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Kinetic analysis of nuclear factor I and its DNA-binding domain with the adenovirus origin of replication

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Nuclear factor I (NFI) is the collective name for a heterogeneous group of proteins, purified from HeLa cells, which stimulate the initiation of adenovirus type 2 DNA replication on binding to the adenovirus origin of replication. Protease treatment of NFI reduced all DNA-protein complexes to a single NFI-DNA complex, suggesting that they share a common DNA-binding domain. We present here a comparison of rate constants for the interaction of the full-length NFI protein and the core DNA-binding domain with their recognition site in the replication origin. The results demonstrate that the core protein alone can bind efficiently to the recognition site and that amino acid sequences outside this domain appear to have minor influence over the binding kinetics of NFI.

Nuclear factor I; Replication, origin of; Adenovirus; DNA-binding domain

1. INTRODUCTION

Adenovirus type 2 DNA can be replicated in vitro by the action of three viral and three cellular proteins [1–3]. Nuclear factor I (NFI) is a protein present in HeLa cells which, on binding to its recognition sequence in the adenovirus origin of replication, stimulates the initiation of adenovirus DNA replication both in vitro [4–8] and in vivo [9–13]. This factor can also function as a transcriptional activator and is in fact a heterogeneous group of polypeptides which can bind to GCCAAT sequences upstream of several eukaryotic genes [14,15], as well as to a specific recognition sequence in the adenovirus replication origin. A single human gene for NFI has recently been identified, which produces multiple mRNAs [15]. These mRNAs are translated into at least three different classes of NFI, probably as a result of differential splicing. The different NFI classes possess a conserved DNA-binding domain as well as additional polypeptide sequences [15]. It now appears possible that alternative splicing may control tissue-specific expression of distinct forms of NFI, as the same conserved DNA-binding domain has been found in cDNAs of a distinct liver-specific rat NFI [16]. Post-translational modifications may increase the heterogeneity of the NFI protein population and it has recently been demonstrated that NFI contains carbohydrate residues that can influence the ability of the protein to activate transcription in vitro [17]. In addition CCAAT box binding proteins have been shown in crude extracts to require cooperative interaction of two or more subunits for high-affinity sequence-specific DNA binding [18].

We have recently demonstrated using DNase I footprinting and methylation protection a specific cooperative interaction between NFI and the adenovirus DNA binding protein (DBP). The binding affinity of NFI for its recognition site in the adenovirus replication origin was shown to be dramatically increased by DBP [19]. Furthermore, DBP altered the binding kinetics of NFI, both by increasing the rate of association and decreasing the rate of dissociation of NFI with its binding site. In the present work we have undertaken a quantitative kinetic analysis of NFI–DNA interactions using the gel electrophoresis DNA binding assay to obtain association and dissociation rate constants and an equilibrium binding constant for the core DNA-binding domain of NFI.

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2. MATERIALS AND METHODS

2.1 *Labelled fragments*

The construction of plasmid pEX containing the adenovirus type 2 origin of DNA replication (Ad-ITR) has been described previously [12]. A 5′-labelled fragment of the top strand of the Ad-ITR containing the NFI recognition site was prepared by digestion of pEX with *Eco*RI followed by treatment with calf alkaline phosphatase and labelling with [γ-^{32}P]ATP (Amersham, specific activity 3000 Ci/mmol) and polynucleotide kinase. DNA was recut with *Bam*HI and the labelled fragment fractionated by electrophoresis in a 6% polyacrylamide gel and recovered by electroelution.

2.2 *Purification of NFI*

HeLa cell nuclear extracts were fractionated by ion exchange

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chromatography on DEAE-sepharose using the gel electrophoresis DNA assay to monitor activity [20]. NFI (0.4 μg/ml) was further purified from the pooled peak of activity by three rounds of DNA recognition site affinity chromatography [21]. Purified proteins were precipitated in 10% trichloroacetic acid, washed with 80% acetone and resuspended in SDS/β-mercaptoethanol. Samples were immersed in a boiling water bath for 3 min prior to electrophoresis in thin 10% SDS-polyacrylamide gels [22], followed by silver staining [23].

