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Thermolabile DNA Binding Proteins from Cells Infected with a Temperature-Sensitive Mutant of Adenovirus Defective in Viral DNA Synthesis

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Infection of African green monkey kidney cells with type 5 adenovirus leads to the synthesis of two infected, cell-specific proteins with approximate molecular weights of 72,000 and 48,000, that bind specifically to single-stranded but not double-stranded DNA. The production of these two proteins was studied after infection with two DNA-negative adenovirus mutants belonging to different complementation groups (H5 ts36 and H5 ts125). Both DNA binding proteins were detected in cells infected with either mutant at the permissive temperature (32 C) and also in H5 ts36-infected cells at the nonpermissive temperature (39.5 C). In H5 ts125-infected cells at 39.5 C, however, less than 5% of the normal wild-type level of these DNA binding proteins was detectable. When H5 ts125-infected cells were labeled with radioactive leucine at 32 C and subsequently shifted to 39.5 C in the presence of unlabeled leucine (chase), the level of DNA binding proteins found in these infected cells was markedly reduced compared to cultures not shifted to 39.5 C. These data suggest that the DNA binding proteins themselves were temperature sensitive. This conclusion was confirmed by experiments in which the DNA binding proteins were eluted from DNA cellulose with buffers of increasing temperatures (thermal elution). The H5 ts125 proteins were shown to elute at lower temperatures than either wild-type or H5 ts36 proteins. These results are taken to indicate that the H5 ts125 mutant codes for a DNA binding protein that is thermolabile for continued binding to single-stranded DNA.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) has been employed to detect three adenovirus-infected, cell-specific proteins that are synthesized at early times after infection (3, 8, 13). These proteins, with molecular weights of approximately 70,000, 20,000 and 10,000, are not found in mature adenovirus virions. Recently, DNA-cellulose chromatography was employed to isolate two early DNA binding proteins found specifically in adenovirus type 5-infected African green monkey kidney (AGMK) cells (11). The molecular weights of these proteins were 72,000 and 48,000. Similar DNA binding proteins have now been detected in Ad2-, Ad5-, and Ad12-infected AGMK and human KB cells (A. J. Levine, P. C. van der Vliet, B. Rosenwirth, J. Rabek, G. Frenkel, and M. Ensinger, Cold Spring Harbor Symp. Quant. Biol., in press; B. Rosenwirth, C. Anderson, R. Gesteland, and A. J. Levine, personal communication). In all cases these DNA binding proteins are synthesized in large amounts after viral infection with up to 10^7 protein molecules made per cell. Neither of these DNA binding proteins was detected in mature adenovirus virions, and both proteins were synthesized in the absence of viral or cellular DNA synthesis (11). These proteins bind specifically to single-stranded DNA from a variety of sources but not to double-stranded DNA or *Escherichia coli* 23*S* ribosomal RNA (Levine et al., Cold Spring Harbor Symp. Quant. Biol., in press; 11). In all of these properties the adenovirus DNA binding proteins resemble T-4 gene 32 protein which is required for T-4 DNA replication and genetic recombination (1). Replicating adenovirus DNA molecules contain large regions of single-stranded DNA and so the function of these proteins may well be related to viral DNA replication (4, 9, 10).

Genetic studies with type 5 adenovirus mu-

tants have revealed the existance of two different complementation groups that code for early viral proteins (5, 14). Temperature-sensitive mutants in each of these two classes do not synthesize viral DNA at the nonpermissive temperature. This paper describes the properties of the adenovirus DNA binding proteins obtained from cells infected with these mutants at the restrictive and permissive temperature.

Both DNA binding proteins are altered after infection with one (H5 ts125) of the two classes of temperature-sensitive mutants. This alteration is expressed as an increase in thermolability of these proteins for continued binding to single-stranded DNA. These data strongly suggest that the adenovirus DNA binding proteins are early proteins coded for by the viral genome and that one or both of these proteins are involved in viral DNA replication.

MATERIALS AND METHODS

Cells and viruses. Primary AGMK cells were grown in Dulbecco modified Eagle medium. H5 ts125 (abbreviations are according to the proposed nomenclature for adenovirus mutants [6]) was isolated and plaque purified as described (5). H5 ts36 (14) was kindly supplied by J. F. Williams. Cells were infected at a multiplicity of 20 to 50 PFU per cell in Dulbecco modified Eagle medium containing 2% calf serum and a low leucine concentration (1 mg per liter). [^{3}H]leucine (10 μCi/ml, 310 mCi/mg) or [^{14}C]leucine (1 μCi/ml, 2.2 mCi/mg) was added at various times after infection. The maintenance and infection conditions for KB cells have been described (12).

