The DNA-binding domain of nuclear factor I is sufficient to cooperate with the adenovirus type 2 DNA-binding protein in viral DNA replication

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Recombinant baculoviruses have been constructed which express the full-length nuclear factor I (NFI) protein or a derivative of NFI that contains only the DNA-binding domain of the protein in infected insect cells. Both proteins were purified from insect cells infected with the respective baculoviruses and tested for their ability to cooperate with the adenovirus type 2 (Ad2) DNA-binding protein during virus replication. DNase I protection experiments demonstrated that the viral DNA-binding protein increased the affinity of both the full-length NFI and the DNA-binding domain of NFI for their recognition site in the Ad2 origin of DNA replication. As a consequence, the NFI-dependent increase in the efficiency of DNA replication observed upon addition of viral DNA-binding protein was the same when the full-length or DNA-binding domain derivative of NFI was added. Thus it appears that all of the activities associated with the ability of NFI to stimulate Ad2 DNA replication are located within the DNA-binding domain of the protein.

Introduction

Nuclear factor I (NFI) or CAAT transcription factor (CTF) is the collective name for a group of proteins that are found in the nucleus of higher eukaryotic cells. A single gene gives rise to differentially spliced mRNAs that are translated into a series of polypeptides ranging in Mr from 55000 to 65000 (Santoro et al., 1988; Meisterernst et al., 1989; Paonessa et al., 1988). This multifunctional protein binds specifically to DNA and stimulates transcription from promoters containing its recognition site (Jones et al., 1987), but was first identified by its ability to stimulate adenovirus type 2 (Ad2) DNA replication in vitro (Nagata et al., 1982). To stimulate DNA replication NFI binds as a dimer (Santoro et al., 1988; Mermod et al., 1989; Gounari et al., 1990) to a palindromic recognition site (TGG-Ns-GCCAAT) located between residues 25 and 39 in the inverted terminal repeat of the linear Ad2 genome (Nagata et al., 1983). Comparison of proteins encoded by three different cDNAs isolated from HeLa cells (CTF1, CTF2 and CTF3) revealed a common N-terminal domain rich in basic amino acids, whereas the C-terminal domains were each distinct, although all were rich in prolines (Santoro et al., 1988). Functional analysis of these domains indicated that the proline-rich regions in the C-terminal region are required for transcriptional activation, whereas the conserved N-terminal region is required for DNA binding and Ad2 DNA replication in vitro (Meisterernst et al., 1989; Mermod et al., 1989; Gounari et al., 1990), although the mechanism of NFI-induced stimulation of Ad2 DNA replication has not been determined. Deletion of the NFI recognition site from the origin of DNA replication reduces the efficiency of Ad2 DNA replication both in vitro (Chalberg & Rawlins, 1984; Guggenheimer et al., 1984; Lally et al., 1984) and in vivo (Hay, 1985a, b; Hay & McDougall, 1986).

The role of NFI in the replication of Ad2 DNA appears, at least in part, to be in the assembly of a preinitiation complex at the origin of DNA replication. This is accomplished by direct protein–protein interactions between NFI and the Ad2-encoded DNA polymerase, which is part of a heterodimeric complex also containing the Ad2 preterminal protein (pTP) (Bosher et al., 1990; Chen et al., 1990; Mul et al., 1990). An additional important component of this preinitiation complex is thought to be the Ad2-encoded DNA-binding protein (DBP), which was originally classified as a single strand-specific binding protein, but is now known to bind dsDNA in a non-specific fashion. One of the functions of DBP in the initiation process is to increase the affinity with which NFI binds to its recognition site in the origin of DNA replication (Cleat & Hay, 1989a; Stuiver & van der Vliet, 1990). Once initiation has taken place DBP is intimately involved in the strand displacement mechanism of Ad2 DNA replication, both by increasing the processivity of the viral DNA polymerase and by binding to displaced single strands (Lindenbaum et al., 1986).
We have constructed recombinant baculoviruses which, after infection of insect cells, express either the full-length NFI protein (CTF1) or the N-terminal DNA-binding domain of CTF1 (NFIDBD). The two proteins were purified from infected insect cells and used in DNase I footprinting and in vitro replication experiments to examine the cooperative interaction between NFI and DBP. Purified DBP could increase the binding affinity of both NFI and NFIDBD for their recognition site in the Ad2 origin of DNA replication, and both proteins could also cooperate with DBP to increase the efficiency of Ad2 DNA replication in vitro.

