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The binding of in vitro synthesized adenovirus DNA binding protein to single-stranded DNA is stimulated by zinc ions

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We have synthesized wild type DNA binding protein (DBP) of adenovirus type 5 (Ad5) and several truncated forms of this protein by a combination of in vitro transcription and translation. The proteins obtained were tested for binding to a single-stranded DNA-cellulose column. It could be shown that for binding of in vitro synthesized wild type DBP, it is necessary to add zinc ions to the in vitro translation system. Binding studies with the truncated proteins revealed that deletion of the carboxyl-terminal 46 amino acids abolishes DNA binding.

Adenovirus; DNA replication; DNA-binding protein

1. INTRODUCTION

Ad5 DBP is a two-domain protein of 529 amino acids [1,2] that is involved in several processes in the infection cycle. The carboxyl-terminal domain (C-domain) binds to DNA [3] and RNA [4] and is essential for adenovirus DNA replication [5]. It binds stoichiometrically to single strands [6] and stimulates DNA replication by interaction with the adenovirus DNA polymerase [7]. The C-domain is also involved in regulation of early gene expression and transformation [8,9]. Mutants in the amino-terminal domain (N-domain) have either an extended host range [10] or are distributed in a late stage of infection [11]. It has been suggested that this domain is involved in splicing [12,13].

Kitchingman [14] has proposed that three highly conserved regions of the C-domain might be involved in DNA binding. Recently, we have identified a fourth conserved region that might be involved in DNA binding [15]. It is a symmetrical arrangement of two cysteine and two histidine residues located between positions 273 and 286 in Ad5 DBP (HisXCysX$_3$GluGlyX$_3$CysXHis). These residues might form a metal-binding site, a so-called zinc-finger, although the sequence motif in DBP does not conform to the consensus sequence [16,17].

To study the DNA binding of DBP we have expressed wild type and several truncated DBPs in a combined in vitro transcription/translation system. The proteins obtained were subsequently tested for DNA binding.

2. MATERIALS AND METHODS

Two types of DNA plasmids (SP64-DBP and SP65-DBP) were constructed in which Ad5 DNA fragments coding for DBP were cloned downstream from the SP6 promoter. The plasmid SP64-DBP consists of the vector SP64 in which a *Sau*3A-*Nco*I DNA fragment of the Ad5 genome (66.5–61.0 map units (mu)) has been cloned carrying the coding region of the DBP gene. The plasmid SP65-DBP contains the vector SP65 and an Ad5 Sau3A-Sau3A DNA fragment (66.5–59.0 mu) (fig.1).

In vitro transcription was performed using 1 μg of predigested plasmid DNA in a total volume of 20 μl containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 5 mM dithiothreitol (DTT), 0.5 mM of each ribonucleoside triphosphate, 2 U/μl RNAsin and 5–10 U SP6

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Abbreviations: DBP, DNA-binding protein; Ad5, adenovirus type 5; ssDNA, single-stranded DNA

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RNA polymerase. Incubation was for 1 h at 37°C. The DNA template was removed with 1–2 U of RQ1 DNase (Promega). The RNA produced was purified by phenol/chloroform (1 : 1, v/v) extraction and isopropanol or ethanol precipitation. It was redissolved in 10–20 μl of RNase free TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 1 U/μl RNAsin.

About 0.5–1.0 μg of RNA was used in a standard in vitro translation. The reaction mix consisted of 14 μl micrococcal nuclease treated rabbit reticulocyte lysate (Promega), 0.4 μl of a 1 mM amino acid mixture (without methionine), 7.5–15 μCi of [^{35}S]methionine (>1200 Ci/mmol) and 1 U/μl RNAsin in a total volume of 20 μl. Incubation was for 1 h at 30°C. Large scale preparations were performed in the same way with the exception that RNAsin was omitted and that RNase was added to a final concentration of 20 μg/ml when the translation reaction was completed. This step was necessary to remove all RNA present in the reticulocyte lysate. The RNase had been heated at 100°C to denature possible contaminating DNase. After RNase treatment for 0.5 h at 30°C, EDTA was added to the mixture to a final concentration of 10 mM.

