI is identical to the CCAAT transcription factor CTF (16) and consists of a family of related proteins with a molecular mass of 52 to 65 kilodaltons. NFI binds as a dimer to its partially symmetric recognition sequence in Ad2, ^{25}TGGATTGAAGCCAA^{39} (see Fig. 2). Analysis of the contacts between NFI and the Ad2 origin revealed that NFI binds to the major groove of DNA and that almost all contacts are confined to one side of the DNA helix (7). NFIII/oct-1 is a 90- to 95-kilodalton protein identical to the octamer-binding transcription factor OTF-I or oct-1 (27, 30, 44a). NFIII/oct-1 binds next to NFI to the sequence ^{40}ATGATAATGA^{49} (31). In contrast to NFI, NFIII/oct-1 has contacts at both sides of the helix, both in the major and the minor grooves (33).

The cDNAs for both proteins have been cloned and sequenced. In agreement with the presence of a family of related proteins, several different NFI/CTF cDNAs were detected, presumably originating from alternative splicing of one or possibly two genes (22, 28, 35). Expression of different domains of either human or rat cDNAs by using a bacterial system or recombinant vaccinia virus has indicated that the conserved N-terminal region, encompassing the DNA-binding domain, is sufficient for dimerization as well as for stimulation of adenovirus DNA replication (13, 23). This domain is distinct from the transcription activation domain.

NFIII/oct-1 is involved in transactivation of several cellular genes like the histone H2B or small nuclear RNA genes (9, 21). Expression of truncated forms of the oct-1 gene or the related oct-2 gene in recombinant vaccinia viruses has indicated that similar to NFI, the DNA-binding region (POU domain), distinct from the transcription activation domain, is sufficient for enhancement of adenovirus DNA replication (41, 42, 44a). The observation that in both cases a DNA-binding core domain suffices for DNA replication and the proximity of both binding sites raises the question of whether NFI and NFIII/oct-1 stimulate DNA replication in a similar way and whether there is any synergism between these two proteins. In previous studies, there has been some uncertainty as to whether NFIII/oct-1 can stimulate adenovirus DNA replication in the absence of NFI or other cellular proteins (32, 33, 34). Moreover, for NFI, levels of stimulation varying between 5- and 30-fold have been reported (1, 6, 46). To resolve these ambiguities, we purified the POU domain and the N-terminal region of NFI (NFI-BD) to

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apparent homogeneity and assayed their role in DNA binding and DNA replication in a well-defined reconstituted system. We demonstrate that the two proteins stimulate replication independently and that the level of stimulation, in particular by NFI, is dependent on the precursor terminal protein-DNA polymerase complex (pTP-pol) concentration. Moreover, we provide evidence for an interaction between pTP-pol and NFI-BD.

MATERIALS AND METHODS

Recombinant vaccinia viruses. The preparation of vaccinia viruses containing the Ad2 pTP and pol genes, respectively, has been described previously (39). To prepare vaccinia virus containing the oct-1 cDNA, the 2.5-kilobase-long *Fnu*DII-*Hin*dIII fragment from pBSoct1$^+$ (40) containing the 743-amino-acids-long oct-1 open reading frame was end repaired and ligated into the filled-in *Eco*RI site of pATA-18, a recombination vector containing the vaccinia virus 11K late promoter (39). For the POU domain, we first constructed mutant 1-23/269-743 by digesting pBSoct-1$^+$ΔBH (41) with *Fnu*dII-*Hin*dIII and ligating in pATA-18. This plasmid was linearized with PflMI, end repaired with T4 DNA polymerase, and cloned into pATA-18-STOP, linearized with *Cla*I and *Bal*I. pATA-18-STOP is a pATA-18 derivative with stop codons in three reading frames directly downstream of a *Bal*I site in the polylinker. This leads to a construct (POU) encoding amino acids 1 to 23 and 269 to 440 to which TIAE was added C terminally. These plasmids were used to prepare recombinant vaccinia viruses as described previously (39). Recombinant vaccinia viruses containing intact rat NFI/CTF cDNA or the N-terminal DNA-binding domain NFI-BD containing amino acids 4 to 240 were prepared as described previously (13).

Purification of replication proteins. The purification of the pTP-pol complex from recombinant vaccinia virus-infected HeLa cells was as described previously (24). One unit (U) is defined as the incorporation of 1 nmol of [α-^{32}P]dCTP (2.5 Ci/mmol) into acid-insoluble material in 60 min at 30°C by using activated calf thymus DNA as a template. This corresponds to the incorporation of 20 pmol of [α-^{32}P]dCTP (5.5 Ci/mmol) into adenovirus DNA under standard replication conditions by using *Xho*I-digested Ad5 DNA-TP as a template, with crude HeLa nuclear extracts as a source of NFI and NFIII/oct-1. Note that the levels can be at least 10-fold higher in the presence of purified nuclear factors under optimal conditions (see Results). One unit equalizes 1 μg of pTP-pol, as determined by silver staining.

For purification of the POU domain, nuclear extracts were prepared from 3×10^9 HeLa cells 24 h after infection with 5 PFU per cell of vaccinia virus containing the POU domain. The cells were washed twice with phosphate-buffered saline containing 0.5 mM $MgCl_2$ and suspended in 30 ml of hypotonic buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40). After swelling, the cells were Dounce homogenized and centrifuged at low speed. The nuclei were resuspended in 10 ml of the same buffer containing 0.3 M NaCl, extracted for 60 min on ice, and centrifuged at low speed. The supernatant was centrifuged at 100,000 × *g* for 30 min and adjusted to 100 mM NaCl with buffer A (25 mM Tris hydrochloride [pH 8.0], 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 0.02% Nonidet P-40, 20% glycerol). The nuclear extract was applied to a 40-ml DEAE Sephacel column equilibrated with buffer A containing 100 mM NaCl. The flowthrough plus 40-ml wash were applied to an 18-ml fast flow S column equilibrated with buffer B (25 mM HEPES-KOH [pH 8.0], 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% Nonidet P-40, 20% glycerol) containing 100 mM NaCl. The column was washed with 90 ml of buffer B-100 mM NaCl and eluted with a 120-ml linear gradient of 100 to 400 mM NaCl. Fractions between 210 and 230 mM NaCl, containing the POU domain, were pooled, diluted to 30 mM NaCl, and loaded onto a 20-ml fast flow Q column equilibrated with buffer A-30 mM NaCl. The flowthrough plus 20-ml wash were loaded onto a 9-ml heparin Sepharose column equilibrated with buffer B-30 mM NaCl. The column was washed with 50 ml of buffer B-100 mM NaCl and eluted with a 54-ml linear gradient of 100 to 600 mM NaCl. POU-containing fractions (290 to 310 mM NaCl) were pooled, diluted to 100 mM, and applied to a 5-ml single-stranded DNA cellulose column equilibrated with buffer B-100 mM NaCl. The column was washed with 100 ml of buffer B-100 mM NaCl followed by a stepwise elution with B-400 mM NaCl, upon which the POU domain eluted.