Isolation of DNA binding proteins. Cells were washed three times with phosphate-buffered saline, collected in a hypotonic buffer (0.02 M Tris-hydrochloride [pH 7.6], 0.01 M NaCl, 1.5 mM $MgCl_2$, and 2.0 mM dithiothreitol) and frozen at −20 C. A DNA-free soluble protein extract was prepared by one of the following methods. (i) After thawing the cell suspension, 500 μg of bovine serum albumin per ml was added and the cells were disrupted by sonic treatment. EDTA was added to 5 mM and NaCl to 1.7 M. After 10 min, 30% polyethylene glycol in 1.7 M NaCl was added to 10% (wt/vol) and 30 min later the mixture was centrifuged for 20 min at 15,000 × *g*. The supernatant was dialyzed against 0.01 M Tris-hydrochloride (pH 7.4), 0.05 M NaCl, 1.0 mM EDTA, 2.0 mM 2-mercaptoethanol, and 10% (wt/vol) glycerol (buffer A). Before applying the extract to a DNA-cellulose column, a small precipitate was removed by centrifugation for 15 min at 10,000 × *g*. (ii) The cell suspension was frozen, thawed, and centrifuged for 20 min at 15,000 × *g*. To the supernatant containing most of the DNA binding proteins, bovine serum albumin was added to 500 μg/ml, EDTA to 5 mM, NaCl to 15 mM, and glycerol to 10% (wt/vol). The supernatant was directly applied to a DNA-cellulose column containing single-stranded calf thymus DNA and eluted at a flow rate of 2 ml/h as described (2, 11). All procedures were performed at 4 C. This latter method gave lower nonspecific binding during DNA-cellulose chromatography. Since high salt conditions were avoided in this procedure, it is possible that only those DNA binding proteins were isolated that were eluted from or not bound to DNA in the cell or extract. For this reason, methods (i) and (ii) were compared for their efficiency of extracting these DNA binding proteins. In an extract of infected cells containing 85 × 10^6 counts/min of total protein ([^{3}H]leucine label) the 15,000 × *g* supernatant of method (ii) contained 5 × 10^5 counts/min of the 72,000- and 48,000-mol-wt DNA binding proteins. When the 15,000 × *g* pellet was extracted by method (i) (polyethylene glycol and high salt) an additional 2.7 × 10^4 counts/min of the 72,000- and 48,000-mol-wt DNA binding protein was obtained. These data demonstrate that only 5.4% of the DNA binding proteins remained in the pellet fraction with method (ii).

Other techniques. SDS-polyacrylamide gel electrophoresis of DNA binding proteins was performed in 10% gels containing 0.1 M sodium phosphate, pH 7.4, and 0.1% SDS. Details of the procedure have been described (11). Viral DNA synthesis was monitored by addition of 10 μCi of [^{3}H]thymidine (52 Ci/mmol) per ml for 2 h. The cells were washed three times with 0.01 M Tris-hydrochloride (pH 8.1) and 0.01 M EDTA, and they suspended in this buffer at 2 × 10^6 cells/ml. Adenovirus DNA was isolated according to a modified Hirt procedure (7, 12). Appropriate samples of the Hirt supernatant were spotted on Whatmann 3MM filters, precipitated with 10% trichloroacetic acid, washed twice with 10% trichloroacetic acid and once with acetone, dried, and counted in a liquid scintillation counter.

RESULTS

DNA binding proteins from cells infected with H5 temperature-sensitive mutants at 39.5 C. Confluent monolayer cultures of AGMK cells were infected with type 5 adenovirus wild-type, H5 ts36, or H5 ts125 in low leucine containing medium at the nonpermissive temperature. Each of these cultures was continuously labeled with [^{3}H]- or [^{14}C]leucine and kept at 39.5 C for the entire period of infection. Viral DNA synthesis, which was monitored in separate cell cultures by labeling viral DNA with [^{3}H]thymidine, was inhibited more than 98% in H5 ts36- and H5 ts125-infected cells at 39.5 C compared to wild-type infected cultures (Table 1). At 16 h after infection, wild-type infected cells labeled with [^{3}H]leucine were mixed with H5 ts36- or H5 ts125-infected cells labeled with [^{14}C]leucine. Protein extracts prepared from these two mixtures were chromatographed on DNA-cellulose and the fractions eluting between 0.5 M NaCl and 1.0 M NaCl were pooled. These fractions normally contain the infected, cell-specific DNA binding proteins (Levine et al., Cold Spring Harbor Symp.

