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Adenovirus-Specific DNA-Binding Protein Inhibits the Hydrolysis of DNA by DNase In Vitro

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The adenovirus-specific DNA-binding protein was isolated from adenovirus type 5-infected KB cells and shown to possess DNase inhibitor activity. The protein decreased the rate of hydrolysis of single-strand DNA proportionately to its concentration in the reaction. Two peaks of activity were obtained upon sedimentation in a glycerol gradient, probably corresponding to the two major adenovirus-specific polypeptides in the preparation (molecular weights, 72,000 and 44,000). The DNase inhibitor activity of the adenovirus DNA-binding protein was distinguishable from that of the cellular DNA-binding protein, which we have described previously (K. Nass and G. D. Frenkel, J. Biol. Chem. **254**:3407-3410, 1979), by its pattern of sedimentation and by the effect of temperature on the two activities. For the adenovirus DNA-binding protein, the ratio of DNase inhibitor activity at 43°C to that at 30°C was approximately 14, whereas for the cellular protein this ratio was less than 3. The DNase inhibitor activity with the temperature coefficient of 14 was absent from cells infected with adenovirus type 5 *ts*125 at 40°C. DNase inhibition is a simple, sensitive, quantitative method for assay of the adenovirus DNA-binding protein.

Infection of cells by any of several adenovirus serotypes results in the appearance of a DNA-binding protein (DBP) with specific affinity for single-stranded DNA (3, 23, 29). The proteins induced by adenovirus type 5 (Ad5) and Ad2 have been extensively characterized (11, 15, 25). They have a molecular weight in sodium dodecyl sulfate (SDS) of 72,000 and are phosphorylated (1, 8, 13, 24). They bind very rapidly to single-stranded DNA in a cooperative fashion to form a stable complex in which the DNA is in an extended conformation. At saturation, one molecule of protein is bound per seven nucleotides of DNA (28).

The Ad5-induced DBP has been shown to be coded by the region of the viral genome located between 0.59 and 0.71 map units from the left end (14). A mutant virus, Ad5 *ts*125 (2), which carries a mutation in this region (5), fails to induce the DBP at the nonpermissive temperature (30). Analysis of the phenotype of the mutant led originally to the conclusion that the DBP is required only for initiation of a round of viral DNA replication (31). However, the subsequent observation that antibody to the DBP inhibits viral DNA synthesis in nuclei demonstrated its involvement in chain elongation as well (32). This conclusion has been supported by the observed temperature sensitivity of DNA replication in soluble extracts of *ts*125-infected cells (6, 7, 9) and by the isolation of the DBP bound to the single-stranded regions of replicating viral DNA molecules (10).

These studies have clearly demonstrated that the adenovirus-specific DBP plays an essential role in viral DNA replication. However, its precise function in the process remains to be determined. We have previously suggested (18) that one role of the protein may be to protect the single-stranded regions of replicating viral DNA molecules (20, 21, 26, 27) from degradation by DNase. This suggestion was prompted by our observation that after adenovirus infection there is a progressive decrease in detectable DNase activity in the cell and that this decrease does not occur after infection by *ts*125 at the nonpermissive temperature (18). In this paper we describe experiments which demonstrate that the DBP can in fact protect single-stranded DNA from hydrolysis by cellular DNase. We also describe experiments which distinguish the DNase inhibitory activity of the adenovirus-specific DBP from that of the cellular DBP which we described previously (19).

MATERIALS AND METHODS

Materials. Adenovirus [^{3}H]DNA, purchased from Bethesda Research Laboratories, Inc., was denatured with alkali. Bovine serum albumin (Miles Laboratories, Inc.) was the crystalline grade. Polyacrylamide gel electrophoresis reagents were obtained from Bio-rad Laboratories, and S1 nuclease was obtained from Calbiochem.

Cells and virus. KB cells were grown in suspension cultures in minimal essential medium (Flow Laboratories, Inc.) containing 10% heat-inactivated fetal calf serum. Adenovirus H5 wild type or *ts*125 (2) was

propagated in KB cells in medium containing 2% heat-inactivated fetal calf serum.

