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Characterization of the non-specific DNA binding properties of the Adenoviral IVa2 protein ☆

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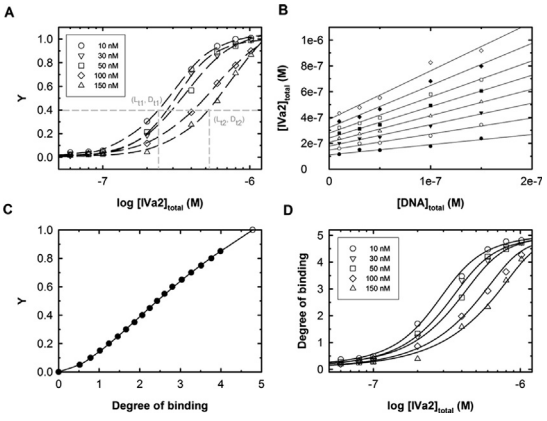
HIGHLIGHTS

- Non-specific DNA binding of the adenoviral IVa2 protein was characterized.
- Novel quantitative approach allowed rigorous characterization of DNA binding.
- IVa2 binds non-specific DNA with strong nearest neighbor cooperativity.
- Proposed IVa2 recruits other viral proteins to initiate virus assembly.

GRAPHICAL ABSTRACT



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ABSTRACT

Human Adenovirus (Ad) is a non-enveloped, icosahedral virus with a linear, double-stranded DNA genome. The Ad IVa2 protein is involved in multiple viral processes including viral late gene transcription and virus assembly. Previous studies have shown that IVa2 loads additional viral proteins onto conserved DNA elements within the Ad genome to regulate these viral processes. IVa2 also possesses strong non-specific DNA binding activity, and it is likely it uses this activity to recruit proteins to the conserved DNA elements. Here we have investigated the non-specific DNA binding activity of IVa2 using nitrocellulose/DEAE filter binding and sedimentation equilibrium techniques. We have analyzed our data using the McGhee and Von Hippel approach [1], and find that IVa2 binds with strong, positive nearest-neighbor cooperativity. In addition, we describe how to apply the McGhee and von Hippel approach to directly analyze sedimentation equilibrium data using non-linear least-squares methods. We discuss the implications of these results with respect to current virus assembly mechanisms.

Abbreviations: Ad, adenovirus; MLP, major late promoter; PS, packaging sequence; EMSA, electrophoresis mobility shift assay; NC, nitrocellulose; MvH, McGhee and von Hippel; NLLS, non-linear least square; MBDF, macromolecular binding density function.

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1. Introduction

Human adenovirus (Ad) is a non-enveloped, icosahedral virus with a linear double stranded DNA genome. Ad infection has been found in several organs and tissues [2,3], and is potentially lethal in immunocompromised patients [4,5].

The Ad IVa2 protein is a multifunctional regulator of the Ad lifecycle. IVa2 is involved in several viral processes including viral late gene transcription and virus assembly/genome packaging. IVa2 was first identified as a transcriptional activator of the viral major late promoter (MLP) which predominantly controls the genes encoding viral structural proteins [6–8]. Additional *in vivo* and *in vitro* studies have shown that the genome packaging process requires at least three viral components: the IVa2 and L4-22 K proteins, and a conserved region within the Ad genome, called packaging sequence (PS) [9–13]. The PS DNA (~200 bp) is composed of multiple recognition elements, called A repeats, which contain the following consensus sequence: 5'-TTTG (N_8) CGXG-3'. The wild type PS DNA contains 4 exact copies of this consensus sequence, called AI, AII, AV, AVI. Additional "half sites," which contain either TTTG or CGXG motifs, are also present. While deletion of the wild type PS DNA is lethal for virus assembly, there are several additional arrangements of A repeats that can support the viral lifecycle. In particular, replacing the PS DNA with two adjacent copies of the A-I-II sequence (i.e. I-II-I-II) rescues wild type viral replication activity [14].

Electrophoresis Mobility Shift Assay (EMSA) studies using Ad-infected nuclear extracts, along with a probe encompassing the A-I-II repeats, showed that the IVa2 and L4-22 K proteins specifically interact with A repeat DNA such that IVa2 recognizes the CGXG motif and L4-22 K *requires* IVa2 to interact with the TTTG motif [9,11,13–15]. Our previous studies, using the same A-I-II DNA, suggest IVa2 binds to the CGXG motif with *apparent* low specificity (~10 fold stronger affinity binding to A-I-II DNA than to a non-specific DNA of *identical length*) [16]. Thus, true specificity requires the presence of both L4-22 K and IVa2 proteins interacting with the consensus 5'-TTTG (N_8) CGXG-3' sequences [17]. While the L4-22 K protein does not bind DNA (i.e. DNA with or without the TTTG sequence) by itself, the IVa2 protein does [17]. Therefore, a tempting hypothesis is that IVa2's non-specific DNA binding activity is required to recruit L4-22 K to the A sequences.

To develop a complete quantitative understanding of how the IVa2 and L4-22 K proteins together specifically recognize the A repeats and therefore regulate the viral lifecycle, we first need to determine the non-specific binding properties of the IVa2 protein. We report here a novel application of nitrocellulose/DEAE filter binding, along with analytical ultracentrifugation, to quantify fundamental thermodynamic parameters such as the non-specific DNA binding site size, affinity and nearest neighbor cooperativity. We discuss these results in the context of current virus assembly hypotheses.