Quant. Biol., in press; 11). The labeled proteins in this eluate were analyzed on SDS-polyacrylamide gel electrophoresis (Fig. 1). Similar amounts of the 72,000- and 48,000-mol-wt DNA binding proteins were detected in wild-type- and H5 ts36-infected cells at 39.5 C (Fig. 1A). In extracts of H5 ts125-infected cells, however, the 72,000- and 48,000-mol-wt DNA binding proteins were either absent or the level of these proteins was markedly reduced when compared to wild-type-infected cell extracts (Fig. 1B). In this experiment, a 58,000-mol-wt DNA binding protein found in roughly equal amounts in infected and uninfected cells (11) as well as in wild-type and both mutant extracts (Fig. 1) is also present. This protein serves as an internal control for the presence of a cellular DNA binding protein found in H5 ts125-infected cells. Normal levels of the two DNA binding proteins were found in H5 ts125-infected cells at the permissive temperature (32 C) (results not presented).

The absence of detectable DNA binding proteins in extracts of H5 ts125-infected cells at nonpermissive temperatures might be explained in one of the following ways. (i) Under permissive conditions the H5 ts125 function might induce the synthesis of the two DNA binding proteins, which may be coded for by the

TABLE 1. *Viral DNA synthesis in mutant-infected cells*[a]

Adenovirus genotype	[^{3}H]thymidine incorporation into viral DNA (counts/min) at:		Ratio of incorporation at 39.5 and 32 C
	39.5 C	32 C	
Wild type	24,500	9,830	2.49
H5 ts36	310	16,300	0.02
H5 ts125	410	9,500	0.04

[a] AGMK cells infected at the indicated temperatures were labeled from 21 to 23 h after infection (39.5 C) or from 42 to 44 h after infection (32 C) with 10 μCi of [^{3}H]thymidine per ml. Viral DNA was isolated and samples were counted as described.

