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Possible Role of the 72,000-Dalton DNA-Binding Protein in Regulation of Adenovirus Type 5 Early Gene Expression

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Relative abundances of early virus RNA species in the cytoplasm of cells infected with wild-type adenovirus type 5 (WT Ad5) and a temperature-sensitive "early" mutant, H5*ts*125 (*ts*125), were compared by hybridization kinetics using separated strands of *Hin*dIII restriction endonuclease fragments of Ad5 DNA. 1-β-D-Arabinofuranosylcytosine (ara-C) was used to limit transcription to early virus genes in cells infected by WT virus. At 40.5°C, a restrictive temperature for *ts*125, three to seven times as much virus RNA from all four early regions of the genome accumulated in the cytoplasm of cells infected by the mutant as accumulated in cells infected by WT. At 32°C, no such difference in the relative abundances of cytoplasmic virus RNA was observed. The capacity to synthesize a 72,000-dalton (72K) virus polypeptide, presumably the single-stranded DNA-binding protein that is defective in *ts*125 at restrictive temperatures, was compared in cells infected at 40.5°C in the presence of ara-C with the mutant or WT Ad5. The rate of 72K polypeptide synthesis, measured by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis of [^{35}S]methionine-labeled polypeptides and autoradiography, was greater at 15 h after infection in *ts*125-infected cells than in cells infected by WT. A time course experiment showed that the rate of synthesis of the 72K polypeptide increased continuously in *ts*125-infected cells during the first 15 h of infection, relative to the rate in WT-infected cells. These data are consistent with the hypothesis that Ad5 early gene expression is modulated by the product of an early gene, the 72K DNA-binding protein.

Productive infection of human cells in tissue culture by human adenovirus type 5 (Ad5) results in the initial ("early") expression of four groups of genes that are located in separate regions of the virus chromosome (10) and that, together, account for about 25% of the coding capacity of the genome. Two of the early gene groups are transcribed from one DNA strand (in the rightward, or *r*, direction as the map is conventionally drawn), and two groups are transcribed from the other (*l*, or leftward) strand (10). This pattern of gene expression resembles that of the closely related Ad2 (7, 32, 35), the serotype that has been most extensively studied. Before virus DNA replication, early RNA is accumulated in the cytoplasm of infected cells in different amounts, depending upon the region of the genome that is transcribed (12). Some of this RNA continues to accumulate throughout infection, while other sequences remain at concentrations similar to those present before virus DNA replication (7).

The products of adenovirus early gene expression have not yet been completely characterized, but some are required for virus DNA replication (9) and for transformation (11, 14, 18). The gene or genes required for virus-induced cell transformation are located at the left end of the genetic map (17) and are transcribed from the *r* strand (11). The only early gene product that has been isolated and studied extensively is a 72,000-dalton (72K) protein that binds to single-stranded DNA (25, 40, 41) and is required for initiation of virus DNA synthesis (16, 42). The gene that codes for this polypeptide is located between 60 and 65% of the genome length from the left end of the genetic map (27) and is transcribed from the *l* strand (11). A temperature-sensitive mutation in this gene, H5*ts*125 (*ts*125), does not replicate virus DNA (9) and does not synthesize late RNA (2, 3) at restrictive temperatures. It also has the unusual phenotype of increased ability to transform cells in culture (16).

In the present paper we have measured the relative amounts of cytoplasmic early virus RNA in cells infected by wild-type (WT) Ad5 and *ts*125 at different temperatures. The data are consistent with the hypothesis that normal function of the 72K protein modulates early gene expression and that the increased trans-

forming ability of *ts*125 can be explained in part by an increased amount of mRNA from genes at the left end of the virus chromosome.

MATERIALS AND METHODS

Cells. KB cells were grown in suspension in Eagle medium with 10% heat-inactivated calf serum, 10 μg of penicillin per ml, 10 μg of streptomycin per ml, and 0.3% $NaHCO_3$. Cultures were diluted each day to a cell concentration of 2×10^5 per ml with fresh medium. HeLa cells (obtained from C. H. S. Young, Columbia University) were grown in monolayer on plastic in Dulbecco medium with the above concentrations of serum and antibiotics plus 50 μg of Tylosin per ml (Flow Laboratories, Inc.) and 0.1% $NaHCO_3$.