KB DNase. A partial purification procedure has been described previously (19). For the present experiments we have used enzyme that was further purified by phosphocellulose chromatography. A complete description of the purification and characterization of the enzyme will appear in a separate publication (N. Berns, K. Randles, and G. D. Frenkel, manuscript in preparation).

Assay of DNA hydrolysis. The reaction mixture (0.3 ml) contained 33 mM Tris-hydrochloride buffer (pH 7.5), 6.7 mM $MgCl_2$, 10 mM mercaptoethanol, 0.2 mg of bovine serum albumin per ml, 0.4 nmol of denatured adenovirus [^{3}H]DNA (10^5 cpm/nmol), and KB DNase. After incubation for 30 min at 37°C, the reaction was chilled, and acid-soluble nucleotide was determined as described previously (18).

Polyacrylamide gel electrophoresis in SDS. Protein samples were prepared and analyzed in slab gels, as described previously (19).

RESULTS

Isolation of the adenovirus DBP. KB cells were infected with Ad5 and incubated at 37°C. At 24 h after infection, an extract was prepared, and the adenovirus-specific DBP was isolated by DNA-cellulose chromatography, by a modification of the method of Levinson et al. (12), as described previously for the cellular DBP (19). The DNA-cellulose eluates were dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.1) containing 20 mM NaCl, 5 mM EDTA, and 10% (wt/vol) glycerol and were stored at 0°C. The adenovirus-specific DBP has previously been shown to elute primarily with 1.0 M NaCl, although a (variable) percentage does elute with 0.5 M NaCl (29). In contrast, the cellular DBP elutes with 0.3 M and 0.5 M NaCl; little if any DBP can be detected eluting with 1.0 M NaCl (19). Analysis of the DNA-cellulose eluates by polyacrylamide gel electrophoresis in SDS is shown in Fig. 1. The 1.0 M NaCl eluate from adenovirus-infected cells (lane B) had two major polypeptides, characteristic of the 72,000- and 44,000-dalton species associated with the adenovirus-specific DBP. The latter polypeptide has been shown to be a proteolytic cleavage product of the former (22). The corresponding 1.0 M NaCl eluate from uninfected cells (lane D) did not contain these polypeptides. The gel of the 0.5 M NaCl eluate from infected cells (lane A) shows the viral DBP superimposed upon the pattern of the 0.5 M eluate of uninfected cells (lane C). Because the 1.0 M NaCl eluate is enriched for the adenovirus-specific DBP (Fig. 1) and contains relatively little of the cellular DBP, it was utilized in the following studies on the effect of the adenovirus DBP on DNase activity.

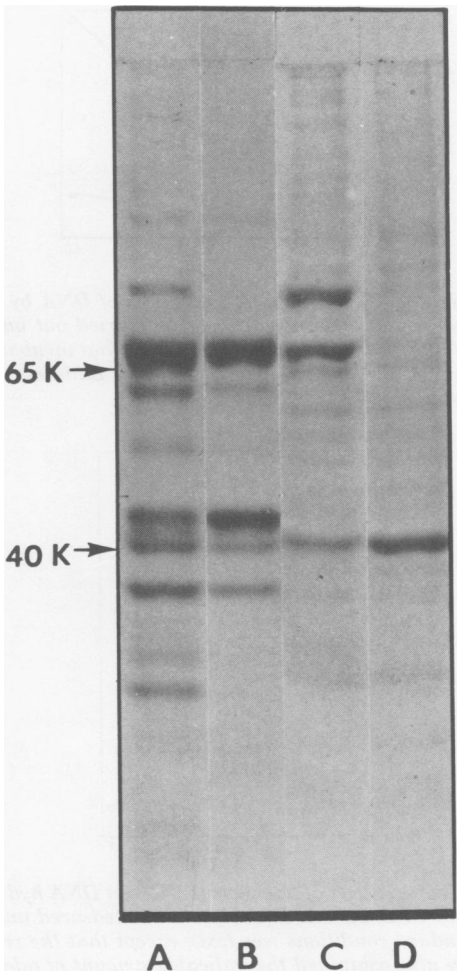

FIG. 1. *Polyacrylamide gel electrophoresis in SDS of eluates of DNA-cellulose chromatography of uninfected and adenovirus-infected KB cells. DNA-cellulose chromatography and polyacrylamide gel electrophoresis were carried out as described previously (19). The arrows indicate the positions of bovine serum albumin and peroxidase markers. Adenovirus-infected eluates: (A) 0.5 M NaCl, (B) 1.0 M NaCl. Uninfected eluates: (C) 0.5 M NaCl, (D) 1.0 M NaCl.*

