Characterization of the chymotryptic core of the adenovirus DNA-binding protein

D. Tsernoglou, A. Tsugita, A.D. Tucker and P.C. van der Vliet*

European Molecular Biology Laboratory, D-6900 Heidelberg, FRG, and **Laboratory for Physiological Chemistry, State University of Utrecht, Vondellaan 24a, 3521 GG Utrecht, The Netherlands*

Received 28 May 1985

A fragment of the DNA-binding protein of adenovirus type 5 has been obtained by controlled chymotryptic digestion of the entire molecule. Partial sequence determination indicates that the fragment consists of amino acids 174–525. The fragment is biologically active as measured by its ability to substitute for the entire molecule in a reconstituted DNA replication system. Crystals have been obtained that show diffraction to 2 Å.

Adenovirus *DNA replication* *DNA-binding protein* *Chymotryptic core* *Crystallization*

1. INTRODUCTION

The adenovirus DNA-binding protein (DBP) is one of the best characterized eucaryotic DNA-binding proteins. It is the major product of the early region E2a and is produced in large amounts, up to 2×10^7 molecules per cell, during lytic infection [1]. The protein has an apparent M_r of 72 000 upon SDS-polyacrylamide gel electrophoresis and it contains 529 amino acids of known sequence [2]. DBP is a multi-functional protein that is necessary for viral DNA replication [3,4] and is involved in regulation of viral RNA synthesis [5,6], host range specificity [7], transformation [8] and possibly virus assembly [9]. The protein binds to single-stranded DNA [10] and interacts with the ends of double-stranded DNA [11].

Evidence has been obtained that DBP contains 2 functional domains which can be separated by chymotrypsin treatment [12,13]. The exact border of these domains is unknown. The N-terminal domain, consisting of about one-third of the molecule, is highly phosphorylated and does not bind to DNA. Mutations in this part of the DBP influence mainly host specificity and late gene expression [2,14]. The C-terminal domain binds to DNA. Mutations located in this part display a DNA replication deficient phenotype and influence early gene expression [5,15].

Here we report the exact composition of a chymotryptic fragment from the C-terminal domain that is biologically active and has been crystallized.

2. EXPERIMENTAL

We have described [16] that, when DBP is set up for crystallization, crystals of a fragment can be obtained. The fragment appeared to have an M_r of about 40 000 and it could also be obtained and crystallized when chymotrypsin was added. We have further examined the digestion of DBP by chymotrypsin with the aim of controlling the reaction to obtain better crystals and to define chemically the fragment crystallized. The time course of digestion of DBP by chymotrypsin is shown in fig.1. The best crystals were obtained during digestion of DBP at 6 mg/ml in 10 mM

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

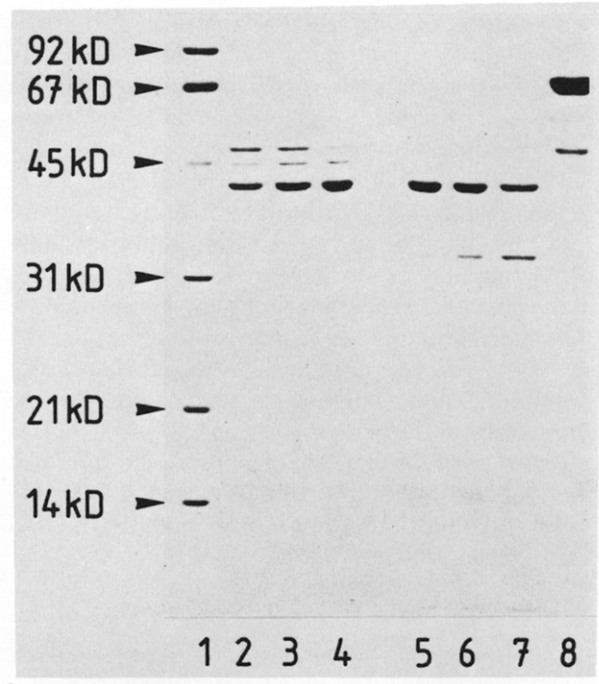

Fig.1. Chymotrypsin digestion of Ad5 DNA binding protein. Ad5 DBP was digested for various times with chymotrypsin under the conditions described in the text. Lane 1, standards. Lanes 2–7, incubation for 1,2,3,4,5,6 h, respectively. Lane 8, starting material.