Virus. WT Ad5 and the temperature-sensitive mutant H5*ts*125 were obtained from H. S. Ginsberg, Columbia University. Virus stocks were titered by plaque assay (9) on HeLa cells (C. S. H. Young, personal communication) or by indirect immunofluorescence (13). Stocks were prepared from twice-plaque-purified virus as described by Lawrence and Ginsberg (24), except that stocks of the mutant were grown at 32°C and harvested 60 h after infection. Virus titers were usually 1×10^{11} to 2×10^{11} PFU/ml for WT and 2×10^{10} to 5×10^{10} PFU/ml for *ts*125.

Virus DNA. Virus DNA was labeled in vivo by addition of 20 μCi of [^{3}H]thymidine per ml (Amersham/Searle; 45 Ci/mmol) to suspension cultures 7 to 8 h after infection. Virions were purified by disruption of infected cells with sodium deoxycholate, DNase treatment, Freon extraction, and two cycles of CsCl centrifugation as described previously (24). DNA was isolated from purified virions by phenol extraction (1). The specific activity ranged from 0.4×10^6 to 2.0×10^6 cpm/μg.

Restriction endonuclease digestion and separation of DNA fragments. Ad5 DNA was digested in 10 mM Tris-hydrochloride (pH 7.6)–7 mM $MgCl_2$–0.1 M NaCl–10% glycerol at 37°C for 3 to 5 h, with 100 μg of virus DNA per ml and 625 U of *Hin*dIII per ml (New England Biolabs). The reaction was stopped by the addition of 0.1 volume of DNA sample buffer containing 100 mM EDTA, 75% sucrose, and 0.1% bromothymol blue. The digest was loaded onto a cylindrical 1.4% agarose gel in E buffer (40 mM Tris-acetate–1 mM EDTA [pH 7.5]) and subjected to electrophoresis in E buffer at 30 V for 12 to 24 h at room temperature in the dark. DNA bands were visualized by fluorescence after staining with ethidium bromide, excised from the gel, and recovered by electrophoresis in a dialysis bag immersed in E buffer at 100 V for 1 to 2 h. The eluted fragments were extracted once with phenol-chloroform-isoamyl alcohol (24:24:1) and precipitated with ethanol at −20°C.

Separation of restriction fragment DNA strands. The method of Hayward (19) and Flint et al. (10) was used, with modifications. Ethanol-precipitated DNA restriction fragments were collected by centrifugation in an SW41 rotor for 2 h at 35,000 rpm at −10°C in polyallomer tubes. The precipitate, containing 1 to 2 μg of DNA, was dissolved in 20 μl of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.6) and denatured by the addition of 5 μl of 1 N NaOH. After 2 min at room temperature, 3 μl of DNA sample buffer was added, and the mixture was loaded onto a cylindrical 0.7% agarose gel (0.7 by 15 cm), prepared in E buffer that had been prerun at 30 V at room temperature for 15 to 30 min (H. Raskas, personal communication). The current was left on during loading, and electrophoresis was continued for 8 to 12 h in the dark. The separated strands were visualized by fluorescence after staining with ethidium bromide and excised with a razor blade. DNA was recovered from the gel slice, denatured, and reannealed as described by Flint et al. (10). Double-stranded DNA was then removed by hydroxylapatite chromatography, and the single-stranded material was dialyzed in the presence of 200 μg of salmon sperm DNA per ml against TE buffer (10 mM Tris-hydrochloride–1 mM EDTA [pH 8.0]).