**Methods**

**Construction of recombinant baculoviruses.** Synthetic adaptors (indicated in Fig. 1) were ligated to the Neol-EcoRI fragment of pCTF1 (Santoro et al., 1988) prior to insertion into the BamHI-cleaved pAcRP23 transfer vector (Possee & Howard, 1987) such that the full-length CTF1 cDNA was under the control of the polyhedrin promoter. The region of the CTF1 cDNA encoding the DNA-binding domain of the protein was isolated on an Neol–SacI fragment from pCTF1. Synthetic adaptors, one of which contained an in-frame stop codon to terminate translation (Fig. 1), were ligated to the fragment, which was inserted into BamHI-cut pAcRP23. Wild-type baculovirus DNA and transfer plasmids were cotransfected into Spodoptera frugiperda (SF9) cells in the presence of lipofectin (Gibco) and recombinant viruses isolated by limiting dilution as described (Watson & Hay, 1990). Virus stocks were prepared and titrated in SF9 cells grown at 28 °C in TC-100 containing 5% fetal calf serum.

**Purification of NFI and NFIDBD.** SF9 cells (110^9 cells) were infected with 2 p.f.u./cell of baculoviruses containing either NFI or NFIDBD cDNA. After 72 h at 28 °C, cells were collected by centrifugation, washed once with PBS, and resuspended in 5 ml 25 mM-HEPES-NaOH pH 8.0, 1 mM-EDTA, 0.4 mM-NaCl, 1 mM-benzamidine, 1 mM-sodium metabisulphite, 0.5% NP-40, and 10 μg/ml antipain, pepstatin and leupeptin. After 30 min on ice, nuclei were removed by low speed centrifugation and the supernatant was clarified by centrifugation at 45000 g for 30 min at 4 °C. To purify NFIDBD, the clarified extract was applied directly to a column of Bio-Rex (Bio-Rad) equilibrated in 25 mM-HEPES-NaOH pH 8.0, 1 mM-EDTA, 2 mM-DTT, 0.4 mM-NaCl and 10% glycerol. Unbound proteins were removed by washing the column with 2 column volumes of the above buffer and NFIDBD was eluted by raising the NaCl concentration to 0.7 M. The full-length NFI protein was purified in a similar manner.

**In vitro DNA replication.** Reactions (30 μl) contained 1 to 2 μg of cytoplasmic extracts from SF9 cells infected with recombinant baculoviruses containing the Ad2 DNA polymerase and pTP genes (Boyer et al., 1990), various amounts of Ad2 DBP purified from Ad2-infected cells (Leith et al., 1989) and NFI, in 25 mM-Tris–HCl pH 8.0, 5 mM-MgCl₂, 2 mM-DTT, 3 mM-ATP, 3 μCi [α-32P]dCTP (specific activity 3000 Ci/mmol) and 50 ng (as DNA) Ad2 cores (Goding & Russell, 1983). The reaction mixture was incubated for 90 min at 37 °C after which the reaction was terminated by heating at 70 °C for 5 min; it was then treated with micrococcal nuclease at 33 units/ml in the presence of 2 mM-CaCl₂ for 30 min at 37 °C. Products were fractionated by SDS-PAGE and the amount of dCMP transferred to pTP was determined as described (Harris & Hay, 1988).