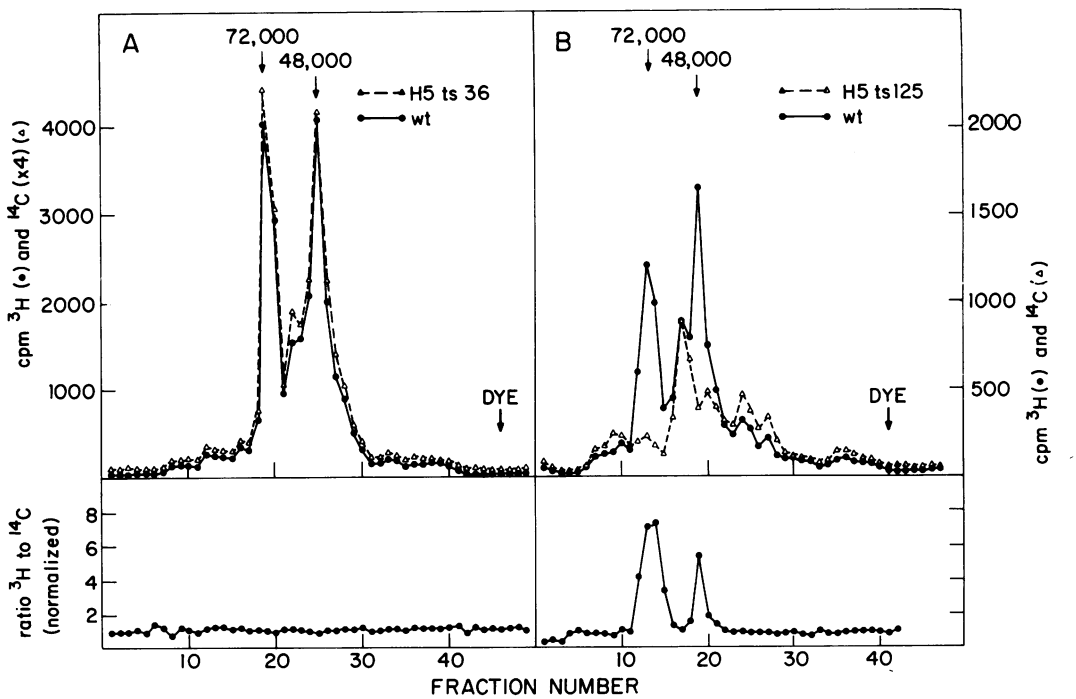

FIG. 1. *SDS-polyacrylamide gel electrophoresis of DNA binding proteins isolated from (A) H5 ts36- or (B) H5 ts125-infected AGMK cells labeled at the nonpermissive temperature. Cells were infected at 39.5 C and kept at that temperature. The radioactive label was added at 2 h postinfection. Wild-type and temperature-sensitive, mutant-infected cells were harvested at 16 h postinfection and mixed, and a protein extract, prepared according to method (i), was chromatographed on DNA-cellulose. The fractions eluting between 0.5 M NaCl and 1.0 M NaCl were pooled and electrophoresed. The ratio of ^{3}H to ^{14}C was normalized with respect to the ratio of the extract before chromatography (●, bottom panel). (A) ●, Wild-type-infected cells labeled with [^{3}H]leucine; △, H5 ts36-infected cells labeled with [^{14}C]leucine. (B) ●, Wild-type-infected cells labeled with [^{3}H]leucine; △, H5 ts125-infected cells labeled with [^{14}C]leucine.*

virus or the cell genome. This would lead to a block in synthesis of these proteins at 39.5 C. In this case, no thermosensitive molecular differences are expected between wild-type and mutant DNA binding proteins. (ii) Alternatively, the H5 ts125 gene(s) may code for altered DNA binding proteins which could then lose their ability to bind to DNA when synthesized at 39.5 C due to irreversible denaturation or denaturation followed by degradation of the proteins in the cell at the nonpermissive temperature. In this case, H5 ts125 could either be a double mutant coding for altered 72,000- and 48,000-mol-wt proteins or the 48,000-mol-wt protein might be a proteolytic breakdown product of the 72,000-mol-wt DNA binding protein. The peptide maps of each of these proteins are most compatible with the notion that the 48,000-mol-wt protein is a breakdown product of the 72,000-mol-wt protein (B. Rosenwirth et al., personal communication).

To distinguish between these alternatives, experiments were designed to determine whether the DNA binding proteins themselves were temperature sensitive. Adenovirus type 5 wild-type- or H5 ts125-infected cells were labeled at 32 C with [^{3}H]- or [^{14}C]leucine, respectively. At 44 h after infection, these cultures were washed with fresh medium containing nonradioactive leucine and shifted to 39.5 C in prewarmed medium. One hour after the shift-up, the wild-type and mutant cultures were harvested and mixed, and the DNA binding proteins were isolated by DNA-cellulose chromatography followed by SDS gel electrophoresis (Fig. 2). After the shift-up to nonpermissive temperatures, a threefold reduction in the level of H5 ts125 DNA binding proteins was observed compared to either wild-type-infected cultures (Fig. 2) or the level of binding proteins found in H5 ts125-infected cultures kept at 32 C (results not presented). This reduction in the level of 72,000- and 48,000-mol-wt proteins binding to DNA was not due to a general decrease in the amounts of all labeled proteins in the H5 ts125-infected cells, because the 58,000-mol-wt cellular DNA binding protein was detected in normal amounts in this experiment after the shift-up in temperature (Fig. 2).

In cultures of H5 ts125-infected cells labeled at 32 C and shifted up for 4 h at 39.5 C, 80 to 90% of both the 72,000- and 48,000-mol-wt DNA binding proteins were no longer detectable. These data demonstrate that the adenovirus DNA binding proteins themselves behave differently in H5 ts125- and wild-type-infected cells. The loss of these DNA binding proteins from infected cell extracts after a shift to the nonpermissive temperature favors the hypothesis that these proteins are coded for by the virus.