For purification of NFI-BD, nuclear extracts were prepared from 2.5×10^9 cells as described above and applied to a 30-ml DEAE Sephacel column equilibrated with buffer A-300 mM NaCl. The flowthrough and 30-ml wash were loaded onto an 18-ml heparin Sepharose column, washed with 100 ml of buffer A-350 mM NaCl, and developed with a 100-ml linear gradient containing 350 to 1,000 mM NaCl. NFI-BD-containing fractions, eluting around 575 mM NaCl, were pooled, diluted with buffer A to 200 mM NaCl, and loaded onto a 1-ml mono S column (Pharmacia) in buffer C (50 mM Bicine [pH 8.7], 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% Nonidet P-40, 20% glycerol) containing 200 mM NaCl. After washing with 10 ml of C-200 mM NaCl, NFI-BD was eluted by using a linear 200 to 600 mM NaCl gradient in buffer C and peaked around 450 mM NaCl. The activity of NFI-BD and the POU domain was monitored by gel retardation. All procedures were performed on ice or at 4°C.

DNA-binding protein (DBP) was purified from Ad5-infected HeLa cells (44). The purification of NFI and NFIII/oct-1 has been described previously (25, 30).

DNase I footprinting. The 338-base-pair (bp) *Acc*I-*Nde*I fragment of pHRI containing the Ad2 origin (33) was 5′-end labeled at the *Acc*I site by polynucleotide kinase to label the bottom strand. For the top strand, the 332-bp *Xba*I-*Nde*I fragment labeled at the *Xba*I site with DNA polymerase I (Klenow fragment) was used. Footprinting was performed by incubating 3 fmol of the end-labeled DNA with various amounts of the purified POU domain or NFI-BD polypeptides in the presence of 100 ng of poly(dI-dC)-poly(dI-dC) in a total volume of 50 μl containing 20 mM HEPES-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, 0.018% Nonidet P-40, 100 mM NaCl, and 1 μg of bovine serum albumin for 30 min at 25°C. Incubation with DNase I and further processing were as described previously (32).

Gel retardation. Binding reactions were performed in 20 μl by incubating 3 fmol (10,000 cpm) of the 110-bp *Eco*RI-*Xho*I fragment from pHRI, containing the Ad2 origin labeled at the *Xho*I site, with varying amounts of protein in the presence of 1 μg of poly(dI-dC)-poly(dI-dC) in a buffer containing 20 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.025% Nonidet P-40, 4% Ficoll, 100 mM NaCl, and 1 μg of bovine serum albumin. After 30 min at 25°C, protein-bound and free DNA were resolved by electrophoresis on a 7% polyacrylamide gel by using 45 mM Tris base–45 mM boric acid–1 mM EDTA–0.01% Nonidet P-40 as a running buffer.

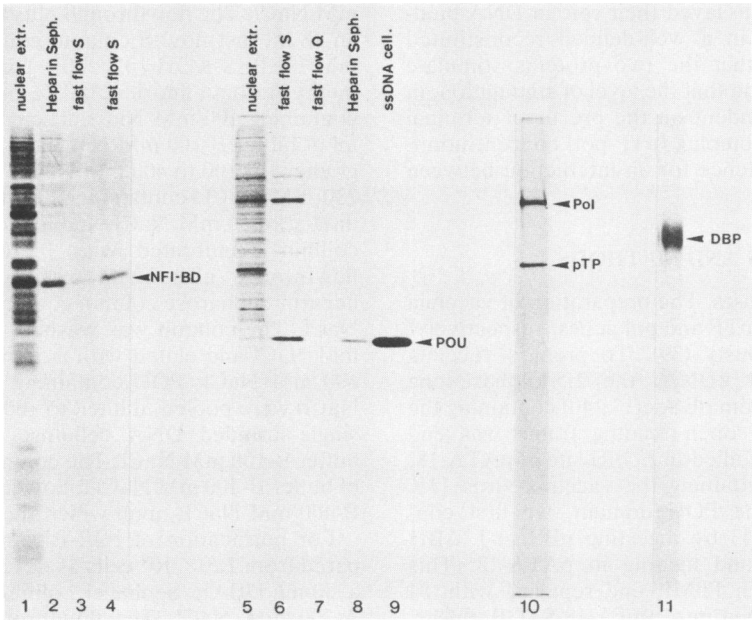


FIG. 1. Purification of NFI-BD and the oct-1 POU domain. An SDS-gel electropherogram stained with silver (lanes 1 to 10) or Coomassie blue (lane 11) is shown. Lanes 1 to 4 indicate various steps of the purification of NFI-BD; lanes 5 to 9 indicate those of the POU domain. From both isolation procedures, the DEAE Sephacel pool is omitted because this column had been coupled to the next one. Lanes 10 and 11 show the pTP-pol complex (24) and DBP, respectively. The arrows indicate the various replication protein bands, the positions of which are not directly comparable due to different electrophoretic conditions.

Both for NFI-BD and the POU domain, one binding unit (bu) is defined as the amount of protein that can bind 50% (1.5 fmol) of a 110-bp Ad2 probe in a gel retardation assay. One binding unit corresponds to 1.4 ng for NFI-BD and 1.1 ng for the POU domain. Under the conditions described above, the K_d values calculated for NFI-BD and the POU domain are approximately 3.10^{-9} M and 10^{-9} M, respectively.

DNA replication in vitro. Reaction mixtures (15 μl) contained 25 ng of *Xho*I-digested Ad5 DNA-TP (2.1 fmol each of NFI- and NFIII/oct-1-binding sites), 0.6 μg of DBP, and various amounts of pTP-pol, NFI, NFI-BD, POU domain, or NFIII/oct-1, as indicated, in a buffer containing 25 mM HEPES-KOH (pH 7.5); 4 mM $MgCl_2$; 0.4 mM DTT; 2 mM ATP; 7.5 mM phosphocreatine; 7.5 μg of creatine kinase per ml; 40 μM of dGTP, dATP, and dTTP; and 2.5 μM [α-^{32}P]dCTP (5.5 Ci/mmol). Incubation was for 1 h at 37°C, and the reaction was stopped by addition of 1.5 μl of stopmix (40% sucrose, 1% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue). The products were analyzed by agarose gel electrophoresis in the presence of SDS as described previously (24), partially dehydrated, and autoradiographed. Incorporation was calculated by measuring Cerenkov counts in gel slices.