Tris-HCl, pH 8.0, 2 M NaCl with 10 μg/ml chymotrypsin (Sigma) for 4 h at 25°C. The reaction was stopped by the addition of excess benzamidine (final concentration 5 mM). Crystals (shown in fig.2) were grown from a solution of 0.5% protein, 100 mM phosphate buffer, pH 7.2, 1.6 M NaCl, 5% ethanol. They grew over a period of 2 weeks to a maximum size of 0.7 mm × 0.5 × 0.4 mm. On 'stills' the crystals show diffraction to about 2 Å. A rotation photograph is given in fig.3. Precession photographs show that the crystals are isomorphous to those obtained earlier [16], i.e. orthorhombic, space group $P2_12_12_1$, with a = 79.1 Å, b = 75.7 Å, and c = 67.4 Å. We have measured a complete set of diffraction data to 2.5 Å resolution by means of rotation photography and we are searching for heavy atom derivatives.

2.1. *Amino acid composition of the fragment*

Biochemical analysis of the fragment was performed on material obtained from dissolved crystals as well as from the appropriate band in SDS gels, corresponding to M_r of 39 000 (see fig.1). The band was cut out and soaked in 10-times its volume of 70% formic acid overnight at room temperature. The extraction was repeated and the formic acid was evaporated. The residue was dissolved in 70% formic acid and chromatographed on Biogel P10 [17]. Amino acid analysis was performed on 5 μg of the fragment [18] and the amino-terminal sequence was analyzed as previously described [19] using 500 pmol of the crystalline protein fragment or 750 pmol of the protein isolated from SDS gels. Both for the crystals and the protein from the gel the amino-terminal sequence was:

SVPIVXAWEKGMEAA (V)

This result places the N-terminus of the fragment at position 174 of the sequence of the entire

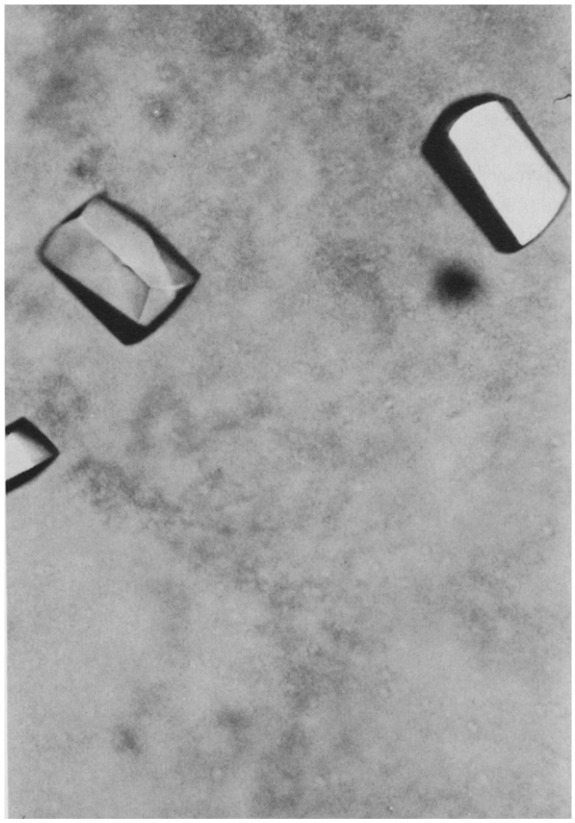

Fig.2. Crystals of the 39 kDa fragment.

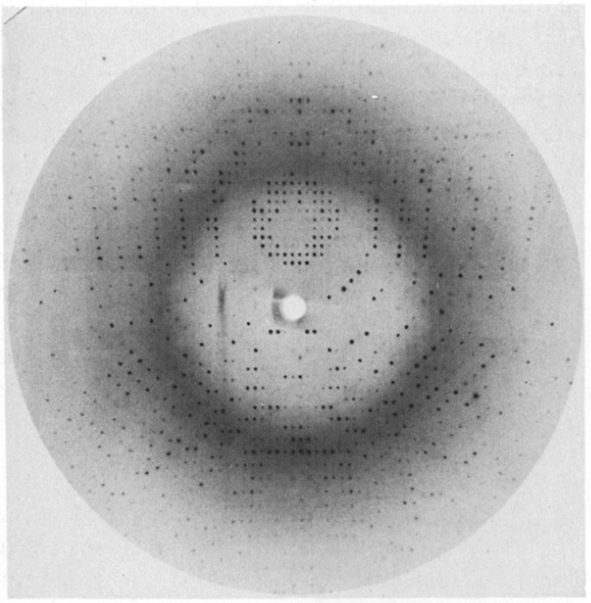

Fig.3. Rotation photograph taken on an Elliott GX21 at 33 kV, 130 mA. Crystal to film distance is 75 mm, temperature 2°C. The edge of the circle corresponds to 2.3 Å.