2. Materials and methods

2.1. IVa2 protein and buffer preparation

The IVa2 protein used in this and all our previous studies is an N-terminal truncated protein starting at amino acid Met 75, which has been shown previously to possess wild type viral activity [18]. This protein was overexpressed in *E. coli.* BL21 (DE3) and purified to homogeneity as described previously [16], and its DNA binding activity was determined as described previously [16]. All experiments were carried out in Buffer H (100 mM NaCl, 40 mM HEPES, pH 7.6 at 25 °C, 10 % (v/v) glycerol, 5 mM 2-mercapato ethanol and 1 mM EDTA).

2.2. DNA preparation

For the preparation of the 109 bp DNA, the pUC19 vector (Invitrogen) was transformed into *E. coli.* DH5 alpha, grown overnight in 1 L LB (37 °C), and purified using QIAGEN Plasmid Maxi kits. The 109 bp DNA was excised from purified pUC19 using BspHI and separated by gel electrophoresis. The final 109 bp DNA has > 99.9% purity as judged by densitometry analysis as well as ^{32}P-labeleing using the Klenow fill-in reaction. The concentration of purified 109 bp DNA was determined by UV spectroscopy using the calculated extinction coefficient of 1.357 μM^{-1} cm^{-1}.

For the preparation of the 241 bp DNA, we constructed a vector (pQY103) containing Ad genomic DNA from position 91 to 300 using pUC19. The DNA was amplified by PCR technique using Ad virus genomic DNA as the template. The primer sequences were as follows: forward: 5'-CCGGATCCGCGGGTGACGTAGTAGTGTGG-3'; reverse: 5'-TCCTGTCGACTCCTCTTATTCAGTTTTCCCGCG-3'. The BamHI and SalI restriction sequence are underlined. The amplified DNA was cleaved by BamHI and SalI, then cloned into pUC19. The sequence fidelity was confirmed by DNA sequencing. The pQY103 was purified as described above and digested with SmaI and HindIII. The purified DNA was labeled and its concentration determined using the calculated extinction coefficient of 3.001 μM^{-1} cm^{-1} at 260 nm.

The 241 bp (Cy3) DNA was prepared using preparative PCR. The pQY103 vector was amplified using Cy3 labeled primers:

forward: 5'Cy3-GGGGATCCGCGGGTGACGTAGTAG-3'

reverse: 5' Cy3-AGCTTGCATGCCTGCAGGTCGAC-3'

The amplified PCR reaction (1 mL) was batch-loaded onto a Q FF resin (500 μL bead volume equilibrated with TE buffer — 10 mM Tris and 1 mM EDTA at pH 8.0, with 500 mM NaCl) (GE). The column was flushed with 1.5 mL of TE buffer with 600 mM NaCl to wash off the unincorporated primers. The 241 bp (Cy3) DNA was eluted with TE buffer plus 700 mM NaCl and then precipitated by ethanol. The DNA was > 99.9% pure as judged by densitometry analysis. The concentration of purified 241 bp (Cy3) DNA was determined by UV spectroscopy using a calculated extinction coefficient of 3.001 μM^{-1} cm^{-1} at 260 nm. The percentage of 241 bp (Cy3) DNA with end-labeled Cy3 dyes was calculated using the extinction coefficient of 0.0869 μM^{-1} cm^{-1} for a single Cy3 dye at 522 nm [16] and the ratio of the absorbance at 522 to 260 nm (A_{522}/A_{260}). For each preparation of the 241 bp (Cy3) DNA, the A_{522}/A_{260} is between 0.046 and 0.049, which returns the average 80%–85% of the 241 bp (Cy3) DNA has end-labeled Cy3 dyes.

2.3. Double membrane filter binding titrations

Filter binding titrations were carried out as described by Wong et al. [19] with the following modifications. Three nitrocellulose (NC) membranes (Hybond-ECL from GE) were layered on top of three DE81 membranes (Whatman). The nitrocellulose membranes will bind protein and protein-DNA complexes, but not the unbound DNA. The DE81 membranes bind the free DNA. Both membranes were incubated in Buffer H for at least 30 min then inserted into a Minifold I Dot-Blot System (96 wells, Schleicher & Schuell). For our system, we found that a single nitrocellulose and DE81 membrane pair was unable to retain all the ^{32}P-labeled materials. Each reaction contained 1200 cpm of ^{32}P-labeled DNA, and when appropriate, non-labeled DNA was added to the indicated final concentration. All reactions were carried out in Buffer H, and were equilibrated at 25 °C for at least 50 min prior to filtration. 150 μL of Buffer H was passed through each well, followed by 25 μL of each reaction mixture, and finally each well was washed with an additional 150 μL of Buffer H. The membranes were exposed overnight to a phosphoimager screen (GE) and the intensity of each spot was quantified by densitometry analysis using Imagequant (GE). The fraction of the signal retained on the NC membranes was calculated using $Y = S_{NC}/(S_{DE} + S_{NC})$, where S_{NC} and S_{DE} are the total intensity from the three NC and DE membranes for each spot. To test whether the experiments reached equilibrium, we doubled the incubation time to 100 min; we saw no difference in the resulting isotherms.