**DNase I footprinting.** Plasmid pHFR1 (Hay et al., 1984), containing the Ad2 inverted terminal repeat, was cleaved with EcoRI and PstI. The EcoRI site was then labelled by incubation with [α-32P]dATP (specific activity 3000 Ci/mmol), the other unlabelled dNTPs and the Klenow fragment of DNA polymerase 1. The labelled DNA fragment was fractionated on an 8% polyacrylamide gel and isolated by electroelution as described previously (Clark et al., 1990). DNase I protection experiments were conducted essentially as described (Clark et al., 1989b). Labelled DNA probe (10000 c.p.m.) in 50 μl 25 mM-HEPES-NaOH pH 8.0, 100 mM-NaCl, 5 mM-MgCl₂, 1 mM-DTT, 20 μg/ml bovine serum albumin (BSA) was incubated for 30 min at 20 °C with various amounts of NFI and DBP. DNase I (0–25 units in 5 μl of the above buffer) was added and incubation continued for a further 60 s at 20 °C, after which the reaction was stopped by the addition of 200 μl...
Adenovirus DNA replication

Expression of NFI in insect cells infected with recombinant baculoviruses

The proteins of the NFI family contain a conserved DNA-binding domain attached to additional protein sequences which appear to be involved in transcriptional regulation. To determine the role of the different NFI domains in Ad2 DNA replication, the CTF1 cDNA (full-length protein) and the DNA-binding domain of CTF1 were inserted into recombinant baculoviruses by homologous recombination after insertion into pAcRP23 (Possee & Howard, 1987) (Fig. 1).

Infection of Sf9 insect cells with the recombinant baculoviruses and analysis of the expressed proteins by SDS-PAGE revealed additional polypeptides of Mr 62000 and 35000, corresponding to the expected MrS of the full-length and DNA-binding domain of CTF1 (data not shown). The DNA-binding activity of these proteins and their ability to form dimers was assessed by a gel electrophoresis DNA-binding assay. Extracts from uninfected Sf9 cells did not appear to contain proteins which bound to the double-stranded oligonucleotide containing the NFI binding site present in the Ad2 origin of DNA replication (Fig. 2a, lane 1). However, extracts from Sf9 cells infected with recombinant baculoviruses expressing the full-length NFI protein generate a slowly migrating, well defined DNA–protein complex (Fig. 2a, lane 2), in contrast to that observed in HeLa cells in which the multiple species of NFI, generated by alternative splicing and modification, generate a heterogeneous

Results

Expression and purification of NFI from insect cells infected with recombinant baculoviruses

of NFI (lane 6), or a mix of NFI and NFIDBD expressing viruses: lane 3, 4 p.f.u./cell NFI and 2 p.f.u./cell NFIDBD; lane 4, 3 p.f.u./cell each virus; lane 5, 2 p.f.u./cell NFI and 4 p.f.u./cell NFIDBD. Cell extracts were made in the presence of 0.5% NP40 and 0.4 M NaCl, and DNA-binding assays were performed as described. The position of DNA–protein complexes containing NFI (●), NFIDBD (●) and the NFI/NFIDBD heterodimer (●) are indicated. (b) Purification of NFI proteins by recognition site affinity chromatography. Extracts from Sf9 cells infected with baculoviruses expressing either NFI or NFIDBD were eluted from Bio-Rex as described and applied to DNA affinity columns which contained a double-stranded synthetic oligonucleotide representing positions 18 to 41 from the Ad2 origin of DNA replication. Unbound proteins were removed by washing with 0.25 M NaCl and specifically bound proteins eluted with 1.0 M NaCl. Fractions (1 ml) were collected and 5 μl of each was analysed by SDS–PAGE and Coomassie blue staining. BSA (10 μg) was coelectrophoresed as a standard.

Fig. 2. Expression of NFI in insect cells. (a) Gel electrophoresis DNA-binding assay for the activity of NFI. Sf9 cells (10⁶) were infected with 5 p.f.u./cell wild-type baculovirus (lane 1), baculovirus expressing full-length NFI (lane 2), baculovirus expressing the DNA-binding domain