Immunoprecipitation. We used anti-pol antiserum directed against a fusion protein (10), anti-pTP antiserum against a fusion protein (47), and anti-pTP antiserum directed against a C-terminal peptide (39). These antisera were coupled to protein-A Sepharose CL-4B by incubation for 45 min at room temperature in buffer D (20 mM HEPES-KOH [pH 7.5], 1 mM DTT, 1 mM EDTA, 0.025% Nonidet P-40, 10% glycerol, 60 mM NaCl) in the presence of 1 μg of poly(dI-dC)-poly(dI-dC), followed by three wash steps. To detect complex formation between pTP-pol and cellular proteins, the 110-bp *Eco*RI-*Xba*I fragment from pHRI, labeled at the *Xba*I site, was incubated for 1 h at 0°C in buffer D in the presence of 1 μg of bovine serum albumin and pTP-pol, NFI-BD, or POU domain in various combinations. Subsequently, the reaction mixtures (20 μl) were brought to 0.001% glutaraldehyde and kept for 1 h at room temperature. Any complexes formed were immunoprecipitated with 1 μl of antiserum coupled to 2 mg of protein-A Sepharose CL-4B for 30 min at 0°C in a total volume of 100 μl. The pellet was washed three times with buffer D, followed by phenol-chloroform extraction in the presence of 0.1% SDS and 1 μg of calf thymus DNA. The DNA was precipitated with isopropanol and analyzed by electrophoresis in an 8% polyacrylamide gel in the presence of 0.1% SDS, followed by autoradiography.

RESULTS

HeLa cells were infected with recombinant vaccinia viruses containing the DNA-binding domain of rat liver NFI (NFI-BD) encoding the N-terminal amino acids 4 to 240 (13, 28) or the oct-1 POU domain, amino acids 269 to 440 (41, 44a). At 24 h after infection, the recombinant proteins were isolated from nuclei (see Methods) and purified to near homogeneity by using several chromatographic steps. Figure 1 shows an SDS-polyacrylamide gel electropherogram of the various fractions of NFI-BD (lanes 1 to 4) and the POU domain (lanes 5 to 9), showing that the most purified fractions are almost homogeneous, similar to the viral proteins pTP-pol and DBP (Fig. 1, lanes 10 and 11). Purification of the DNA-binding domains appeared to be easier than that of the intact transcription factors. The results of the purification procedure are summarized in Table 1.

To establish the DNA-binding properties in more detail, we compared the borders of the recognition sequences in the adenovirus origin of NFI-BD and the POU domain with those of intact NFI and NFIII/oct-1, respectively, by DNase I footprinting. As shown in Fig. 2A, the protected regions

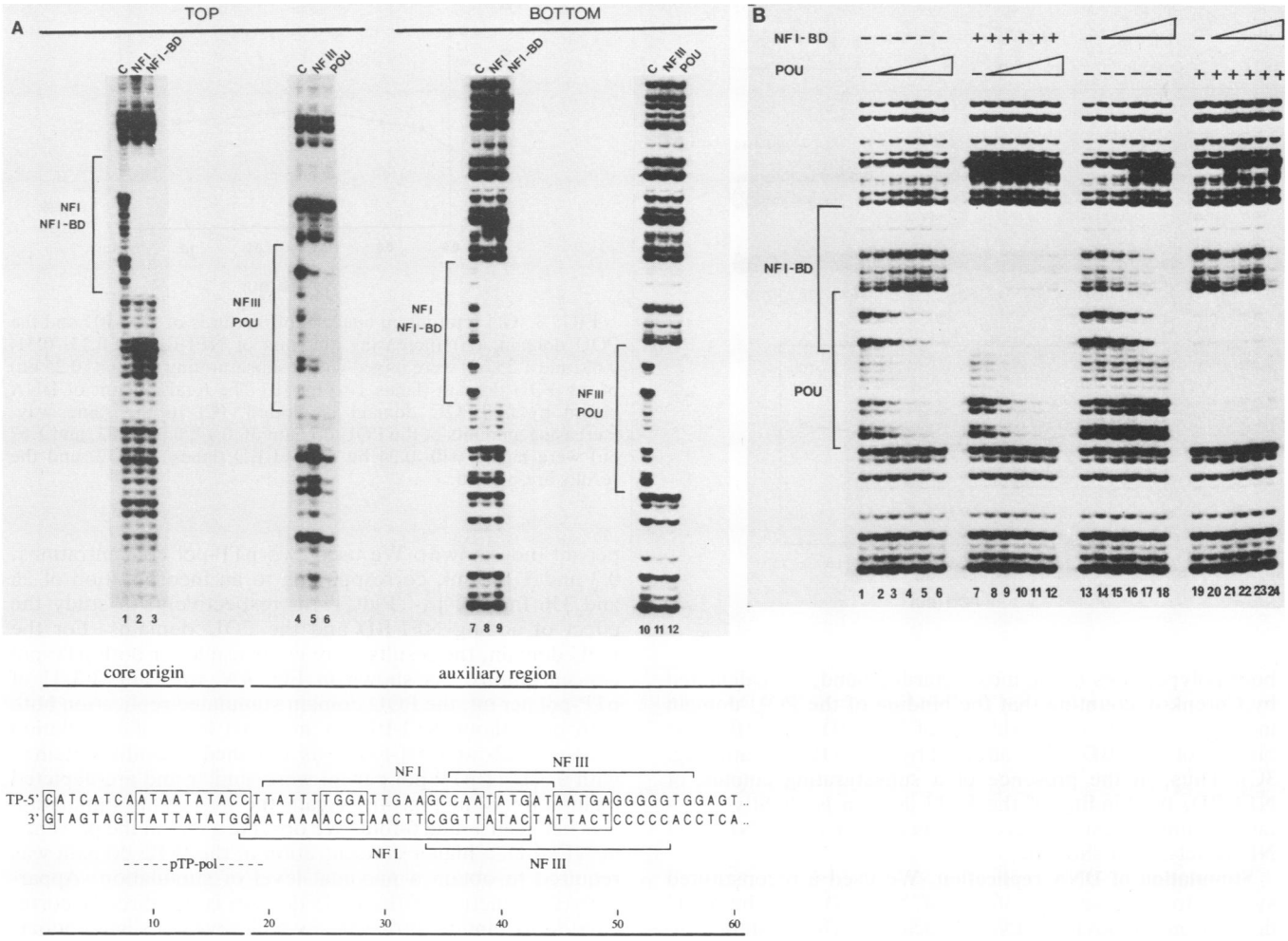
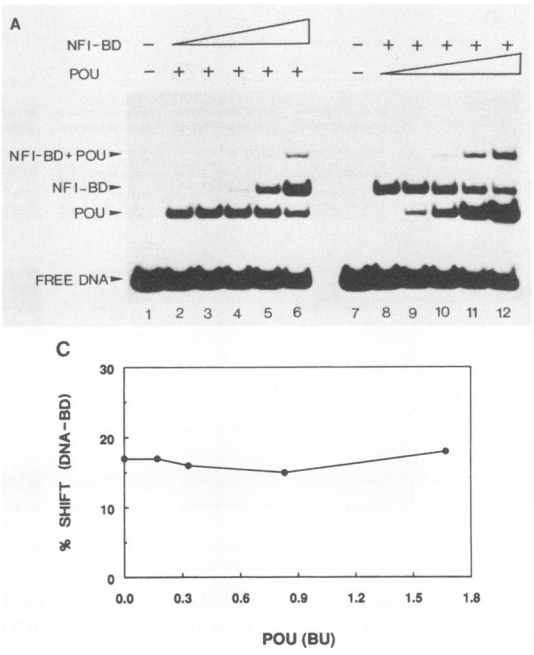