DNase inhibition by the adenovirus DBP. The hydrolysis of denatured adenovirus DNA by KB DNase was measured by the production of acid-soluble nucleotide as described above. As shown in Fig. 2, the hydrolysis was linear with time to at least 60 min. In the presence of the DBP, the reaction still proceeded linearly but at a significantly reduced rate. Under the conditions of this experiment the rate of hydrolysis was reduced approximately fivefold by the DBP.

The rate of hydrolysis of DNA was measured in the presence of increasing amounts of DBP. The results (Fig. 3) show that the rate of hydrolysis was inversely proportional to the

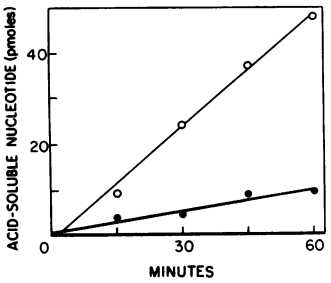

FIG. 2. *Time course of hydrolysis of DNA by KB DNase. KB DNase reaction was carried out under standard conditions (see text), except that incubation was for the indicated times.* (○) *No addition;* (●) *addition of 3 μg of adenovirus DBP.*

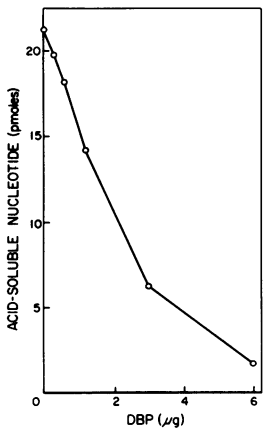

FIG. 3. *Effect of adenovirus DBP on DNA hydrolysis by KB DNase. Hydrolysis was measured under standard conditions (see text), except that the reaction also contained the indicated amount of adenovirus DBP.*

amount of DBP present in the reaction, or, stated differently, the inhibition of DNase activity was proportional to the amount of DBP present.

Comparison with cellular DNase inhibitor. In some preparations of cellular DBP, a small amount of cellular DNase inhibitor activity has been detected in the 1.0 NaCl eluate. Since the DNase inhibitor activity that we find in this eluate of adenovirus-infected cells resembles the cellular activity (19), it is essential to be able to distinguish between these two activities.

(i) Sedimentation analysis. The sedimentation rate of the DNase inhibitor activity in the 1.0 M NaCl eluate from infected cells was determined in a glycerol gradient. Two peaks of activity were obtained at approximately the positions expected for proteins of molecular weights 72,000 and 44,000 (Fig. 4A). DNase inhibitor activity associated with the 44,000-dalton proteolysis fragment of the DBP would not be surprising, in view of the fact that the fragment retains DNA-binding capacity (29).

Sedimentation analysis of the cellular DNase inhibitor activity is shown in Fig. 4B. The two peaks of activity correspond reasonably well to the expected sedimentation rate of the major polypeptides seen in SDS-polyacrylamide gels (Fig. 1, lane C). The sedimentation pattern of the DNase inhibitor activity of the adenovirus DBP preparation (Fig. 4A) is clearly distinguishable from that of the cellular DBP (Fig. 4B) (see below).