2.3. *Gel electrophoresis DNA binding assay*

A fixed amount of NFI (2 ng) was digested with 10 μg/ml Protease XIV (Sigma) before or after incubation with labelled DNA (Ad-ITR) prior to electrophoresis through a 6% polyacrylamide gel. To determine the association rate constant for NFI binding to the adenovirus origin of replication, labelled DNA (1.5 ng) was incubated with a saturating amount of purified NFI (2 ng) in a final volume of 20 μl containing 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 4 mM DTT, 100 mM NaCl and 0.5 μg BSA in the absence of carrier DNA. Incubations were carried out at 20°C for increasing time periods. A large excess of unlabelled competitor DNA (25 ng of a double-stranded oligonucleotide of the NFI recognition site) was then added to each reaction mixture and incubated for 3 min [24,25]. Reaction mixtures were loaded directly onto alternate tracks of a running 6% (60:1 acrylamide:bis-acrylamide) polyacrylamide gel and electrophoresis carried out at 150 V for 2 h. Gels were fixed in 10% acetic acid, dried and exposed to Kodak X-ray film at −70°C with an intensifying screen. Alignment of autoradiographs with dried gels allowed free and complexed DNA to be excised from individual complexes and ^{32}P radioactivity measured on an SL30 liquid scintillation spectrometer.

The NFI dissociation rate constant was measured by incubating labelled DNA (1.5 ng) with a saturating amount of purified NFI (2 ng) as described previously for 60 min at 0°C. Excess competitor DNA (25 ng) was then added and incubation continued at 20°C. At various times after addition of competitor DNA, free and bound DNA were separated by electrophoresis in a native polyacrylamide gel as described above. Further processing of the gels was also carried out as described previously.

2.4. *DNase I footprinting*

Labelled DNA (1.5 ng) was incubated with purified NFI for 60 min at 0°C in a final volume of 50 μl containing 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 4 mM DTT, 100 mM NaCl, 0.5 μg BSA and 100 ng poly dAdT + poly dGdC. Incubation mixtures were then treated with 0.25 U DNase I (Amersham) for 60 s at 24°C, and the reaction was stopped by the addition of 200 μl 1.5 M mercaptoethanol–0.3 M sodium acetate containing 100 μg/ml tRNA. DNA was phenol- and chloroform-extracted, ethanol-precipitated, and separated on a 12% polyacrylamide 50% urea sequencing gel.

3. RESULTS AND DISCUSSION

3.1. *Protease treatment of NFI*

Incubation of the full-length NFI protein with a ^{32}P-labelled fragment containing the NFI recognition site in the Ad-ITR results in the formation of multiple DNA-protein complexes as shown in fig.1A. Protease-treatment of NFI before or after incubation with labelled DNA reduced all complexes to a single NFI-DNA complex, suggesting that share a common DNA-binding domain (fig.1A). The results in fig.1B of DNase I footprinting of the full-length protein and the core DNA-binding domain reveal that both states of the protein will effectively protect the recognition site in the Ad-ITR and produce identical DNase cleavage patterns.