FIG. 2. DNase I footprints of NFI, NFIII/oct-1, NFI-BD, the POU domain, and mixtures of these proteins. (A) The footprints of intact NFI and NFIII/oct-1 are identical to those of the DNA-binding domains. Either the top strand (lanes 1 to 6) or the bottom strand (lanes 7 to 12) were used as probes, and saturating levels of protein were used. The binding borders are indicated in brackets. C, Without protein. The Ad2 sequence is shown, with boxes showing regions conserved among several human adenovirus serotypes. (B) Noncooperative binding of NFI-BD and the POU domain. Footprints were performed with increasing amounts of the POU domain (3.3, 10, 20, 30, and 67 bu) in the absence (lanes 1 to 6) or in the presence (lanes 7 to 12) of a saturating amount of NFI-BD (2.5 bu). In the reverse situation, increasing amounts of NFI-BD (0.15, 0.3, 0.83, 1.25, and 2.5 bu) were used in the absence (lanes 13 to 18) or in the presence (lanes 19 to 24) of a saturating amount of the POU domain (67 bu).

were identical and correlate with published data for NFI and NFIII/oct-1 (7, 31). Previously, it was shown that upon adding identical amounts of DNA-binding units, both NFI-BD (13) and the POU domain (44a) stimulated adenovirus DNA replication to the same extent as the intact proteins did. Since BD and the POU domain were easier to purify and were more stable, we performed most other experiments with these polypeptides.

Nonsynergistic binding of NFI and NFIII/oct-1. Contact point analysis has indicated that NFI and NFIII/oct-1 bind closely together. They even share an AT base pair at position 39, of which the T residue is contacted by NFI, while the A residue as well as the phosphate backbone make contact with NFIII/oct-1. Mutation studies confirmed the importance of this AT base pair both for NFI and NFIII/oct-1 binding (20, 33). This proximity of the two proteins suggests that they might influence each other by cooperative binding. To test this, we studied the effect of adding a saturating amount of NFI-BD to increasing concentrations of the POU domain and vice versa by DNase I footprinting. As shown in Fig. 2B (lanes 1 to 12), the concentration required for maximal protection of the Ad2 origin by the POU domain was independent of the presence of NFI-BD. In the reverse situation, even a slightly higher amount of NFI-BD was required for maximal protection (lanes 13 to 24) when the POU domain was present.

The need for a saturating amount of NFI-BD or POU domain makes the DNase I protection assay less suitable to detect slight differences in binding affinity. Therefore similar experiments under nonsaturating conditions were performed by gel retardation with a 110-bp Ad2 origin probe (Fig. 3A). The DNA concentration used is well below the K_d values observed for both the POU domain and NFI-BD under these conditions (see Materials and Methods). Therefore, this system is suitable to detect possible cooperativity. Three retarded bands can be observed, containing, in decreasing order of mobility, the POU domain, NFI-BD, and a combination of both (Fig. 3A). Taking into account the presence of



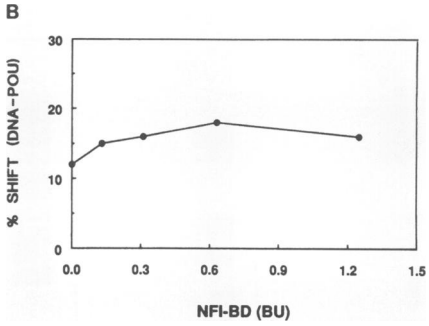


FIG. 3. Gel retardation analysis of mixtures of NFI-BD and the POU domain. (A) Increasing amounts of NFI-BD (0, 0.13, 0.31, 0.63, and 1.25 bu) were mixed with a subsaturating amount (0.25 bu) of the POU domain (lanes 1 to 6). (B) The total amount of DNA bound by the POU domain is plotted. (C) In the same way, increasing amounts of the POU domain (0, 0.17, 0.33, 0.83, and 1.67 bu) were mixed with 0.34 bu of NFI-BD (lanes 7 to 12) and the results are plotted.

both polypeptides in the most retarded band, we calculated by Cerenkov counting that the binding of the POU domain increases 1.5-fold upon addition of NFI-BD (Fig. 3B). The binding of NFI-BD is not affected by the POU domain (Fig. 3C). Thus, in the presence of a subsaturating amount of NFI-BD, the binding of the POU domain is slightly facilitated. Similar results were obtained with intact NFI and NFIII/oct-1 (not shown).

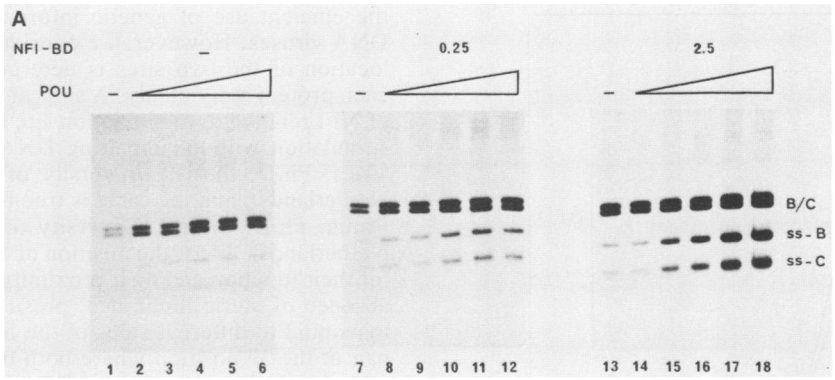
Stimulation of DNA replication. We used a reconstituted system to analyze the effects of NFI-BD and the POU domain on adenovirus DNA replication. This system consisted of the purified pTP-pol complex, DBP, and the Ad5 DNA-TP complex digested with *Xho*I as template. As shown in Fig. 4A, incubation of these components under replication conditions leads to specific labeling of the B and C fragments containing the origin. In addition, labeled, single-stranded B and C strands were observed originating from second rounds of displacement synthesis, indicating very efficient replication. In the absence of transcription factors, a low level of replication was observed, as expected. This level was dependent upon the pTP-pol concentration, with a maximum around 0.5 pmol of dCTP/h incorporated at 10 U of pTP-pol per ml (not shown). We used two pTP-pol concentrations, 0.3 and 3.3 U/ml, corresponding to an incorporation of 26 and 356 fmol of [α-^{32}P]dCTP/h, respectively, to study the effect of adding NFI-BD and the POU domains. For the POU domain, the results were comparable for both pTP-pol concentrations. As shown in Fig. 4A, by using 3.3 U of pTP-pol per ml, the POU domain stimulated replication both with or without NFI-BD. A maximal level of stimulation between 4.2- and 5.1-fold was obtained. Results obtained with 0.3 U of pTP-pol per ml were similar and are depicted quantitatively in Fig. 4B. Again the maximum stimulation was between 4- and 6-fold. We observed that in the presence of NFI-BD, a higher concentration of the POU domain was required to obtain a maximal level of stimulation. Apparently the function of the POU domain is not directly correlated to its binding properties, which reveal a slight cooperativity with NFI-BD (Fig. 3B).