A

	Crystal		39 kDa protein (gel) 40 min	DNA sequence 174–525
	20 min	40 min		
D + N	32.6	35.8	32.5	34
T	9.4	11.4	12.8	13
S	21.3	23.4	25.3	24
E + Q	39.9	39.7	37.8	37
P	17.2	17.6	17.8	17
G	22.2	22.8	28.2	18
A	35.0	35.0	35.0	35
V	18.9	19.4	19.4	24
M	8.2	7.9	7.9	11
I	8.4	8.7	9.0	11
L	29.4	31.6	31.2	33
Y	4.3	4.6	5.5	6
F	14.6	15.6	15.2	16
H	14.9	14.8	15.0	15
K	19.4	20.0	21.0	23
R	15.4	14.8	15.4	16
C	12.6		12.5	13
W	–		–	6
Σ				352 (M_2 39.391)

molecule [2] (see fig.4B). The amino acid composition (fig.4A), in conjunction with the M_r of 39 000, suggests that 4–5 amino acids are missing from the carboxy-terminus. To determine the C-terminus, 250 pmol of the crystalline fragment or the chymotryptic fragment from the SDS gel were dissolved in 0.1 M pyridine acetate buffer containing 0.5% oligo-oxyethylene. 5 μg carboxypeptidase A (Sigma, diisopropylphosphate-treated) was added and incubated for various times. This procedure liberated Asn and Glu. Since carboxypeptidase A will not hydrolyze arginine, this means that the C-terminal amino acid both in the crystal and in the protein from the gel is Asn at position 525. This is supported by the fact that the amino acid composition of 174–525 shows the best match with the results obtained by amino acid analysis (fig.4A) and the observed M_r.

2.2. *The fragment is active in DNA replication*

We compared the biological activity of the fragment with that of the intact DBP by analysis in a reconstituted DNA replication system. Ad5 DNA, isolated from virions and complexed with the ter-

```
  1  MASREEEQRETIPERGRGAARRPPIMEDVSSPSPSPPPPRAPPKKRMRRR
 51  IESEDEEDSSQDALVPRIPSPRPSTSAADLAIAPKKKKKRPSPKPERPPS
101  PEVIVDSEEEREDVALQMVGFSNPPVLIKHGKGGKRIVRRLNEDDPVARG
151  MRIQEEEEEPSEAESEITVMNPLSVPIVSAWEKGMEAARALMDKYHVDND
201  LKAMFKLLPDQVEALAAVCKTWLNEEHRGLQLTFISNKIFVTMMGRFLQA
251  YLQSFAEVTYKHHEPIGCALWLHRCAEIEGELKCLHGSIMINKEHVIEMD
301  VISENGQRALKEQSSKAKIVKNRWGRNVVQISNTDARCCVHDAACPANQF
351  SGKSCGMFFSEGAKAQVAFKQIKAFMQALYPNAQTGHGHLLMPLRCECNS
401  KPGHAPFLGRQLPKLTPFALSNAEDLDADLISDKSVLASVHHPALIVFQC
451  CNPVYRNSRAQGGGPNCDFKISAPDLLNALVMVRSLWSENFTELPRMVVP
501  EFKWSTKHQYRNVSLPVAHSDARQNPFDF-COOH
```

Fig.4.(A) Amino acid composition (mol%) of the crystalline DBP fragment and the corresponding 39 kDa protein from SDS-polyacrylamide gels. (B) Amino acid composition of Ad5 DBP (from [2]). The arrows indicate the borders of the crystallized fragment.