0.3 M sodium acetate, 20 mM EDTA and 100 μg/ml tRNA. DNA was isolated by organic extraction followed by ethanol precipitation, and resuspended in formamide prior to analysis on 8% polyacrylamide–urea gels. Gels were fixed in acetic acid, dried and exposed to X-ray film at −70 °C in the presence of an intensifying screen.
Fig. 3. Influence of DBP on DNA binding of NFI and NFI\(_{\text{DBD}}\). The EcoRI–PstI fragment from pHRI, containing the Ad2 origin of DNA replication, was 3' end-labelled at the EcoRI site and incubated with various concentrations of NFI and DBP prior to DNase I digestion. (a) DNase I cleavage pattern produced in the absence of NFI and DBP (lane 0), and increasing concentrations of NFI (lane 1, 1.6 ng; lane 2, 4.8 ng; lane 3, 16 ng) or NFI\(_{\text{DBD}}\) (lane 1, 1.5 ng; lane 2, 5 ng; lane 3, 15 ng). (b) Labelled DNA was incubated alone (lanes 0) or in the presence of NFI (0-8 ng) or NFI\(_{\text{DBD}}\) (0-6 ng), and increasing concentrations of DBP (lanes 1, 10 ng; lanes 2, 20 ng; lanes 3, 100 ng; lanes 4, 200 ng; lanes 5, 250 ng) prior to DNase I digestion.

series of DNA–protein complexes (Cleat & Hay, 1989a). Extracts from cells infected with the virus expressing the DNA-binding domain generate a more rapidly migrating DNA–protein complex (Fig. 2a, lane 6). Extracts of cells coinfected with both viruses revealed the presence of an additional DNA–protein complex of intermediate migration, which is consistent with the formation of a heterodimer containing one full-length monomer and one monomer of the DNA-binding domain (Fig. 2a, lanes 3 to 5). Similar conclusions were reached for rat NFI expressed in HeLa cells by Gounari et al. (1990).

Both the full-length protein and the DNA-binding domain were purified from infected cell extracts by a combination of ion exchange chromatography and DNA affinity chromatography. Protein eluting from the DNA affinity columns was analysed by SDS-PAGE and staining with Coomassie blue. The full-length protein was predominantly a single species of \(M_r\) 62000, whereas the DNA-binding domain elutes from the column as two species, one of which appears to be generated from the other by proteolysis (Fig. 2b). This is suggested by the observation that the more slowly migrating component can be converted into the faster migrating component by limited proteolysis in vitro (data not shown). It has been demonstrated previously that the NFI DNA-binding activity is present in a highly protease-resistant domain (Cleat & Hay, 1989b). It was possible to purify 1 to 2 mg of each protein from a 1 l culture of infected Sf9 cells.

Stimulation of binding of NFI to DNA by Ad2 DBP

Ad2 DBP can increase the affinity of NFI for its recognition site in the Ad2 origin of DNA replication (Cleat & Hay, 1989a; Stuiver & van der Vliet, 1990). To determine whether the full-length protein or only the DNA-binding domain of NFI was required for this, DNase I footprinting analysis was carried out. The full-length protein and the DNA-binding domain appeared to bind to the Ad2 origin of DNA replication with similar affinity and indistinguishable patterns of DNase I protection (Fig. 3). Based on this experiment full-length NFI and NFI\(_{\text{DBD}}\) were added to DNA fragments containing the Ad2 origin of DNA replication at concentrations giving only partial protection of the binding site from cleavage with DNase I. Addition of increasing concentrations of DBP to full-length NFI and NFI\(_{\text{DBD}}\) resulted in an increase in NFI site occupancy in each case (Fig. 3), indicating that DBP could cooperate

![Fig. 4. In vitro replication with NFI and NFI\(_{\text{DBD}}\). Cytoplasmic extract (1 \(\mu\)g) from Sf9 cells infected with recombinant baculoviruses expressing Ad2 DNA polymerase and pTP was incubated with Ad2 cores (50 ng DNA), DBP (1 \(\mu\)g), 0, 2, 4 or 8 ng (lanes 1 to 4) of NFI or NFI\(_{\text{DBD}}\), and [\(\alpha\)\(^{32}\)P]dCTP under conditions specified in the text. After 90 min at 37 °C, samples were digested with micrococcal nuclease and analysed on SDS–polyacrylamide gels, followed by autoradiography. Positions of the pTP–dCMP complex and the protease-cleaved TP–dCMP complex are indicated.](image-url)
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Fig. 5. Stimulation of initiation of Ad2 DNA replication by DBP.
(a) Cytoplasmic extract (1 μg) from Sf9 cells infected with recombinant baculoviruses expressing Ad2 DNA polymerase and pTP was incubated with Ad2 cores (50 ng DNA), NFI (2 ng) or NFI_{DBD} (2 ng), [γ-^{32}P]dCTP and 0, 0.12, 0.25, 0.5 or 1.0 μg (lanes 1 to 5) of DBP. Incubation conditions and analysis of products were as described in the legend to Fig. 4. (b) To determine dCMP transfer, the regions of the gel corresponding to the positions of pTP–dCMP and TP–dCMP were excised and the radioactivity was determined by liquid scintillation counting. For each point the total dCMP transfer was determined as the sum of the transfer to TP and pTP. ■, NFI_{DBD}; □, NFI.

with the DNA-binding domain of NFI to increase its affinity for its recognition site.