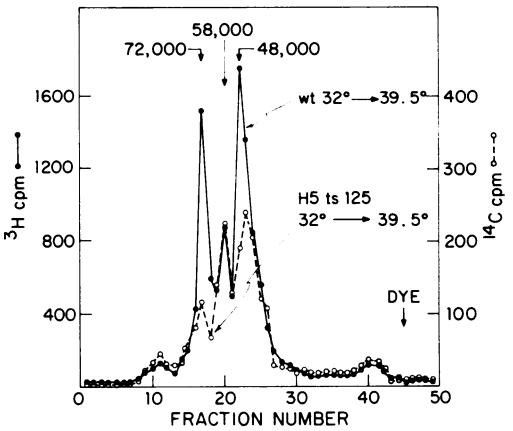

FIG. 2. *SDS-polyacrylamide gel electrophoresis of wild-type and H5 ts125 DNA binding proteins labeled at 32 C and subsequently shifted to 39.5 C for 1 h. AGMK cells were infected with H5 ts125 or wild-type adenovirus at 32 C and labeled from 2 to 44 h postinfection with [^{14}C]leucine or [^{3}H]leucine, respectively. The medium was then removed, and the cultures were washed with prewarmed medium containing 100-fold excess of nonradioactive leucine and were further incubated at 39.5 C. After 1 h, the cultures were harvested and mixed, and DNA binding proteins were isolated and analyzed as described in Fig. 1. Before the shift to 39.5 C, the ratio of ^{3}H to ^{14}C in the 72,000- and 48,000-mol-wt proteins was the same as the ratio in the crude extract (^{3}H/^{14}C = 4). After the shift-up, this ratio increased to about 8 for the 48,000-mol-wt protein and 16 for the 72,000-mol-wt protein. The level of the 58,000-mol-wt protein found in uninfected, wild-type- or mutant-infected cells serves as an internal control for a thermostable cellular protein with a ^{3}H/^{14}C ratio of 4 before and after the shift to 39.5 C. The level of labeled proteins not specific for infected cells, that coelectrophorese at the positions of the DNA binding proteins, can be neglected (11). These data demonstrate a two- to fourfold reduction of the amount of H5 ts125 DNA binding proteins after the shift-up to 39.5 C. Symbols: ●, wild-type-infected cells labeled with [^{3}H]leucine; ○, H5 ts125-infected cells labeled with [^{14}C]leucine.*

Thermolabile DNA binding of H5 ts125 proteins. To test whether H5 ts125-mutant proteins were also thermosensitive in their DNA binding properties in vitro, the following experiment was performed. Cell extracts, prepared after infection with wild type or H5 ts125 at 32 C, were mixed with mock-infected cell extracts and passed over a single-stranded DNA-cellulose column at 4 C in a buffer containing 0.15 M NaCl. The bound proteins were then thermally eluted with a buffer containing 0.25 M NaCl by increasing the temperature of the buffer in a water jacketed column (Fig. 3). The

majority of the H5 ts125 DNA binding proteins eluted in the 4 to 20 C wash, whereas the majority of the wild-type proteins eluted in the 40 C wash (Fig. 3C). A mixture of wild-type proteins and H5 ts125 DNA binding proteins, each prepared at 32 C, gave similar results (Fig. 4).

To confirm that these differences in thermal elution were due to the 72,000- and 48,000-mol-wt proteins, the fractions from the DNA-cellulose column in Fig. 4 that were eluted at 4, 20, 40, and 50 C were collected and analyzed by SDS-polyacrylamide gel electrophoresis. The relative amounts of 72,000- and 48,000-mol-wt proteins eluted at different temperatures were calculated from the electrophorograms. The results (Fig. 5) confirm that the two DNA binding proteins from H5 ts125-infected cells are more thermolabile for continued DNA binding than the wild-type proteins. A similar experiment performed with mixed extracts from H5 ts125- and H5 ts36-infected cells (Fig. 5) demonstrates that the 72,000-mol-wt DNA binding protein from H5 ts36 behaves like wild-type, 72,000-mol-wt protein during thermal elution. The H5 ts36 48,000-mol-wt protein was eluted from DNA in equal amounts above and below 40 C, but was certainly more thermostabile than H5 ts125 48,000-mol-wt protein, 90% of which was released from DNA below 40 C. A greater variability in elution temperatures of this 48,000-mol-wt protein might be expected. This component is really a collection of several protein species of similar molecular weights (45,000 to 50,000 mol wt) which have recently been resolved from each other by higher SDS-polyacrylamide gel concentrations and autoradiographic analysis of the gel itself. These multiple species appear to be proteolytic breakdown products of the 72,000-mol-wt proteins (B. Rosenwirth et al., personal communication).