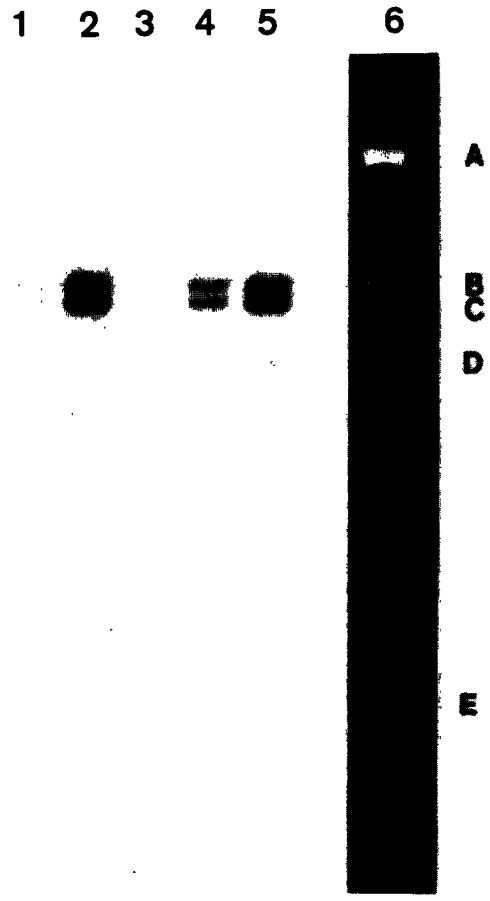

Fig.5. The fragment supports DNA replication in vitro. Reaction mixtures (30 μl) contained 4.5 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2.0 mM ATP, 7.5 mM creatine phosphate, 7.5 μg/ml creatine kinase, 20 μM each of dATP, dGTP and dTTP, 2.5 μM [α-^{32}p]dCTP (16 Ci/mmol), 3 mU pTP-pol, 2 μl nuclear extract, 1 μg cytosol RNA [21], 30 ng DNA-TP previously digested with *Xho*I, and variable amounts of DBP. After 60 min at 37°C the mixture was analyzed by agarose gel electrophoresis in 1% gels containing 0.1% SDS [20]. Lanes 1–5 show an autoradiogram and lane 6 the input DNA, stained with ethidium bromide. Lane 1, 0.3 μg DBP; lane 2, 1 μg DBP, lane 3, no DBP; lane 4. 0.3 μg fragment; lane 5, 1 μg fragment.

minal protein (DNA-TP) was digested with *Xho*I to produce two terminal fragments B and C, of 6146 and 5644 base-pairs, respectively. The mixture of fragments was incubated with purified DBP or fragment, the complex of preterminal protein and DNA polymerase (pTP-pol) and a nuclear extract from uninfected cells under conditions of DNA replication, in the presence of [α-^{32}P]dCTP. After 60 min at 37°C the reaction mixture was analyzed on an agarose-SDS gel as described [20]. The results (fig.5) show that without DBP, no replication is observed. Both in the presence of DBP and of the fragment, specific labeling of the origin containing fragments B and C is observed, indicating that the fragment can support DNA replication as well as the intact molecule. The activity of the 39 kDa fragment was 82% of that of the 72 kDa protein at 30 μg/ml, while at 10 μg/ml the 39 kDa fragment was 1.3-times more active.

3. DISCUSSION

The functions of DBP in DNA replication and regulation of late transcription are located in physically separated domains and may act independently [15,22]. Several reports have shown that DBP can be degraded using chymotrypsin [12,13,23], trypsin [23] or pronase [23] producing relatively stable fragments. For chymotrypsin digestion, C-terminal fragments with M_r ranging from 34000 to 45000 have been reported [13,24]. We show here that under high salt digestion conditions a major chymotryptic fragment of 352 amino acids is obtained with a calculated M_r of 39391. A minor degradation product of about 34 kDa is found also, while upon longer incubation smaller fragments are produced (see fig.1), but only the 39 kDa fragment forms crystals. The preferred chymotryptic site at position 173/174 is located within a region (amino acids 167–181) which is very hydrophobic and for the most part conserved in Ad5, Ad7, and Ad12 [15,25]. The remaining N-terminal part of DBP is less conserved than the C-terminal part. It has an aberrant amino acid composition, containing the majority of the Pro and Glu residues, no Cys, Tyr or Trp, and only one His and Phe residue out of the 16 and 19 that are present in the entire molecule. This part may account for the aberrant mobility of the DBP upon electrophoresis in SDS-polyacrylamide gels and could be responsible for the highly asymmetric configuration of the native molecule as concluded from hydrodynamic studies [10,23,26]. The presence of the N-terminal part probably prevents crystallization of the intact protein. Interestingly, crystallization of a fragment of the molecule has

also been observed for T4 gene 32 helix destabilizing protein [27,28] and the *E. coli* single-stranded DBP [29,30]. The 174–525 fragment could substitute for the intact protein in DNA replication. The specific activity which we observed was dependent on the concentration but of the same magnitude as that of the intact molecule.

We also attempted to assay the activity of the crystalline material but the crystals could only be dissolved under denaturing conditions, such as high pH. Our results fit with those of others showing that C-terminal fragments could complement DNA-negative H5ts125 mutant extracts [24,31]. Apparently this fragment is functional in the absence of the N-terminal domain. Its 3-dimensional structure is currently under investigation.