During these studies, we noted a substantial difference between the degree of stimulation by NFI-BD at low and high pTP-pol levels. For 2.5 bu NFI-BD, the stimulation was 7.4-fold at 3.3 U of pTP-pol/ml and 46-fold at 0.33 U of pTP-pol/ml. Therefore, we investigated the stimulation by NFI-BD and the POU domain as a function of the pTP-pol concentration in more detail. First we varied the NFI-BD concentrations at the same pTP-pol levels (Fig. 5A). The results confirm our initial observation that the level of stimulation is strongly dependent on the pTP-pol concentra-

TABLE 1. Purification of replication proteins

Fraction	Total protein (mg)	Vol (ml)	Total binding units ($\times 10^5$)	Purification (fold)	Yield (%)
NFI-BD					
HeLa nuclear extract	75	15	7.1	1.0	100
DEAE-Sephacel + heparin Sepharose	6.3	24	5.9	9.9	84
Mono S	0.75	5	5.4	76	75
POU domain					
HeLa nuclear extract	90	30	5.1	1	100
DEAE-Sephacel + Fast flow S	4.3	24	4.2	17.2	82
DEAE-Sephacel + Fast flow Q	3.6	400	2.5	12.3	49
Heparin Sepharose	0.57	19	2.0	62	40
ssDNA[a] cellulose	0.14	2	1.3	164	26

[a] ssDNA, Single-stranded DNA.



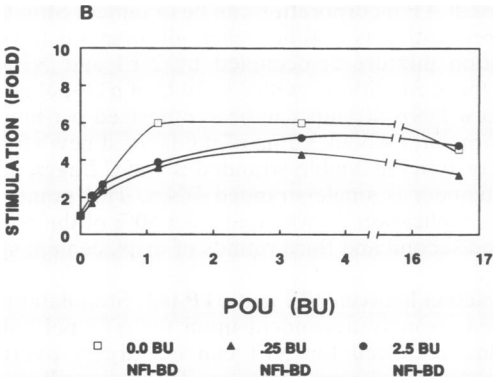


FIG. 4. Effect of NFI-BD on stimulation by the POU domain. (A) To a replication mixture containing 3.3 U of pTP-pol per ml, 0, 0.17, 0.33, 1.2, 3.3, and 16.7 bu of the POU domain were added (lanes 1 to 6, 7 to 12, and 13 to 18) in the absence or presence of 0.25 or 2.5 bu of NFI-BD. The basal levels of replication (lanes 1, 7, and 13) were 250, 475, and 2,000 fmol of [α-^{32}P]dCTP, respectively, in 15 μl. Note that the exposure time for lanes 1 to 6 was twice as long as for the rest of the figure. (B) Under the same conditions as in panel A but with 0.33 U of pTP-pol per ml, the stimulation by the POU domain is plotted against its amount of binding units. The basal levels of replication were 20, 120, and 920 fmol of [α-^{32}P]dCTP, respectively, in 15 μl.

tion. This was not true for the POU domain (Fig. 5B). These plots also indicate that the concentrations required for maximal stimulation were close to the saturation point of the template DNA (2 bu). Figure 6 shows the maximal stimulation as a function of the pTP-pol concentration. A clear difference in pTP-pol dependency is observed between NFI-BD and the POU domain. At 10 U/ml, the stimulation of NFI-BD was 10% of the level obtained at 0.3 U/ml, while the POU domain still stimulated 53%. At a large excess of pTP-pol (30 U/ml), the stimulation by NFI-BD was only 3% of that obtained at 0.3 U of pTP-pol per ml, while for the POU domain this was 25%. Furthermore, just as we observed for the POU domain, the degree of stimulation by NFI-BD was not changed in the presence of an optimal amount of POU domain. This shows that there is no synergism between the two proteins. We performed the same experiments with partly purified (50% pure) NFI and NFIII/oct-1 with similar results (not shown).

NFI interacts with pTP-pol. The results described above could be explained by a direct interaction between pTP-pol and NFI, which can lead to an increased binding of pTP-pol to the origin or to an increased stability of the initiation complex. To study the formation of a pTP-pol-NFI complex, we incubated ^{32}P-labeled DNA containing bp 1 to 110 of the Ad2 origin with pTP-pol in a binding buffer containing 60 mM NaCl (based on standard replication conditions) and looked at the effect of adding NFI-BD. Protein complexes that had been formed were fixed with glutaraldehyde and immunoprecipitated with anti-pTP or anti-pol antisera. Labeled DNA bound to the precipitates was detected by gel electrophoresis. Figure 7 shows an experiment performed with an anti-pTP antiserum (47). A small amount of DNA was bound in the absence of proteins (lane 1). The addition of NFI-BD reduced this background (lane 2). The amount of DNA detected for pTP-pol alone (lane 6) was three times the background level, indicating binding of pTP-pol to the origin. When NFI-BD and pTP-pol were mixed, the amount of DNA was considerably higher and dependent on the NFI-BD concentration (lanes 3 to 5). No such effect was observed for the POU domain either in the absence (compare lanes 8 and 9 to lane 6) or in the presence of NFI-BD (compare lane 10 to lanes 3 to 5). In this case, a reduction

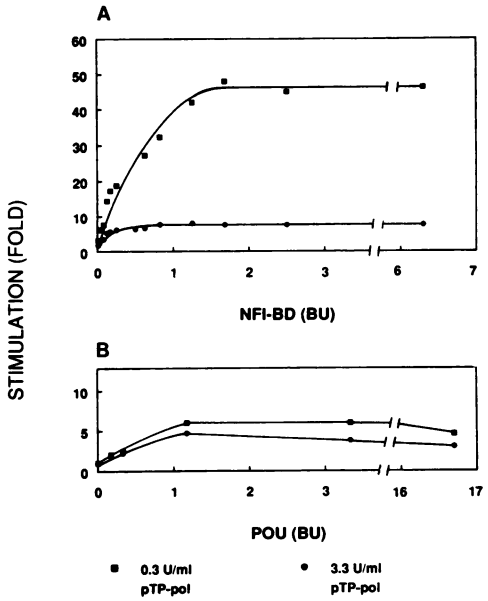


FIG. 5. Stimulation by NFI-BD or the POU domain at two pTP-pol concentrations. The stimulation of DNA replication as a function of NFI-BD (A) or the POU domain (B) is plotted for 0.3 or 3.3 U of pTP-pol per ml. Basal levels of replication were 25 and 336 fmol of [α-^{32}P]dCTP for NFI-BD and 20 and 250 fmol of [α-^{32}P]dCTP for the POU domain, respectively.