10 μl of each translation reaction was layered on a standard 10% SDS-polyacrylamide gel [18]. Electrophoresis was for 16 h at 60 V in a Tris-glycine buffer (50 mM Tris, 400 mM glycine) containing 0.1% SDS. Autoradiography of the dried gel was usually for 18 h at −80°C using an intensifying screen and Fuji film.

ssDNA-cellulose chromatography was performed as described [19] using a small column with a bed volume of 2.0 ml. Proteins were eluted using a stepwise NaCl gradient from 50 mM to 2 M in 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/l L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) and 1 mM β-mercaptoethanol. Addition of EDTA was necessary to counteract the effects of the nuclease in the reticulocyte lysate. Samples of 30 μl of each gradient fraction were assayed by electrophoresis.

3. RESULTS AND DISCUSSION

For the synthesis of wild type and truncated DBPs the plasmids SP64-DBP and SP65-DBP were digested prior to the transcription reaction with restriction enzymes that cleaved the DBP coding region once (*Bgl*I, *Hin*dII, *Eco*RV and *Aos*I) or not at all (*Eco*RI and *Hin*dIII) (fig.1). The main protein product of the full-length coding region (*Eco*RI or *Hin*dIII cleaved plasmids) had an estimated size of 70 kDa (fig.2). The difference with the expected apparent molecular mass of 72 kDa might be explained by the absence of phosphates on the in vitro generated DBP.

To study their DNA binding capacity the DBPs were subjected to ssDNA-cellulose chromatography. In initial experiments with wild type DBP, most of the protein did not bind to the column (fig.2). This is probably due to an incorrect conformation of the molecules that had been synthesized in the in vitro translation system. A similar lack of DNA binding of DBP synthesized in a reticulocyte lysate system was observed by Asselbergs et al. [20]. Since we have tentatively identified a zinc-binding domain in the DBP molecule on the basis of comparisons of the amino acid sequences of dif-

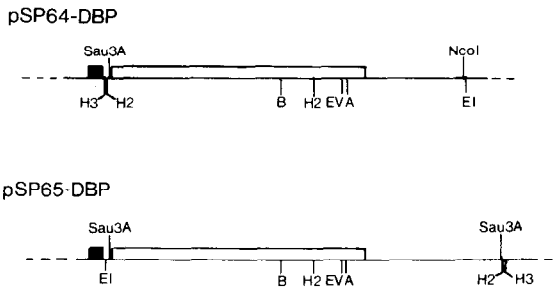

Fig.1. Schematic presentation of the plasmids SP64-DBP and SP65-DBP. The black boxes represent the SP6 promoter, the open bar the coding region of the Ad5 DBP gene. Relevant restriction sites are indicated: A, *Aos*I; B, *Bgl*I; EI, *Eco*RI; EV, *Eco*RV; H2, *Hin*dII; H3, *Hin*dIII.

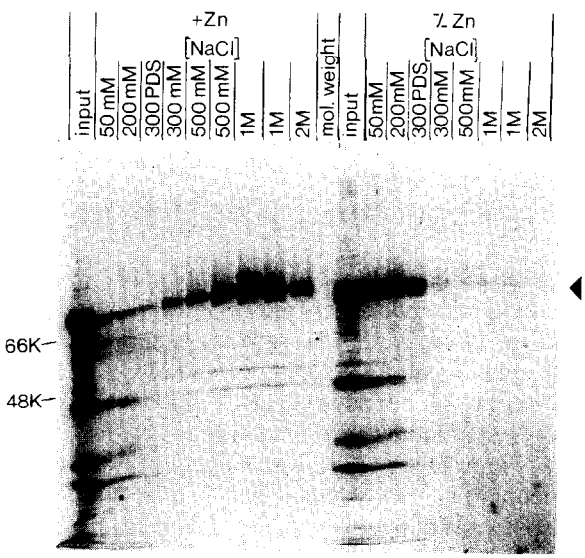

Fig.2. ssDNA-cellulose chromatography of ^{35}S-labeled wild type Ad5 DBP synthesized in vitro in the presence (+Zn) or in the absence (−/−Zn) of 1 mM zinc acetate. The proteins bound to the ssDNA-cellulose were stepwise eluted at increasing NaCl concentration. PDS = 0.2% potassium dextran sulphate. Samples of 30 μl of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis. The arrowhead indicates the position of the synthesized full length DBP.