(ii) Effect of temperature on activity. The two DBPs also differ in the effect of temperature on their respective DNase inhibitor activities. DNase inhibition by the cellular and adenovirus DBPs was measured at 43 and 30°C. The results show that the adenovirus DBP has more than 10-fold greater inhibition activity at 43 than at

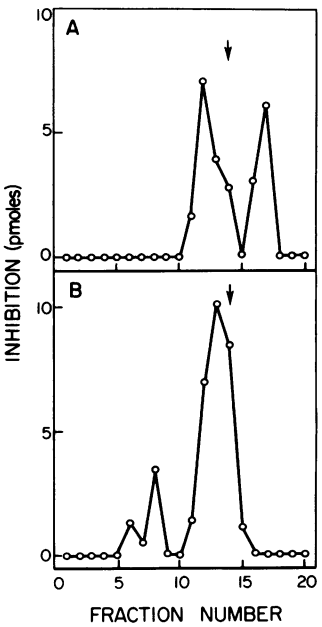

FIG. 4. *Glycerol-gradient sedimentation of the DNase inhibitor activities of uninfected and adenovirus-infected KB cells. The proteins of the 1.0 M NaCl eluate from adenovirus-infected cells (A) and the 0.5 M NaCl eluate from uninfected cells (B) were sedimented in 10 to 30% glycerol gradients containing 10 mM Tris-hydrochloride buffer (pH 8.1) and 10 mM mercaptoethanol. After centrifugation for 16 h at 38,000 rpm at 4°C in a SW50.1 rotor, fractions were collected and assayed for DNase inhibitor activity using S1 nuclease under the conditions described previously (19). The arrow indicates the approximate position of sedimentation of bovine serum albumin, as determined by absorbance at 260 nm in a parallel gradient.*

30°C (Table 1, experiments 1 and 2). For the cellular DBP, in contrast, this ratio is less than 3.

DNase inhibitor activity of Ad5 *ts*125-infected cells. Extracts of cells infected with Ad5 *ts*125 at 40°C have been shown not to contain the adenovirus DBP (30). It has been suggested that this is the result of greater sensitivity of the *ts*125 DBP to proteolytic breakdown (4). When extracts of cells infected with *ts*125 at 40°C were analyzed by DNA-cellulose chromatography, polyacrylamide gel electrophoresis of the column eluates (Fig. 5) confirmed the absence of the adenovirus DBP. DNase inhibitor assays on the eluates showed that although some activity was present, it had the temperature coefficient characteristic of the cellular DBP (Table 1, experiments 3 and 4).

DISCUSSION

We have presented evidence that the adenovirus-specific DBP is able to inhibit the hydrolysis of DNA by DNase. The ability of some DBPs to affect DNase activity was first demonstrated for the *Escherichia coli* helix-destabilizing protein (16, 17). The effect of this protein depended upon the DNase; in some cases the protein actually stimulated the DNase activity (17). At least some of the effects of the *E. coli* DBP on DNase appear to result from direct interaction between the protein and the DNase (17). We are currently investigating the mechanism of DNase inhibition by the adenovirus DBP.

TABLE 1. *Effect of temperature on DNase inhibitor activities*

Expt	DBP	Inhibition (pmol)[a]		Q_{30}^{43}[b]
		30°C	43°C	
1	Cellular	10.5	26.6	2.5
	Adenovirus (wild type)	2.3	29.4	13
2	Cellular	6.5	19.0	2.9
	Adenovirus (wild type)	1.3	20.3	16
3	Adenovirus (*ts*125)[c]	4.2	9.0	2.1
4	Adenovirus (*ts*125)[c]	0.9	3.3	3.7

[a] Inhibition is defined as the difference between the acid-soluble nucleotide produced by KB DNase in the absence and in the presence of 1.2 μg of the indicated DBP under standard conditions (see text), except that the incubation was at the indicated temperature. Cellular DBP was the 0.5 M NaCl DNA-cellulose eluate (19); viral DBPs were the 1.0 M NaCl eluates (see text).

[b] Q_{30}^{43} Ratio of inhibition at 43°C to that at 30°C.

[c] Infection was carried out at 40°C. Extract was prepared at 19 h after infection.