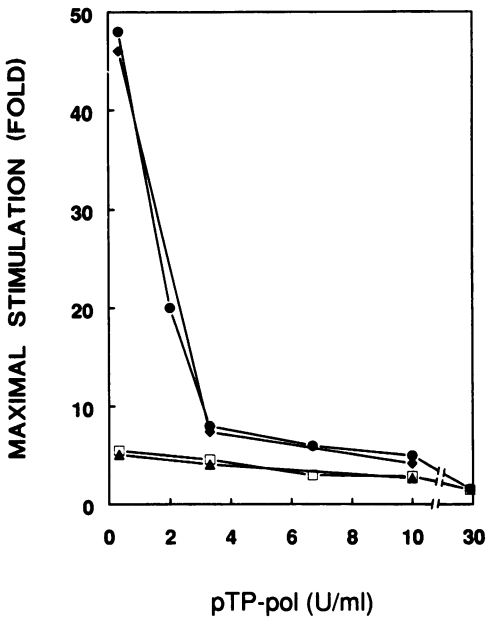


FIG. 6. Stimulation as a function of the pTP-pol concentration. The level of stimulation of DNA replication is given for a saturating level of NFI-BD (2.5 bu) in the absence (●) or presence (◆) of 3.3 bu of POU. The same is given for a saturating level of the POU domain (bu) without (□) or with (▲) 2.5 bu NFI-BD.

rather than an increase was observed. The same results were obtained for another anti-pTP antiserum (39). In three independent experiments, 2.5 bu of NFI-BD increased the binding 4.2- ± 1-fold. With an anti-pol antiserum (10), the increase in amount of DNA precipitated, caused by NFI-BD, was only 1.6-fold, but this may have been due to the low activity of the serum. Omitting glutaraldehyde reduced the NFI-BD effect from 4.2- to 1.4-fold. This suggests that NFI-BD interacts directly with pTP-pol, enabling protein-protein cross-linking. With intact NFI, identical results were obtained. We interpret these results to mean that NFI and pTP-pol can form a metastable complex leading to an increased binding of origin DNA and that cross-linking is essential to detect the interaction under our conditions.

DISCUSSION

NFI and NFIII/oct-1 function independently. We show here that despite the proximity of NFI and NFIII/oct-1, when bound to DNA, the two proteins hardly affect each other's binding properties. This lack of cooperativity agrees with previous results obtained when studying the effect of the viral DBP on the binding kinetics (3, 38). DBP enhances binding of NFI but not of NFIII/oct-1, showing a different response to this viral protein.

Also in their stimulation of DNA replication, NFI and NFIII/oct-1 behave independently and in a different manner. One argument is that the maximal level of stimulation by NFI and NFIII/oct-1 is additive, i.e., the degree of stimulation obtained is the product of the values obtained for each protein alone. Besides, a different response to variation in the pTP-pol concentration is found, which can be explained by an interaction between NFI and pTP-pol but not NFIII/oct-1 and pTP-pol.

What then is the significance of the close spacing of these two sites in the adenovirus origin? One explanation could be the efficient use of genetic information common to many DNA viruses. However, we consider it more likely that the location of the two sites is determined by constraints for each protein individually. A shift of only 2 bp in the position of NFI relative to the initiation site already severely inhibits stimulation without impairing DNA binding (1, 46; E. De Vries, Ph.D. thesis, University of Utrecht, Utrecht, The Netherlands), and the same is true for NFIII/oct-1 (G. J. M. Pruijn, Ph.D. thesis, University of Utrecht, Utrecht, The Netherlands). Thus, the position of both proteins is essential for their function and their proximity is made possible by the absence of steric hindrance, presumably because the proteins bind to different sides of the helix around an AT base pair at the 39 position where both binding sites overlap.

By using the reconstituted DNA replication system, high levels of dCTP incorporation can be obtained. Stimulation of DNA replication is optimal when all template DNA in the replication mixture is occupied by NFI and NFIII/oct-1. Under these conditions, with 3 to 10 U of pTP-pol per ml, 9.3 ng of new DNA strands can be synthesized per hour on 4.2 ng of template. About 3.5 ng of the labeled new strands can be detected in the double-stranded B and C fragments, while the remainder is single-stranded DNA. This points to very efficient replication, in which at least 80% of the template is used and second and third rounds of displacement synthesis occur.

Interaction between NFI and pTP-pol. Stimulation by NFI appeared strongly dependent upon the pTP-pol concentration, and the need for NFI can be largely overcome at increased pTP-pol concentrations. This may well explain the variable values reported in the literature for stimulation by NFI, as the pTP-pol concentration in these experiments was not always identical. We calculate an intranuclear concentration of 0.5 U of pTP-pol per ml, taking a value of 10^{-9} ml for the nuclear volume and assuming a random distribution. This indicates that the lower pTP-pol concentrations used here are in the range of those present in infected-cell nuclei. Therefore, under physiological conditions, NFI will have a considerable stimulating effect, in agreement with the stimulation by an NFI site in vivo (14, 45).

We interpret our immune precipitation data to show that

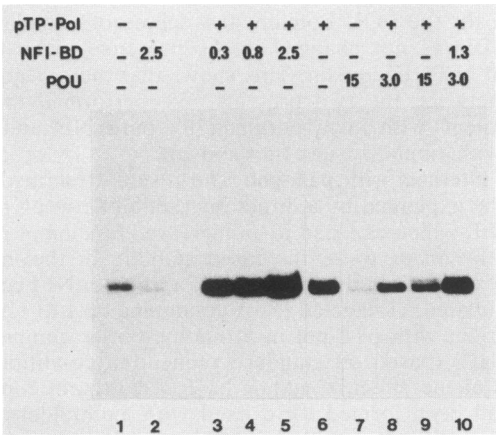


FIG. 7. NFI-BD interacts with the pTP-pol complex. Various amounts of NFI-BD and the POU domain were mixed with 30 U of pTP-pol per ml as indicated and added to ^{32}P-labeled origin DNA. Any complexes that were formed were fixed with 0.001% glutaraldehyde followed by immunoprecipitation with anti-pTP antibodies (see Materials and Methods). The DNA was recovered and analyzed on SDS gels.