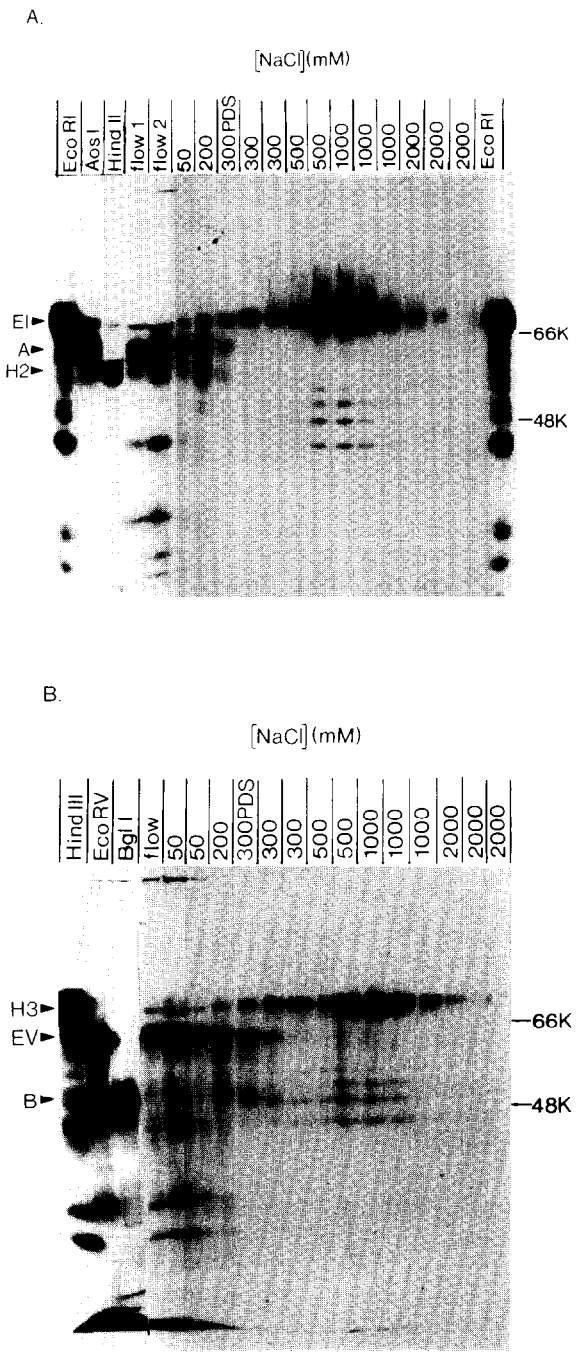

Fig.3. ssDNA-cellulose chromatography of ^{35}S-labeled DBPs synthesized in vitro in the presence of 1 mM zinc acetate. The expression plasmids were cleaved with (A) *Eco*RI (EI), *Aos*I (A) and *Hin*dII (H2) leading to full-length DBP (529 amino acids) and truncated proteins of 483 and 414 amino acids, respectively and with (B) *Hin*dIII (H3), *Eco*RV (EV) and *Bgl*I (B) leading to synthesis of full-length DBP and truncated DBPs of 470 and 345 amino acids, respectively. The arrows indicate the positions of the various proteins. Elution was performed stepwise at increasing NaCl concentrations. PDS = 0.2% potassium dextran sulphate. Samples of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis. The input preparations are shown in the left lanes.

ferent DBPs [15], zinc acetate was added to the in vitro translation reaction to a concentration of 1 mM. The addition of zinc improves the binding of DBP to ssDNA dramatically, suggesting that the presence of zinc is required for the formation of native DBP (fig.2). Therefore, zinc was included in all subsequent translation reactions. Addition of zinc acetate to the translation mixture after translation did not lead to ssDNA binding of the synthesized DBP (not shown).

The most attractive explanation for the zinc effect is that the addition of Zn^{2+} to the translation reaction allows the DBP to fold in its native conformation probably by the formation of a zinc-binding domain. Several nucleic acid binding proteins carry a so-called zinc finger, that has been shown to be absolutely necessary for binding of the protein to DNA [21–23], while in many other DNA and RNA binding proteins a putative zinc binding domain can be recognized ($CysX_{2-4}CysX_n$-$PheX_nLeuX_nHisX_3His$, X is any amino acid) [16]. In contrast to these domains, the putative zinc binding domain of DBP ($HisXCysX_3GluGlyX_3$-$CysXHis$) contains only one amino acid residue between the two Cys/His ligands at either side of the structure. A possible structure for the DBP Zn-binding domain might be a combination of two beta sheets separated by a turn. The putative Zn-binding domain is exceptionally stable, since the DNA binding of DBP is resistant to prolonged exposure to 10 mM EDTA [6].