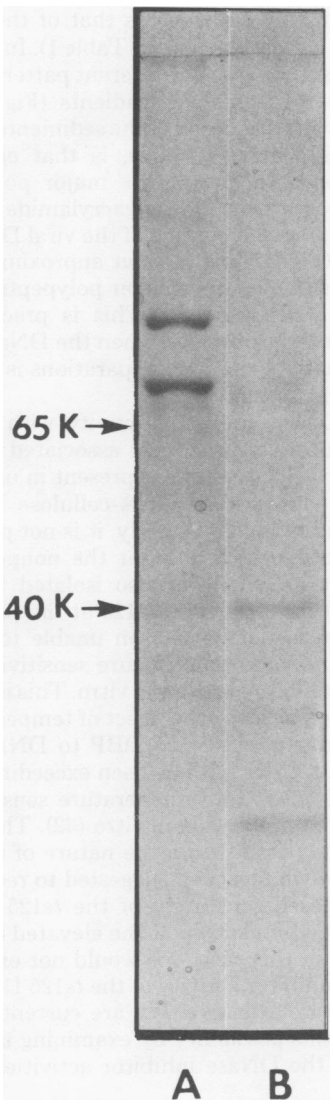

FIG. 5. *Polyacrylamide gel electrophoresis in SDS of DNA-cellulose eluates from KB cells infected with Ad5 ts125 and maintained at 40°C. At 19 h after infection, an extract was prepared, chromatographed on DNA-cellulose, and analyzed by polyacrylamide gel electrophoresis as described previously (19). The markers are the same as in Fig. 1. (A) 0.5 M NaCl eluate; (B) 1.0 M NaCl eluate.*

Although the DNase-inhibitor activity of the adenovirus DBP is similar to that of the cellular DBP (19), it is clearly distinguishable from it by the effect of temperature on the two activities. The activity of the adenovirus DBP has a ratio of DNase inhibition at 43°C to that at 30°C of

approximately 14, whereas that of the cellular DBP activity is less than 3 (Table 1). In addition, the two activities show different patterns of sedimentation in glycerol gradients (Fig. 4). The simplest interpretation of the sedimentation patterns, as described above, is that each peak corresponds to one of the major polypeptide bands seen in the SDS-polyacrylamide gels (Fig. 1). The large polypeptide of the viral DBP preparation (Fig. 1, lane B) is at approximately the same position as the smaller polypeptide of the cellular DBP (lane C). This is precisely the result which is obtained when the DNase inhibitor activity of the two preparations is analyzed by sedimentation (Fig. 4).

The DNase inhibitor activity with a 43°C/30°C ratio of 14 is clearly associated with the adenovirus DBP. It is not present in uninfected cells, it elutes from DNA-cellulose with the DBP, and most importantly, it is not present in cells infected with *ts*125 at the nonpermissive temperature. We have also isolated the DBP from cells infected with *ts*125 at the permissive temperature but have been unable to demonstrate significant temperature sensitivity of the DNase-inhibitor activity in vitro. This is perhaps not surprising since the effect of temperature on the binding of the *ts*125 DBP to DNA is only slight (30). In fact, it has been exceedingly difficult to demonstrate temperature sensitivity of *ts*125 DNA replication in vitro (32). These difficulties may result from the nature of the *ts*125 defect, which has been suggested to result from the increased sensitivity of the *ts*125 DBP to proteolytic breakdown at the elevated temperature (4). In this case, one would not expect the DNase-inhibitor activity of the *ts*125 DBP to be temperature sensitive. We are currently investigating this possibility by examining the sensitivity of the DNase inhibitor activities to protease.

Our earlier observation that the specific activity of the DNase decreases after wild-type adenovirus infection, but not after infection with *ts*125 at the nonpermissive temperature, led us to suggest that the adenovirus DBP may be able to inhibit the hydrolysis of DNA by DNase (18). Our present results confirm this suggestion but do not prove that this is the actual function of the DBP in vivo. Hydrolysis of the single-stranded regions of replicating viral DNA molecules would presumably abort replication; our results demonstrate that the DBP can protect the replicating molecules, but they do not demonstrate that this is in fact the function of the DBP that makes it indispensable for viral DNA replication.

Regardless of whether it turns out to be the primary function of the adenovirus DBP in vivo, DNase inhibition nevertheless provides a simple and sensitive quantitative assay for active protein. Up to now, the presence of the DBP could be determined only by polyacrylamide gel electrophoresis, and its activity could only be measured by DNA binding. This simple assay for active DBP should prove to be a useful tool in further studies on the role of the protein in adenovirus DNA replication.

ACKNOWLEDGMENTS

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