Preparation of cytoplasmic RNA. The method of Craig and Raskas (5) was used, with modifications. Three-liter spinner cultures of KB cells were infected with 100 PFU/cell, and 25 μg of 1-β-D-arabinofuranosylcytosine (ara-C) was added at 1 h and again at 12 h after infection for early RNA preparations from both *ts*125-infected and WT-infected cells. The cells were harvested by centrifugation and washed once with cold TBS buffer (0.14 M NaCl–1.5 mM $MgCl_2$–10 mM Tris-hydrochloride [pH 7.6]). The cell pellet was resuspended in three times its volume of ice-cold TBS and then rapidly mixed with an equal volume of cold 0.2% Nonidet P-40 in TBS (final concentration, 0.1% Nonidet P-40). Disruption of cell membranes was checked by phase-contrast microscopy, and the suspension was immediately centrifuged at $250 \times g$ for 10 min at 4°C to remove cell debris and most of the nuclei. The supernatant solution was then centrifuged at $18{,}000 \times g$ for 20 min at 4°C. The resulting supernatant solution was made 0.5% in sodium dodecyl sulfate (SDS) and frozen at −20°C until used. RNA was first purified by extraction with phenol-chloroform-isoamyl alcohol essentially as described by Craig and Raskas (5). After ethanol precipitation, the RNA was dissolved in 2 ml of 0.1 M NaCl–10 mM $MgCl_2$–10 mM Tris-hydrochloride (pH 7.6) and digested with 25 μg of electrophoretically purified DNase (Worthington Biochemicals Corp.) per ml for 30 min at 37°C. The solution was then made 0.5% in SDS and incubated with 0.5 mg of autodigested Pronase (Calbiochem) per ml at 37°C for 30 min (38). The digest was extracted twice with phenol-chloroform-isoamyl alcohol, made 0.3 M in NaCl, and precipitated with 3 volumes of ethanol at −20°C. After a wash with 70% ethanol at −10°C, the RNA pellet was dried under a stream of nitrogen and dissoved in TE. The yield of cytoplasmic RNA from 3 liters of cells was 3 to 6 mg, determined by optical density at 260 nm (assuming an extinction coefficient of 0.023 $\mu g^{-1} ml^{-1}$). The preparations typically had an absorbance ratio, A_{260}/A_{280}, of greater than 2.1.

Hybridization kinetics. Hybridizations were done in 1.0 M NaCl–1 mM EDTA–100 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (pH 7.0) at 67°C. The RNA concentration was 1 mg/ml, and the DNA concentration was either 75 ng equivalents per ml (for [^{3}H]DNA with a specific activity of $>10^6$ cpm/μg) or 200 ng equivalents per ml (for [^{3}H]DNA with a specific activ-

ity of $<10^6$ cpm/μg). The concentration of nanogram equivalents of single-stranded virus DNA from each fragment was calculated by dividing the amount of radioactivity per milliliter by the specific activity of the whole-virus DNA in nanograms per milliliter (calculated before restriction endonuclease digestion) and dividing the result by the fractional length of the particular fragment. For each hybridization reaction, 60-μl samples were taken at various times and added to 140 μl of ice-cold buffer to obtain a final concentration of 0.1 M sodium acetate–2 mM $ZnCl_2$–10% glycerol–20 μg each of native and heat-denatured salmon sperm DNA per ml and a final pH of 4.5. The samples were held at 4°C until the conclusion of the experiment. S_1 endonuclease digestion was then carried out at 37°C for 1 h, with enough enzyme to digest more than 98% of the single-stranded DNA. S_1 endonuclease was purified from crude α-amylase (Sigma Chemical Co.) by the method of Sutton (37). After S_1 digestion, duplicate 90-μl portions were spotted onto 1-inch (ca. 2.54-cm) squares of Whatman DE81 ion-exchange paper that were then rinsed batchwise five times in 0.5 M Na_2HPO_4 at room temperature, twice in distilled water, and twice in 95% ethanol, dried in an oven at 80°C, and counted in toluene-based scintillation fluid (R. Weinmann, personal communication). For each hybridization reaction, two samples were taken before incubation at 67°C; one was digested with S_1 to determine the amount of S_1-resistant probe (always less than 2% of the total input DNA), and one was incubated at 37°C in digestion buffer without enzyme to determine the total amount of DNA in each reaction.

Labeling of infected-cell polypeptides. HeLa or KB monolayers in 35-mm-diameter plastic petri dishes (approximately 10^6 cells per dish) were infected with virus suspension for 1 h at 37°C in 5% CO_2. Unadsorbed virus was removed by aspiration and replaced with 3 ml of Eagle medium containing 5% calf serum, with or without 25 μg of ara-C (Sigma) per ml, and the plates were incubated at either 32 or 40.5°C. At the time of labeling, the medium was replaced with 1 ml of Eagle medium with or without ara-C, but without serum or unlabeled methionine. Two μCi of [^{35}S]methionine (Amersham/Searle, 330 Ci/mmol) per ml was added, and after either 30 min or 1 h, labeling was stopped by removing the medium and washing twice with Tris-buffered saline at room temperature. The cell monolayers were then washed twice with ice-cold 10% trichloroacetic acid, once with cold 5% trichloroacetic acid, and twice with cold 0.05% trichloroacetic acid. The cell sheet was scraped off in the final wash and collected by centrifugation.