ACKNOWLEDGEMENTS

We thank R. Schiphof for growing cells, M.M. Kwant for expert technical assistance, and L. Philipson for advice. This work was supported in part by The Netherlands Foundation for Chemical Research (SON) with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO).

REFERENCES

[1] Van der Vliet, P.C. and Levine, A.J. (1973) Nat. New Biol. 246, 170–174.
[2] Kruijer, A., Van Schaik, F.M.A. and Sussenbach, J.S. (1981) Nucleic Acids Res. 9, 4439–4457.
[3] Van der Vliet, P.C., Zandberg, J. and Jansz, H.S. (1977) Virology 80, 98–110.
[4] Horwitz, M.S. (1978) Proc. Natl. Acad. Sci. USA 75, 4291–4295.
[5] Carter, T.H. and Blanton, R.A. (1978) J. Virol. 25, 664–674.
[6] Babich, A. and Nevins, J.R. (1981) Cell 26, 371–379.
[7] Klessig, D.F. and Grodzicker, T. (1979) Cell 17, 957–966.
[8] Ginsberg, H.S., Ensinger, M.J., Kauffman, R.S., Mayer, A.J. and Lundholm, U. (1974) Cold Spring Harbor Symp. Quant. Biol. 39, 419–426.
[9] Nicolas, J.C., Sarnow, P., Girard, M. and Levine, A.J. (1983) Virology 126, 228–239.
[10] Van der Vliet, P.C., Keegstra, W. and Jansz, H.S. (1978) Eur. J. Biochem. 86, 389–398.
[11] Fowlkes, D.M., Lord, S.T., Linné, T., Pettersson, U. and Philipson, L. (1979) J. Mol. Biol. 132, 163–180.
[12] Klein, H., Maltzman, W. and Levine, A.J. (1979) J. Biol. Chem. 254. 11051–11060.
[13] Linné, T. and Philipson, L. (1980) Eur. J. Biochem. 103, 259–270.
[14] Anderson, C.W., Hardy, M.H., Dunn, J.J. and Klessig, D.F. (1983) J. Virol. 48, 31–39.
[15] Kruijer, W., Van Schaik, F.M.A., Speyer, J.G. and Sussenbach, J.S. (1983) Virology 128, 140–153.
[16] Tsernoglou, D., Tucker, A.D. and Van der Vliet, P.C. (1984) J. Mol. Biol. 172, 237–239.
[17] Dianoux, A.-C., Vignais, P.V., Tsugita, A. (1981) FEBS Lett. 130, 119–124.
[18] Tsugita, A. and Scheffler, J.J. (1982) Eur. J. Biochem. 124, 585–588.
[19] Riccio, P., Tsugita, A., Bolba, A., Vilbois, F. and Quagliariello, E. (1985) Biochem. Biophys. Res. Commun., in press.
[20] Van Bergen, B.G.M., Van der Leij, P.A., Van Driel, W., Van Mansfeld, A.D.M. and Van der Vliet, P.C. (1983) Nucleic Acids Res. 11, 1975–1989.
[21] Van der Vliet, P.C., Van Dam, D. and Kwant, M.M. (1984) FEBS Lett. 171, 5–8.
[22] Rice, S.A. and Klessig, D.F. (1984) J. Virol. 49, 35–49.
[23] Schechter, N.M., Davies, W. and Anderson, C.W. (1980) Biochemistry 19, 2802–2810.
[24] Friefeld, B.R., Krevolin, M.D. and Horwitz, M.S. (1983) Virology 124, 380–389.
[25] Quinn, C.O. and Kitchingham, G.R. (1984) J. Biol. Chem. 259, 5003–5009.
[26] Sugawara, K., Gilead, Z. and Green, M. (1977) J. Virol. 21, 338–346.
[27] Cohen, J., Chiu, W. and Hosoda, J. (1983) J. Mol. Biol. 169, 235–248.
[28] McKay, D.B. and Williams, K.R. (1982) J. Mol. Biol. 160, 659–661.
[29] Ollis, D., Brick, P., Abdel-Meguid, S.S., Murthy, K., Chase, J.W. and Steitz, T.A. (1983) J. Mol. Biol. 170, 797–801.
[30] Hilgenfeld, R., Saenger, W., Schomburg, O. and Krauss, G. (1984) FEBS Lett. 170, 143–151.
[31] Van Bergen, B.G.M. and Van der Vliet, P.C. (1983) J. Virol. 46, 642–648.