NFI enhances the binding of pTP-pol to DNA containing the origin of replication. Only in the presence of glutaraldehyde was a significant effect observed. Apparently, there is only a weak or transient interaction between NFI and pTP-pol, comparable to, e.g., the interaction between SP1 and TFIID (4). A weak interaction may suffice, in view of the local high concentration of the two proteins when brought together through DNA binding. The interacting domain of NFI is located in the first 240 amino acids, in accordance with the notion that the interaction is important for DNA replication. Interaction of pTP-pol with this NFI domain apparently does not interfere with its DNA binding or dimerization capacity. So far we have not further delimited the site of interaction, either on NFI or on pTP-pol, but such studies are now feasible.

What properties of pTP-pol could be changed by interaction with NFI? We envisage at least three possibilities. One is that NFI directs pTP-pol to the core origin. Alternatively, by interacting with NFI, the stability of an initiation complex including pTP-pol is enhanced. Also, the kinetics of formation of a pTP-dCMP complex could be influenced directly by a modification of the structure of the active site of pTP-pol. Since these reactions are coupled during initiation, it will be difficult to discriminate between the latter two mechanisms. Our assay is not stringent enough to establish that we measure the binding to the origin of pTP-pol alone. In this respect, it is interesting that a direct interaction between pol and NFI was observed in the absence of origin DNA (M. S. Horwitz, personal communication).

Role of NFIII/oct-1. No complex was found between NFIII/oct-1 and pTP-pol. One explanation is that we cannot detect any interaction with NFIII/oct-1 by this method because of the high dissociation rate of a NFIII/oct-1-DNA complex relative to a NFI-DNA complex (C. P. Verrijzer and P. C. Van der Vliet, unpublished data). However, the relatively low pTP-pol dependency and the noncooperative binding with NFI make it more plausible that NFIII/oct-1 stimulates initiation by another mechanism, without direct interactions with either NFI or pTP-pol. An attractive hypothesis is that NFIII/oct-1 changes the structure of the DNA in such a way that initiation is favored. This effect should require a fixed position of the NFIII/oct-1-binding site. It could also explain why the homeodomain inhibits DNA replication (44a) without directly interacting with other viral proteins. Since the homeodomain makes less contacts with the origin than the POU domain does, this may lead to an abberant DNA structure not fit to sustain initiation, e.g., bending in the wrong direction. Studies to investigate this directly are underway.

In contrast to the lack of protein-protein interactions observed here, NFIII/oct-1 is well capable of interacting with other proteins or even with itself in another context. For instance, NFIII/oct-1 as well as the POU domain can bind cooperatively to adjacent octamer sites or to a heptamer site next to the octamer in the immunoglobulin H enhancer (17, 19, 29). Moreover, NFIII/oct-1 and the POU domain interact with the herpes simplex virus protein VP16 (Vmw65) to form a strong transcription-activating complex (11, 12, 18, 26, 43). This ability is located in the homeodomain (36).

The dispensability of the transcription activation domains may indicate the use of different mechanisms to activate transcription (8) and adenovirus DNA replication as suggested before (23, 44a). A likely explanation is that adenovirus adapted to existing conserved regions of families of DNA-binding proteins to enhance its own replication rather than using exactly the same mechanism as used in transcription activation. Nevertheless, we show that stimulation is not just related to DNA binding but also involves a specific interaction between a viral and a cellular protein.

ACKNOWLEDGMENTS

We are grateful to Winship Herr for providing us with pBSoct1$^+$ and pBSoct1$^+\Delta$BH, to Henk G. Stunnenberg for a gift of recombinant vaccinia virus expressing NFI-BD, to L. Zhao and R. Padmanabhan for anti-pTP antiserum and to Marshall S. Horwitz for anti-pol antiserum and for communicating results prior to publication. We thank Wim van Driel for purification of NFI-BD.

This work was supported in part by the Netherlands Foundation for Chemical Research (SON), with financial support from the Netherlands Organization for Scientific Research (NWO).

LITERATURE CITED

1. **Adhya, S., P. S. Shneidman, and J. Hurwitz.** 1986. Reconstruction of adenovirus replication origins with a human nuclear factor I binding site. J. Biol. Chem. **261**:3339–3344.
2. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58**:671–717.
3. **Cleat, P. H., and R. T. Hay.** 1989. Co-operative interactions between NFI and the adenovirus DNA binding protein at the adenovirus origin of replication. EMBO J. **8**:1841–1848.
4. **Courey, A. J., D. A. Holtzman, S. P. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59**:827–836.
5. **De Pamphilis, M. L.** 1988. Transcription elements as components of eukaryotic origins of DNA replication. Cell **52**:635–638.
6. **De Vries, E., W. Van Driel, M. Tromp, J. Van Boom, and P. C. Van der Vliet.** 1985. Adenovirus DNA replication in vitro: site-directed mutagenesis of the nuclear factor I binding site of the Ad2 origin. Nucleic Acid Res. **13**:4935–4952.
7. **De Vries, E., W. Van Driel, S. J. L. Van den Heuvel, and P. C. Van der Vliet.** 1987. Contact point analysis of the HeLa nuclear factor I recognition site reveals symmetrical binding at one side of the DNA helix. EMBO J. **6**:161–168.
8. **Dynan, W. S.** 1989. Modularity in promoters and enhancers. Cell **58**:1–4.
9. **Fletcher, C., N. Heintz, and R. G. Roeder.** 1987. Purification and characterization of OTF-1, a transcription factor regulating the cell cycle expression of a human histone H2b gene. Cell **51**:773–781.
10. **Friefeld, B. R., R. Korn, P. J. de Jong, J. J. Sninsky, and M. S. Horwitz.** 1985. The 140-kDa adenovirus DNA polymerase is recognized by antibodies to Escherichia coli-synthesized determinants predicted from an open reading frame on the adenovirus genome. Proc. Natl. Acad. Sci. USA **82**:2652–2656.
11. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus transactivating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85**:6347–6351.
12. **Goding, C. R., and P. O'Hare.** 1989. Herpes simplex virus Vmw65-octamer binding protein interactions: a paradigm for combinatorial control of transcription. Virology **173**:363–367.
13. **Gounari, F., R. de Francesco, J. Schmidt, P. C. Van der Vliet, R. Cortese, and H. G. Stunnenberg.** 1990. Amino terminal domain of NFI binds to DNA as a dimer and activates adenovirus DNA replication. EMBO J. **9**:559–566.
14. **Hay, R. T.** 1985. Origin of adenovirus DNA replication. Role of nuclear factor I binding site in vivo. J. Mol. Biol. **186**:129–136.
15. **Hay, R. T., and W. C. Russell.** 1989. Recognition mechanism in the synthesis of animal virus DNA. Biochem. J. **238**:3–16.
16. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
17. **Kemler, I., E. Schreiber, M. M. Müller, P. Matthias, and W. Schaffner.** 1989. Octamer transcription factors bind to two different sequence motifs of the immunoglobulin heavy chain promoter. EMBO J. **8**:2001–2008.
18. **Kristie, T. M., J. H. LeBowitz, and P. A. Sharp.** 1989. The

octamer-binding proteins form multi-protein-DNA complexes with HSV αTIF regulatory protein. EMBO J. **8**:4229–4238.