Previous experiments with chymotrypsin-cleaved DBP have placed the amino-terminal border of the DNA binding domain of DBP around amino acid 174 [3,24]. We have attempted to establish the carboxyl-terminal border of the region of DBP involved in DNA binding by analysis of full-length DBP and different truncated DBPs on a single ssDNA-cellulose column (fig.3). Only the full-length DBP molecules and some of their degradation products were able to bind to the

column, while all truncated proteins did not bind to the column at all or could be eluted from the columns with the low salt washes. The experiment with the *AosI* cleaved expression plasmid shows that even the carboxyl-terminal 46 residues of the DBP molecule are absolutely required for binding to ssDNA-cellulose since the corresponding protein is not able to bind to DNA, despite the fact that it still contains all the conserved regions as identified by Kitchingman [14] and us [15]. Clearly, these regions are not sufficient for DNA binding. Interestingly, the secondary mutation of the r13 pseudo-revertant, that compensates the defect of the original mutation in the H5ts125 mutant at position 413, is located in this region, at 21 residues from the C-terminus of the molecule [2]. It is possible that the 46 residue long C-terminal region is directly involved in DNA binding, since in analogy with the T_4 gene 32 protein it contains several aromatic residues that might participate in the binding of DBP to DNA [25]. Alternatively, the loss of this region might result in a change in the tertiary structure of a large part of the DBP molecule, leading to loss of the ability to bind to DNA.

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REFERENCES

[1] Kruijer, W., Van Schaik, F.M.A. and Sussenbach, J.S. (1981) Nucleic Acids Res. 9, 4439–4457.
[2] Kruijer, W., Nicolas, J.C., Van Schaik, F.W.A. and Sussenbach, J.S. (1983) Virology 124, 425–433.
[3] Ariga, H., Klein, H., Levine, A.J. and Horwitz, M.S. (1980) Virology 101, 415–424.
[4] Cleghon, V.G. and Klessig, D.F. (1986) Proc. Natl. Acad. Sci. USA 83, 8947–8951.
[5] Van der Vliet, P.C. and Sussenbach, J.S. (1975) Virology 67, 415–426.
[6] Van der Vliet, P.C., Keegstra, W. and Jansz, H.S. (1978) Eur. J. Biochem. 86, 389–398.
[7] Lindenbaum, J.O., Field, J. and Hurwitz, J. (1986) J. Biol. Chem. 261, 10218–10227.
[8] Carter, T.H. and Blanton, R.A. (1978) J. Virol. 25, 664–674.
[9] Ginsberg, H.S., Ensinger, M.J., Kaufmann, R.S., Mayer, A.J. and Lundholm, U. (1974) Cold Spring Harbor Symp. Quant. Biol. 39, 419–426.
[10] Klessig, D.F. and Grodzicker, T. (1979) Cell 17, 957–966.
[11] Edvardsson, B., Ustacelebi, S., Williams, J. and Philipson, L. (1978) J. Virol. 25, 641–651.
[12] Klessig, D.F. and Chow, L.T. (1980) J. Mol. Biol. 139, 221–242.
[13] Andersson, K.P. and Klessig, D.F. (1984) Proc. Natl. Acad. Sci. USA 81, 4023–4027.
[14] Kitchingman, G.R. (1985) Virology 146, 90–101.
[15] Vos, H.L., Van der Lee, F.M., Reemst, A.M.C.B., Van Loon, A.E. and Sussenbach, J.S. (1988) Virology 163, 1–10.
[16] Berg, J.M. (1986) Science 232, 485–487.
[17] Berg, J.M. (1988) Proc. Natl. Acad. Sci. USA 85, 99–102.
[18] Laemmli, U. (1970) Nature 227, 680–685.
[19] Van der Vliet, P.C., Zandberg, J. and Jansz, H.S. (1977) Virology 80, 98–110.
[20] Asselbergs, F.A.M., Mathews, M.B. and Smart, J.E. (1983) J. Mol. Biol. 163, 177–207.
[21] Miller, J., McLachlan, A.D. and Klug, A. (1985) EMBO J. 4, 1609–1610.
[22] Kadonaga, J.T., Carner, K.R., Masiarz, F.R. and Tjian, R. (1987) Cell 51, 1079–1090.
[23] Nagai, K., Nakaseko, Y., Nasmyth, K. and Rhodes, D. (1988) Nature 332, 284–286.
[24] Tsernoglou, D., Tsugita, A., Tucker, A.D. and Van der Vliet, P.C. (1985) FEBS Lett. 188, 248–252.
[25] Prigodich, R.V., Casas-Finet, J., Williams, K.R., Konigsberg, W. and Coleman, J.E. (1984) Biochemistry 23, 522–529.