**Ability of NFI and the DNA-binding domain of NFI to cooperate with DBP in the stimulation of Ad2 DNA replication**

To determine whether the increase in DNA-binding activity of NFI_{DBD} was manifested in an increase in initiation of Ad2 DNA replication, an *in vitro* system dependent on the presence of DBP for efficient replication was employed. In the presence of DBP both NFI and NFI_{DBD} stimulated the transfer of \[^{32}P\]dCMP to Ad2 pTP (Fig. 4), which represents the first synthetic step in Ad2 DNA replication. Using a suboptimal amount of NFI and NFI_{DBD}, increasing concentrations of DBP were added to the *in vitro* reactions. Initiation activity was assayed by the transfer of \[^{32}P\]dCMP to the Ad2 preterminal and subsequently processed terminal proteins, the latter being generated by the action of the Ad2-encoded protease, which also cleaves pTP *in vivo* and is an integral component of the Ad cores used as template in these experiments. In each case a fourfold increase in initiation activity was observed in the presence of 1 μg of DBP (Fig. 5). This increase in activity was dependent on the presence of NFI as very little initiation activity was detected when experiments contained 1 μg DBP but no NFI (Fig. 4, lanes 1). Thus only the DNA-binding domain of NFI is required to cooperate with DBP during Ad2 DNA replication.

**Discussion**

A large body of genetic and biochemical evidence (reviewed in Hay & Russell, 1989) indicates that DBP is intimately involved in the elongation stage of Ad2 DNA replication. However, the role of DBP in the initiation process is less clear cut, with some investigators reporting that DBP does not stimulate initiation of Ad2 DNA replication (Rosenfeld et al., 1987) and others reporting that DBP was responsible for a two- to threefold increase in the frequency of initiation (Nagata et al., 1982; Kenny & Hurwitz, 1988). Relevant to these findings is the observation that the ability of NFI to stimulate the Ad2 DNA replication is related directly to the concentration of DBP in the reaction (De Vries et al., 1985). It has been noted subsequently that at low NFI concentrations DBP increases the affinity of NFI for its recognition site, with a resultant increase in the frequency of initiation (Cleat & Hay, 1989a; Stuiver & van der Vliet, 1990). These findings are supported and extended by the observations reported here. Thus, a fourfold increase in the frequency of initiation is observed in the presence of optimal amounts of DBP, and this stimulation is observed in the presence of either complete NFI or the DNA-binding domain of the protein. Therefore, at one level the role of DBP is to enhance binding of NFI to its recognition site in the Ad2 origin of DNA replication, which in turn serves, by direct protein–protein interactions, to facilitate formation of a preinitiation complex also containing the pTP/polymerase heterodimer (Bosher et al., 1990; Chen et al.,...
However, the stimulatory effect of DBP on initiation frequency is unlikely to be confined to increased NFI binding. This is suggested by observations made with the related Ad4. The Ad4 origin of DNA replication consists of only the terminal 18 bp of the viral genome and as such does not contain an NFI binding site, but DBP can still stimulate the frequency of the initiation reaction (Temperley & Hay, 1991). Therefore it appears that all the activities associated with the ability of NFI to stimulate Ad2 DNA replication are confined to the DNA-binding domain of the protein, and that these effects may be manifested by direct protein–protein interactions with the DNA polymerase, cooperative interactions with DBP or alterations in DNA structure that accompany NFI binding.

We would like to thank John Nicholson and Ian Armitt for excellent tissue culture backup. We are indebted to Professor R. Tjian for providing CTF cDNAs. This work was supported by the Cancer Research Campaign.

References