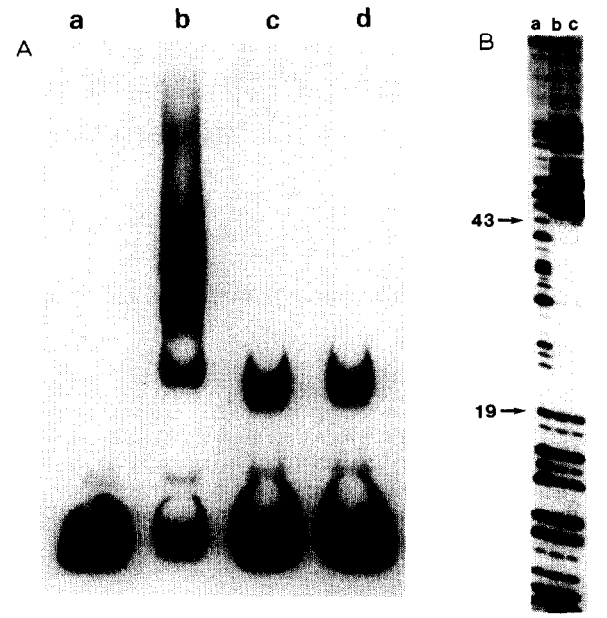

Fig.1. Protease treatment of NFI. (A) NFI was digested with 10 μg/ml Protease XIV (Sigma) before (c) or after (d) incubation with labelled DNA (Ad-ITR) prior to electrophoresis through a 6% polyacrylamide gel. a, no NFI; b, untreated NFI. (B) DNase I footprint of the core DNA-binding domain (b) and the full-length NFI protein (c) on the Ad-ITR. a, no NFI. Numbers to the left of the figure indicate bases at the boundary of the NFI recognition site.

3.2. *Determination of NFI association rate constants*

The association rate constant of NFI was determined by incubating a ^{32}P-labelled fragment of the Ad-ITR with a saturating amount of the full-length protein or the core DNA-binding domain for increasing time periods as described in section 2. The measurement of ^{32}P radioactivity in free and bound DNA demonstrated in fig.2A,B was used to plot the graph shown in fig.2C. The slopes of lines of best fit are equal to the association rate constants (k_a) of the major full-length protein complex formed ($k_{a1} = 3.2 \times 10^7\ M^{-1}s^{-1}$), the multimeric complexes (see below) formed on increased incubation ($k_{a2} = 1.8 \times 10^7\ M^{-1}s^{-1}$), and the core DNA-binding domain ($k_{a3} = 2.7 \times 10^7\ M^{-1}s^{-1}$). The additional radioactive species evident in all tracks between the specific complex and free DNA is a minor contaminant in the DNA preparation which is unaffected during the time course of the experiment.

3.3. *Determination of NFI dissociation rate constant*

We further investigated the binding kinetics of NFI interaction with its recognition site by determining the NFI dissociation rate constant using gel electrophoresis to separate DNA-protein complexes from free DNA. Increased incubation of full-length NFI and the DNA probe produces more slowly migrating multimeric species (fig.3A), which may result from interactions between DNA-bound NFI molecules. It is apparent from fig.3 that dissociation of higher order complexes