SDS-polyacrylamide gel electrophoresis. Samples were solubilized in 50 μl of sample buffer (1% SDS–5% β-mercaptoethanol–50 mM Tris [pH 6.8]–10% glycerol) by boiling for 3 min, and samples containing 1×10^5 to 2×10^5 cpm were analyzed by electrophoresis on a slab gel (1.5 by 10 by 15 cm) composed of a linear 7 to 20% gradient polyacrylamide-SDS separation gel with a 1- to 2-cm stacking gel of 3% polyacrylamide (23). The acrylamide-bisacrylamide ratio was 30:0.8. Gels were run at 100 V for 4 h, fixed overnight in 7.5% acetic acid–25% methanol, and dried. Autoradiographs were made with Kodak No-Screen X-ray film by exposing for 24 to 48 h. Densitometer tracings were made on a Gilson recording spectrophotometer equipped with a linear transport attachment.

Immunoprecipitation. Whole-cell extracts labeled with [^{35}S]methionine were treated sequentially with antisera and *Staphylococcus* as described by Kessler (20) and modified by Levine (personal communication). Extracts were made from 10^7 KB cells by freezing and thawing in 1 ml of reticulocyte standard buffer (RSB; 10 mM NaCl–1.5 mM $MgCl_2$–10 mM Tris-chloride [pH 7.6]) containing 0.5% Nonidet P-40 and 0.3 mg of phenylmethylsulfonyl fluoride (Sigma Chemical Co.) per ml, disrupting briefly by sonic treatment, and centrifuging at $17,000 \times g$ for 15 min to remove debris. Twenty-microliter portions were incubated with either control hamster serum or with antiserum against Ad2-transformed hamster cells (Ad2-HK-A2325 line, isolated by Lewis et al. [26]) produced by newborn hamsters that developed tumors after injection with the transformed cells (Levine, personal communication). The adenovirus-specific antiserum precipitates a 72K polypeptide from Ad5-infected cells, and this polypeptide has been shown to be the virus-coded, DNA-binding protein (Levine, personal communication). After antibody adsorption for 15 min at 4°C, 30 μl of washed *Staphylococcus aureus*, strain Cowan I (20), was added to each reaction. After 15 min at room temperature, the reactions were centrifuged at $2,000 \times g$ for 10 min, and the supernatant solutions were denatured and analyzed directly by polyacrylamide-SDS gel electrophoresis. The precipitates were washed by four centrifugations through 0.5 M NaCl–5 mM EDTA–50 mM Tris-chloride (pH 7.8)–1% Nonidet P-40–5% sucrose, and the bound material was then released by boiling with gel sample buffer. After removal of the *Staphylococcus* by centrifugation, the supernatant solution was analyzed by gel electrophoresis as above.

RESULTS

Restriction endonuclease *Hin*dIII digestion of Ad5 DNA. Complete digestion of Ad5 DNA by *Hin*dIII yielded a mixture of nine fragments that were resolved completely by electrophoresis on agarose gels. The size of each fragment was calculated from its mobility on a 1% agarose gel, assuming a logarithmic relationship between molecular weight and mobility (29) and using *Eco*R · RI fragments B and C as standards (10). The *Hin*dIII fragment A was too large to migrate according to the above relationship, and therefore its size was estimated by summing the sizes of the other fragments and subtracting this total from the molecular weight of intact Ad5 DNA, which was taken to be 23×10^6 (39). The order of *Hin*dIII fragments on the Ad5 genetic map (36; C. Mulder, personal communication) is shown in Fig. 1, with the calculated sizes indicated as the fraction of the genome length corresponding to the *Hin*dIII cleavage sites.