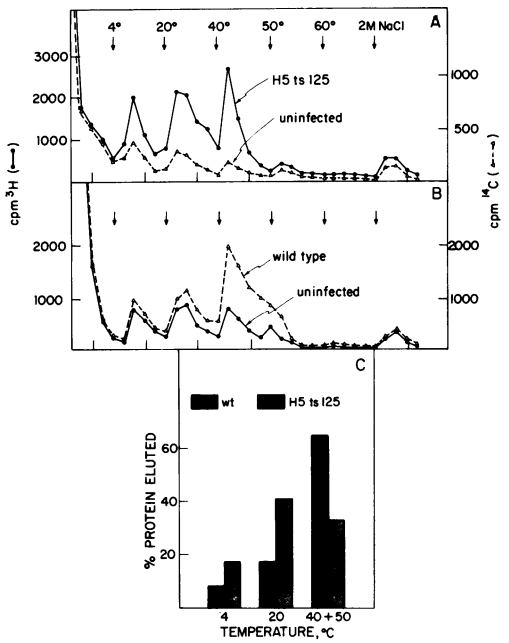

FIG. 3. *Thermal elution of wild-type and H5 ts125 proteins bound to DNA-cellulose. AGMK cells were infected at 32 C with H5 ts125 or wild-type adenovirus and labeled from 16 to 42 h after infection with [³H]leucine and [¹⁴C]leucine, respectively. Mock-infected cells were also labeled with [³H]leucine or [¹⁴C]leucine during this period. Protein extracts were made according to method (ii). Crude extracts from H5 ts125- and mock-infected cells (A) or wild-type- and mock-infected cells (B) were applied to a water jacketed DNA-cellulose column in buffer A containing 0.15 M NaCl. After a rinse with this buffer, the DNA binding proteins were eluted with the same buffer containing 0.25 M NaCl by raising the temperature stepwise as indicated. Fractions of 0.5 ml were collected and 50-μliter samples were counted. The percentage of the total infected, cell-specific DNA binding protein eluted at 4, 20, or 40 to 50 C with the wild-type or H5 ts125 infected cell extracts is presented in (C). (A) ●, H5 ts125 DNA binding proteins labeled with [³H]leucine; Δ, mock-infected cell DNA binding proteins labeled with [¹⁴C]leucine. (B) ●, Mock-infected cell DNA binding proteins labeled with [³H]leucine; Δ, wild-type infected cell DNA binding proteins labeled with [³H]leucine. (C) ■, Wild-type proteins eluted from DNA-cellulose; □, H5 ts125 proteins eluted from DNA-cellulose.*

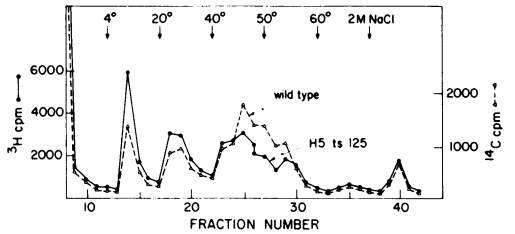

FIG. 4. *Thermal elution from DNA-cellulose of a mixture of wild-type and H5 ts125 DNA binding proteins. Extracts from wild-type- and H5 ts125-infected cells were prepared, mixed, and loaded onto DNA-cellulose as described in Fig. 3. This double labeling procedure shows the elution of H5 ts125 DNA binding proteins at a lower temperature than wild-type proteins. Symbols: Δ, wild-type DNA binding proteins labeled with [¹⁴C]leucine; ●, H5 ts125 DNA binding proteins labeled with [³H]leucine.*

DISCUSSION

Infection of monkey or human cells with type 5 adenovirus results in the synthesis of two species of single-stranded-specific DNA binding proteins shortly before viral DNA replication commences (11). These proteins are not detect-