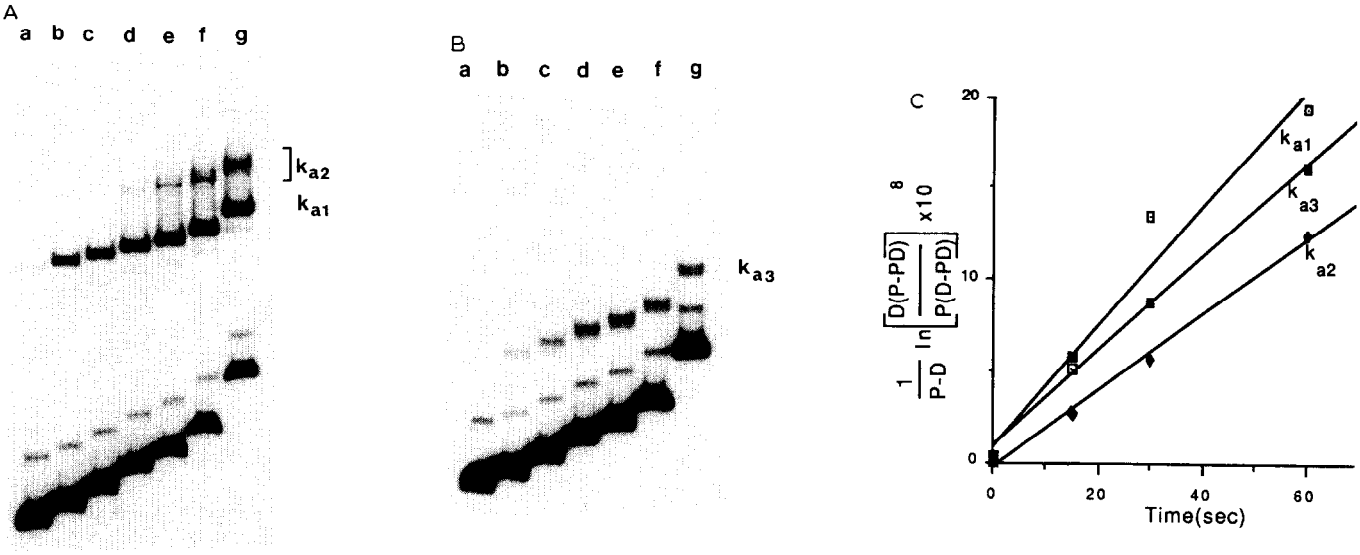

Fig.2. Determination of the NFI association rate constants. Labelled DNA (Ad-ITR) was incubated with a saturating amount of purified NFI over an increasing time period, as described in section 2, prior to electrophoresis through a 6% polyacrylamide gel. Reaction times represented are (a) 0 s, (b) 15 s, (c) 30 s, (d) 60 s, (e) 2.5 min, (f) 5 min, and (g) 15 min. (A) Full-length NFI protein. (B) Core DNA-binding domain. (C) Calculation of NFI association rate constants. Lines of best fit of mean values ($n = 3$) of

$$\frac{1}{P-D} \ln \left[\frac{D(P-PD)}{P(D-PD)}\right]$$

against time [25] were plotted where P = conc. of protein, D = conc. of DNA and PD = conc. of protein-DNA complex. The slopes of the lines (k_a) are equal to the association rate constant for the major full-length protein complex (⊡, $k_{a1} = 3.2 \times 10^7\ M^{-1}\ s^{-1}$), the multimeric NFI complexes (♦, $k_{a2} = 1.8 \times 10^7\ M^{-1}\ s^{-1}$), and the core DNA-binding-domain (■, $k_{a3} = 2.7 \times 10^7\ M^{-1}\ s^{-1}$).