Strand separation of *Hin*dIII fragments. We were able to separate the DNA strands of *Hin*dIII fragments A, B, C, E, F, and G, but not D, by gel electrophoresis after alkaline de-

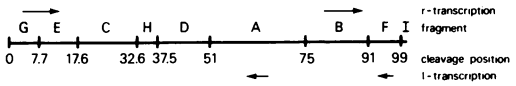


FIG. 1. *HindIII restriction endonuclease cleavage map of Ad5 DNA. Fragments are lettered according to their relative rates of migration during electrophoresis on a 1% agarose gel, from A (slowest) to I (fastest). The fragment order was determined by Mulder (personal communication). Fragment sizes were calculated from electrophoretic mobility as described in the text. The direction and approximate extent of early transcription are indicated by arrows and were determined by comparing the data in Tables 1 and 2 with those published by others (10).*

naturation as described above. We did not isolate the smallest fragments, H and I. From 5 to 25% of the isolated single strands were retained on hydroxylapatite in 0.14 M phosphate after elution from the gel and self-annealing for 24 to 48 h. Self-annealing the DNA that was not retained on the column in 0.14 M phosphate resulted in less than 1% S_1-resistant DNA in 24 h, indicating little or no contamination of this fraction with complementary strands.

Strand assignments were made according to the direction of transcription (35) by hybridizing early and late cytoplasmic RNA to the separated strands of four *Hin*dIII fragments that contain early genes, A, B, G, and F. Early cytoplasmic RNA was obtained from cells infected by *ts*125 at 40.5°C in the presence of ara-C (2, 3). This RNA hybridized to only one strand of each fragment; late RNA gave a different pattern of hybridization (Table 1). Comparison of our results to published data for Ad5 transcription that used fragments generated by different restriction enzymes (10) allowed unambiguous assignment of transcriptional direction to the fast- and slow-migrating strands of the *Hin*dIII fragments tested, because the direction of transcription of each early region of the genome had already been established (10). Our results for the A fragment agree with those of Sussenbach and Kuijk (36).

Virus DNA sequences transcribed at 40.5°C. Competition hybridization with whole-cell RNA has demonstrated that the early mutant, *ts*125, transcribes only early sequences at a restrictive temperature (3). This was corroborated by hybridization to separated strands of the three Ad5 *Eco*R · RI restriction endonuclease fragments (2). To compare the relative cytoplasmic abundance of virus RNA in cells infected by *ts*125 and by WT virus, we first examined in more detail the DNA sequences transcribed. Total cytoplasmic RNA was prepared from cells infected with *ts*125 or WT virus in the presence of ara-C at either 40.5 or 32°C. Ara-C was added to prevent late gene expression by the WT (28), and, therefore, always was present in *ts*125 infection as a control. The RNA was then hybridized to separated strands of *Hin*dIII fragments in liquid phase. Samples were taken at intervals, and the amount of hybridization was determined by S_1 endonuclease digestion. Maximum hybridization for RNA from the *Hin*dIII A_l and B_r regions was determined experimentally by sampling at 24 h; maximum hybridization for all fragments tested was also calculated from the kinetic data by extrapolation of a double-reciprocal plot to infinite time. In most cases, the 24-h hybridization value for the A_l and B_r fragments was greater than 90% of the calculated maximum hybridization. Although different RNA preparations gave somewhat variable results, all early virus sequences appeared to be transcribed in cells infected with *ts*125 at a restrictive temperature (Table 2) within the sensitivity of the method. Our results are similar to those reported by others for Ad5 early transcription (10), taking into account differences in the position of the fragments generated by different restriction enzymes. However, we consistently observed more hybridization to F_l with RNA from cells infected at 40.5°C by either WT or *ts*125.

Relative abundances of early RNA. The abundance of virus RNA transcripts that hy-

TABLE 1. *Strand assignment by hybridization of Ad5 early and late cytoplasmic RNA to separated strands of HindIII fragments*

DNA		RNA[a] hybridization (%)[b]		Strand assignment[d]
Fragment	Migration[c]	Early	Late	
A	Fast	0	71	*r*
A	Slow	19	17	*l*
B	Fast	58	100	*r*
B	Slow	0	1	*l*
F	Fast	4	11	*r*
F	Slow	33	37	*l*
G	Fast	0	7	*l*
G	Slow	64	46	*r*

[a] Early cytoplasmic RNA was extracted from cells infected with *ts*125 at 40.5°C in the presence of ara-C for 15 h; late RNA was extracted from cells infected with WT Ad5 at 37°C for 18 h.

[b] Hybridization expressed as percentage of DNA fragment single strand hybridized within 18 h under standard conditions (see text), using 1 mg of *ts*125 RNA per ml and 200 μg of late RNA per ml.

[c] Relative strand migration rates on 1% agarose gels.