19. **Lebowitz, J. H., R. G. Clerc, M. Brenowitz, and P. A. Sharp.** 1989. The oct-2 protein binds cooperatively to adjacent octamer sites. Genes Dev. **3**:1625–1638.
20. **Leegwater, P. A. J., W. van Driel, and P. C. van der Vliet.** 1985. Recognition site of nuclear factor I, a sequence specific DNA-binding protein from HeLa cells that stimulates adenovirus DNA replication. EMBO J. **4**:1515–1521.
21. **Mattaj, I. W., S. Lienhard, J. Jiricny, and E. M. DeRobertis.** 1985. An enhancer-like sequence within the *Xenopus* U2 gene promoter facilitates the formation of stable transcription complexes. Nature (London) **316**:163–167.
22. **Meisterernst, M., L. Rogge, R. Foechler, M. Karaghiosoff, and E. L. Winnacker.** 1989. Structural and functional organization of a porcine gene coding for nuclear factor I. Biochemistry **28**: 8191–8200.
23. **Mermod, N., E. A. O'Neill, T. J. Kelly, and R. Tjian.** 1989. The proline-rich transcriptional activator of CTF/NFI is distinct from the replication and DNA binding domain. Cell **58**:741–753.
24. **Mul, Y. M., R. T. Van Miltenburg, E. De Clercq, and P. C. Van der Vliet.** 1989. The mechanism of inhibition of adenovirus DNA replication by the acyclic nucleoside triphosphate analogue (*S*)-HPMPApp: influence of the adenovirus DNA binding protein. Nucleic Acids Res. **17**:8917–8929.
25. **Nagata, K., R. A. Guggenheimer, T. Enomoto, J. H. Lichy, and J. Hurwitz.** 1982. Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA **79**:6438–6442.
26. **O'Hare, P., C. R. Goding, and A. Haigh.** 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. EMBO J. **7**:4231–4238.
27. **O'Neill, E. A., C. Fletcher, C. R. Burrow, N. Heintz, R. G. Roeder, and T. J. Kelly.** 1988. Transcriptional factor OTF-1 is functionally identical to the DNA replication factor NFIII. Science **241**:1210–1213.
28. **Paonessa, G., F. Gounari, R. Frank, and R. Cortese.** 1988. Purification of a NFI-like DNA-binding protein from rat liver and cloning of the corresponding cDNA. EMBO J. **7**:3115–3123.
29. **Poellinger, L., and R. G. Roeder.** 1989. Octamer transcription factors 1 and 2 each bind to two different functional elements in the immunoglobulin heavy chain promoter. Mol. Cell. Biol. **9**:747–756.
30. **Pruijn, G. J. M., P. C. Van der Vliet, N. A. Dathan, and I. W. Mattaj.** 1989. Anti-OTF-1 antibodies inhibit NFIII stimulation of in vitro adenovirus DNA replication. Nucleic Acids Res. **17**:1845–1863.
31. **Pruijn, G. J. M., W. Van Driel, and P. C. Van der Vliet.** 1986. Nuclear factor III, a novel sequence-specific DNA binding protein from HeLa cells stimulating adenovirus DNA replication. Nature (London) **332**:656–659.
32. **Pruijn, G. J. M., W. Van Driel, R. T. Van Miltenburg, and P. C. Van der Vliet.** 1987. Promoter and enhancer elements containing a conserved sequence motif are recognized by nuclear factor III, a protein stimulating adenovirus DNA replication. EMBO J. **6**:3771–3778.
33. **Pruijn, G. J. M., R. T. Van Miltenburg, J. A. J. Claessens, and P. C. Van der Vliet.** 1988. Interaction between the octamer-binding protein nuclear factor III and the adenovirus origin of DNA replication. J. Virol. **62**:3092–3102.
34. **Rosenfeld, P. J., E. A. O'Neill, R. J. Wides, and T. J. Kelly.** 1987. Sequence-specific interactions between cellular DNA-binding proteins and the adenovirus origin of DNA replication. Mol. Cell. Biol. **7**:875–886.
35. **Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian.** 1988. A family of human CAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature (London) **334**:218–224.
36. **Stern, S., M. Tanaka, and W. Herr.** 1989. The oct-1 homeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature (London) **341**:624–630.
37. **Stillman, B. M.** 1989. Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. **5**:197–245.
38. **Stuiver, M. H., and P. C. van der Vliet.** 1990. Adenovirus DNA-binding protein forms a multimeric protein complex with double-stranded DNA and enhances binding of nuclear factor I. J. Virol. **64**:379–386.
39. **Stunnenberg, H. G., H. Lange, L. Philipson, R. T. Van Miltenburg, and P. C. Van der Vliet.** 1988. High expression of functional adenovirus DNA polymerase and precursor terminal protein using recombinant vaccinia virus. Nucleic Acids Res. **16**:2431–2444.
40. **Sturm, R. A., G. Das, and W. Herr.** 1988. The ubiquitous octamer-binding protein oct-1 contains a POU domain with a homeo box subdomain. Genes Dev. **2**:1582–1599.
41. **Sturm, R. A., and W. Herr.** 1988. The POU domain is a bipartite DNA-binding structure. Nature (London) **336**:601–604.
42. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by oct-1 and oct-2: interdependent activation domains induce oct-2 phosphorylation. Cell **60**:375–386.
43. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression. Genes Dev. **2**:718–729.
44. **Tsernoglou, D., A. D. Tucker, and P. C. Van der Vliet.** 1985. Crystallization of a fragment of the adenovirus DNA binding protein. J. Mol. Biol. **172**:237–239.

44a. **Verrijzer, C. P., A. J. Kal, and P. C. Van der Vliet.** 1990. The DNA binding domain (POU domain) of transcription factor oct-1 suffices for stimulation of DNA replication. EMBO J. **6**: 1883–1888.

45. **Wang, K., and G. D. Pearson.** 1985. Adenovirus sequences required for replication in vivo. Nucleic Acids Res. **13**:5173–5178.
46. **Wides, R. J., M. D. Challberg, D. R. Rawlins, and T. J. Kelly.** 1987. Adenovirus origin of DNA replication: sequence requirements for replication in vitro. Mol. Cell. Biol. **7**:864–874.
47. **Zhao, L., and R. Padmanabhan.** 1988. Nuclear transport of adenovirus DNA polymerase is facilitated by interaction with preterminal protein. Cell **55**:1005–1015.