able in uninfected cells and are produced after viral infection even in the absence of viral or cellular DNA synthesis (Levine et al., Cold Spring Harbor Symp. Quant. Biol., in press; 11). Thus, these proteins are properly named early infected, cell-specific viral proteins. The experiments presented in this paper demonstrate that these DNA binding proteins are likely coded for by the type 5 adenovirus gene prescribed by the mutant H5 ts125. Little or no 72,000- and 48,000-mol-wt DNA binding proteins can be detected in extracts of H5 ts125-infected cells prepared at 39.5 C. These DNA binding proteins present in H5 ts125-infected cells grown at 32 C are thermolabile in their continued binding to single-strand DNA when compared to the wild-type or H5 ts36 proteins. The DNA binding proteins synthesized in H5 ts125-infected cells at 32 C are present in much reduced amounts after these cells are shifted (for 1 h) to 39.5 C. If these cells are kept at the nonpermissive temperature for a longer time (4 h), lower levels of these DNA binding proteins were detected. When H5 ts125-infected monkey or human cells are shifted up in temperature from 32 to 39.5 C, the rate of viral DNA synthesis declines rapidly and is reduced to 5 to 25% of its original rate by 30 min at 39.5 C

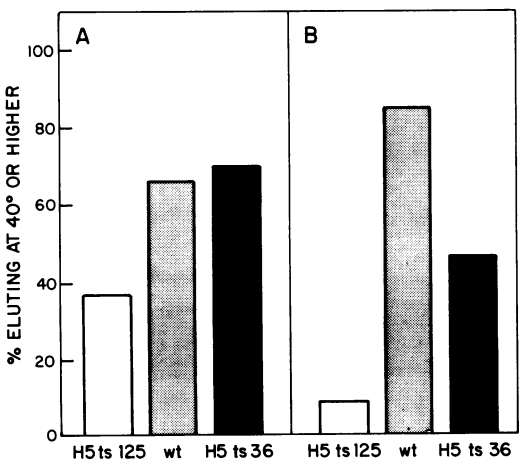

FIG. 5. *Thermosensitive release from single-stranded DNA-cellulose of temperature-sensitive mutant and wild-type DNA binding proteins with molecular weights of 72,000 (A) and 48,000 (B), respectively. Fractions 14-21 and 23-29 from Fig. 4 were pooled and electrophoresed on SDS-polyacrylamide gels. From the total radioactivity at the 72,000- and 48,000-mol-wt position in the gel, the percentage of each protein eluting at 40 C or higher was calculated. A similar experiment was performed after thermal elution of a mixture of H5 ts125- and H5 ts36-infected cells. The results of both H5 ts125 extracts were averaged.*

(Levine et al., Cold Spring Harbor Symp. Quant. Biol., in press).

These results are most simply interpreted by assuming that the H5 ts125 mutant codes for an altered DNA binding protein. In all these experiments, both the 72,000- and 48,000-mol-wt proteins appear to be altered by the H5 ts125 mutant. This may be due to the possibility that (i) H5 ts125 is a double mutant, or (ii) the 48,000-mol-wt protein(s) is a proteolytic breakdown product of the 72,000-mol-wt protein. Recent peptide maps of the 72,000- and 48,000-mol-wt proteins favor the second possibility (B. Rosenwirth et al., personal communication). Whether or not the 48,000-mol-wt protein(s) is a physiologically functional intermediate or is just an artifact of proteolytic digestion in vitro is not at present clear.

The assignment of the 72,000- and 48,000-mol-wt proteins to adenovirus mutant H5 ts125 indicates that one or both of these proteins is indeed involved in viral DNA replication. Adenovirus replicating intermediates contain extensive amounts of single-stranded DNA (4, 9, 10). These adenovirus DNA binding proteins share a number of properties with T-4 gene 32 protein which is required for viral DNA synthesis and genetic recombination (1). Both gene 32 protein and the adenovirus proteins bind specifically, in solution, to single-stranded DNA. Both are produced in very large amounts, even though they are early proteins, suggesting a stoichiometric rather than a catalytic function. Genetic dominance studies with H5 ts125 show that indeed the protein coded for by this mutant is required in stoichiometric levels whereas the H5 ts36 function is employed in catalytic fashion (C. S. H. Young and J. F. Williams, personal communication). In addition, H5 ts125 and H5 ts36 have very different phenotypes after shift up in temperature from 32 to 39.5 C (Levine et al., Cold Spring Harbor Symp. Quant. Biol., in press; and J. S. Sussenbach, personal communication). Thus, both the genetic and biochemical studies support the view that these two mutants, involved in adenovirus DNA replication, have different functions (Levine et al., Cold Spring Harbor Symp. Quant. Biol., in press; 5, 14). More information concerning the role of these adenovirus DNA binding proteins in viral DNA replication will await the further characterization of the phenotypes of H5 ts125 and similar mutants.

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