[d] Strand assignment according to direction of transcription (see text; J. Virol. 22:830–831, 1977).

bridize to a given strand of a *Hin*dIII fragment should be proportional to the slope of a plot of C_0/C as a function of C_rt, where C_0 is the concentration of DNA sequences complementary to the RNA, C is the concentration of these DNA sequences remaining unhybridized at any time, and C_r is the total concentration of RNA (33). The simplest case is assumed, in which the rate constant for hybridization is the same for all RNA species, and all transcripts from a given fragment of DNA are present in equal amounts. If these conditions are not met, then the initial rate of hybridization will measure either the most abundant RNA or the RNA with the greatest hybridization rate constant or both. For comparative purposes, the relative abundance of a virus RNA species is expressed here simply as the slope of the curve, because it was not feasible to determine the rate constants directly.

In a reconstruction experiment, the relative abundances calculated from the plots of C_0/C versus C_rt agreed well with the actual abundances over a fivefold range of virus RNA concentrations (Fig. 2). Whereas the rate was not linear with concentration between 0.5 and 1.0 mg of infected-cell RNA per ml, none of the rates of hybridization observed exceeded that of the highest concentration of B_r RNA used in the reconstruction (Fig. 3 and 4).

To compare the amounts of early virus RNA in the cytoplasm of cells infected by *ts*125 and WT at 40.5°C, a single culture of KB cells was divided into two parts, which were then infected with either virus under indentical conditions. Cytoplasmic RNA was extracted and purified from both cultures in parallel and was then hybridized to a set of four Ad5 DNA probes for early RNA: A_l, B_r, F_l, and G_r. The kinetics of hybridization are shown in Fig. 3, and the abundances calculated from two experiments are listed in Table 3. Three to seven times as much RNA from each fragment was present in the cytoplasm of cells infected by *ts*125 as in the cytoplasm of cells infected by WT. In contrast, the rates of hybridization of RNA from cells infected with either virus at 32°C were almost identical when any DNA fragment was used (Fig. 4). The apparent small differences in the rate of hybridization of 32°C RNA to G_r and F_l were not reproducible in three separate experiments (Table 3). The abundances calculated from the data at 32°C were generally higher than those in cells infected with WT virus at 40.5°C (Table 3).

TABLE 2. *Saturation of HindIII fragment early strands by cytoplasmic RNA*

Fragment	Strand	Saturation[a]				Early WT[b]
		32°C		40.5°C		
		WT	*ts*125	WT	*ts*125	
A	*l*	19	27	24.6 ± 5.5[c]	25.5 ± 6.6	18
B	*r*	56	56	54	56 ± 7.2	52
F	*l*	45	52	77	73.5 ± 2.1	63
G	*r*	68	63	57	47.5 ± 16.3	58

[a] Saturation expressed as maximum percentage of single-stranded fragment hybridized, calculated from a plot of 1/fraction hybridized versus 1/time.

[b] Calculated from data of Flint et al. (10).

[c] Averages of five to six independent measurements, using different preparations of RNA, are shown with standard deviations. Other data represent averages of two or three determinations.

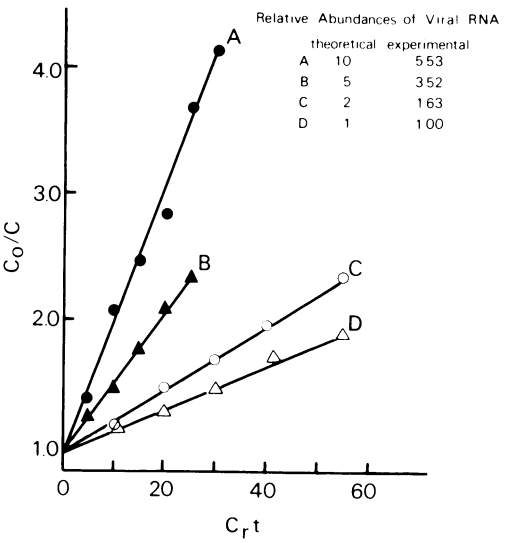


FIG. 2. *Hybridization kinetics reconstruction. Four dilutions of cytoplasmic RNA from cells infected with ts125 at 40.5°C in the presence of ara-C were hybridized to the r strand of HindIII fragment B in solution, as described in the text: (A) 1 mg/ml (●); (B) 0.5 mg/ml (▲); (C) 0.2 mg/ml (○); (D) 0.1 mg/ml (△). The final RNA concentration was adjusted to 1 mg/ml in each case with cytoplasmic RNA from uninfected cells. C_r, the total RNA concentration, was 1 mg/ml or approximately 2.8 mM in nucleoside monophosphate. Therefore, C_rt = 10 mol · s/liter at 1 h. The slopes of the hybridization curves were calculated by linear regression analysis. (Insert) Theoretical abundances of infected-cell RNAs are tabulated relative to the greatest dilution, and experimentally determined abundances are expressed as the ratios of the slopes of the hybridization curves.*