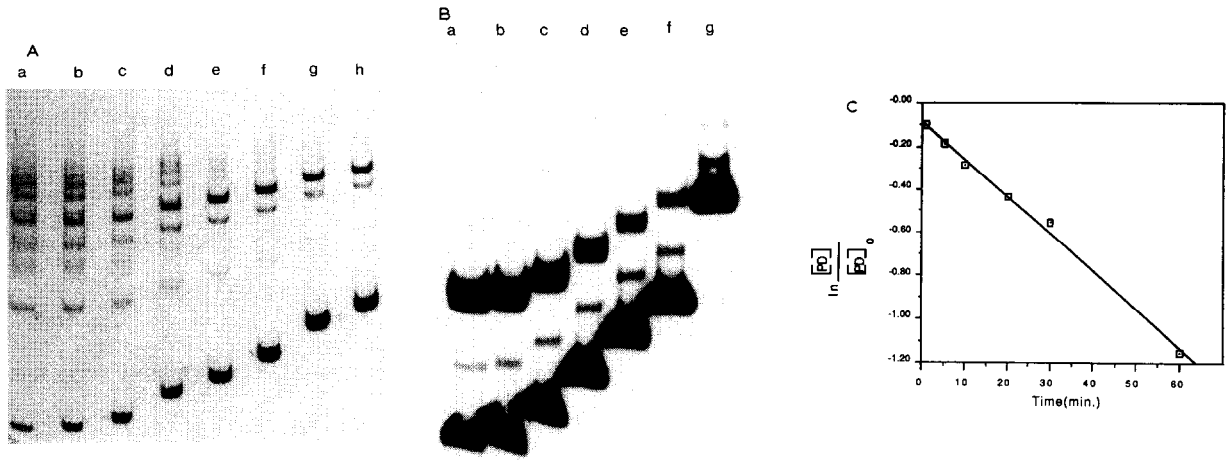

Fig.3. Determination of the core DNA-binding domain dissociation rate constant. Labelled DNA (Ad-ITR) was incubated with a saturating amount of purified NFI for 60 min at 0°C, as described in section 2. After addition of excess unlabelled competitor DNA for an increasing time period at 20°C, samples were loaded onto a running 6% polyacrylamide gel. Reaction times are (a) 0 min, (b) 1 min, (c) 5 min, (d) 10 min, (e) 20 min, (f) 30 min, (g) 60 min, and (h) 90 min. (A) Full-length NFI protein. (B) Core DNA-binding domain. (C) Calculation of the core DNA-binding domain dissociation rate constant. A line of best fit of mean values ($n = 3$) of

$$\ln \frac{[PD]}{[PD]_0}$$

against time [25] was plotted where [PD] = conc. of protein-DNA complex at time t and $[PD]_0$ = conc. of protein-DNA complex at time 0. The slope of the line is equal to the dissociation rate constant ($k_{diss} = 3.2 \times 10^{-4}\ s^{-1}$).

of the full-length protein results in an increase in the amount of lower molecular weight complexes formed with time. It is not possible, therefore, to measure a dissociation rate constant for the full-length protein over the time course of the experiment. A graphic representation of the results of total ^{32}P radioactivity in the bound and free DNA fractions using the core DNA-binding domain (fig.3B) is illustrated in fig.3C. The slope of the line of best fit is equal to the dissociation rate constant for the core protein ($k_{diss} = 3.2 \times 10^{-4}$ s^{-1}). The measurement of association and dissociation rate constants for the core DNA-binding domain permits the calculation of an equilibrium binding constant for this domain ($K_{eq} = k_a/k_{diss}$, [25]) of 8.4×10^{10} M^{-1}. The values for the association rate constants, the dissociation rate constant and the equilibrium binding constant of the core DNA-binding domain of NFI are similar to those between the CAP protein and the *lac* promoter ($k_a = 6.7 \times 10^6$ $M^{-1}s^{-1}$, $k_{diss} = 1.2 \times 10^{-4}$ s^{-1}, $K_{eq} = 5 \times 10^{10}$ M^{-1} [25]. In addition the equilibrium binding constant obtained here for human NFI recognition of the Ad-ITR is in the same range as those for porcine liver NFI binding to a variety of different recognition sites [26]. There exists a wide range of porcine NFI equilibrium binding constants, however, ranging between non-specific binding of 10^7 M^{-1} for the HSV-tk gene to 2×10^{11} M^{-1} for a fully symmetrical NFI binding site near the mouse adenovirus FL ITR [26]. It seems probable that such a range also occurs with the binding of human NFI to different recognition sites.

The reduction of all DNA-protein complexes of the full-length NFI protein to a single NFI-DNA complex after treatment with protease suggests that all the NFI species share a common DNA-binding domain and that this domain can bind efficiently to its recognition site. Recently, mutational analysis of NFI cDNAs has revealed that different regions of the protein are involved in replication and transcriptional regulation and that the N-terminal region of the protein is sufficient for both DNA binding and DNA replication [27]. The size of the core DNA-binding domain produced by protease treatment is smaller, however, than the DNA-binding domain obtained previously by mutational analysis [data not shown]. The measurement of association rate constants for the full-length protein and the core DNA-binding domain used in the present series of experiments demonstrates that the full-length protein binds at a faster rate than the core protein. The difference is minimal, however, and it appears that amino acid sequences outside the core DNA-binding domain do not strongly influence the binding of NFI to its recognition site.

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