Rate of 72K polypeptide synthesis. If the increased amount of virus RNA in the cytoplasm of cells infected by *ts*125 at 40.5°C reflects an increase in functional mRNA, then the rate of synthesis of virus polypeptides should also be greater in these cells than in those infected by WT virus. We therefore measured the amount

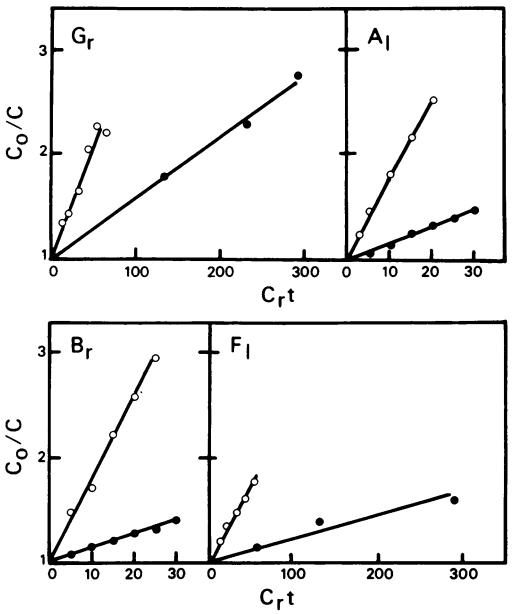


FIG. 3. *Hybridization kinetics of cytoplasmic RNA from cultures infected at 40.5°C. Two 3-liter cultures from the same pool of KB cells were infected with 100 PFU of either WT Ad5 or ts125 per cell in the presence of 25 μg of ara-C per ml added twice, at 1 and 12 h postinfection. Cultures were incubated at 40.5°C for 15 h, at which time the cells were harvested and cytoplasmic RNA was extracted as described in the text. The rates of hybridization to the indicated single strands of HindIII fragments in liquid were determined for WT RNA (●) and ts125 RNA (○) at the same time and with the same batch of Ad5 [³H]-DNA fragments.*

of [^{35}S]methionine incorporated into infected-cell polypeptides during a short pulse under these conditions. The only virus-induced band that could be discerned in cells infected in the presence of ara-C was the 72K polypeptide (Fig. 5). A comparison of the amount of labeling in the 72K region by a densitometer scan (Fig. 6) revealed that twice as much label was incorporated in *ts*125-infected cells as in cells infected by WT. The results of eight such determinations are also shown; in every case the amount of label in the 72K region was greater in *ts*125-infected cells than in cells infected by WT.

Similar results were obtained when KB cells, instead of HeLa cells, were the host and when infected cultures were shifted to 32°C before labeling (Fig. 7); when cells were infected at 32°C for 15 or 24 h, no difference was observed (data not shown).

The 72K polypeptide that was overproduced in cells infected by *ts*125 at 40.5°C and shifted to 32°C before labeling was removed from cell extracts in progressively larger amounts by increasing concentrations of antiserum to hamster cells transformed by Ad2 (26) (Fig. 7A). This antiserum is specific for several early Ad5 proteins, including the 72K DNA-binding protein (Levine, personal communication). Nonimmune serum did not cause this dose-dependent reduction in the 72K band. Analysis of the polypeptides removed from cell extracts by the immune and control sera showed differential precipitation of a 72K polypeptide by immune serum, as well as polypeptides with estimated molecular weights of 60K, 50K, 39K, and 14K (Fig. 7B).

The time course of 72K protein synthesis was investigated in pairs of cultures infected by WT and *ts*125 at 40.5°C in the presence of ara-C. The ratios of the 72K peaks in *ts*125-infected cells to those in WT-infected cells at each time are shown in Fig. 8. At 6 h after infection, only half as much label was incorporated into the 72K polypeptide in *ts*125-infected cells as in WT-infected cells. However, the ratio continued to

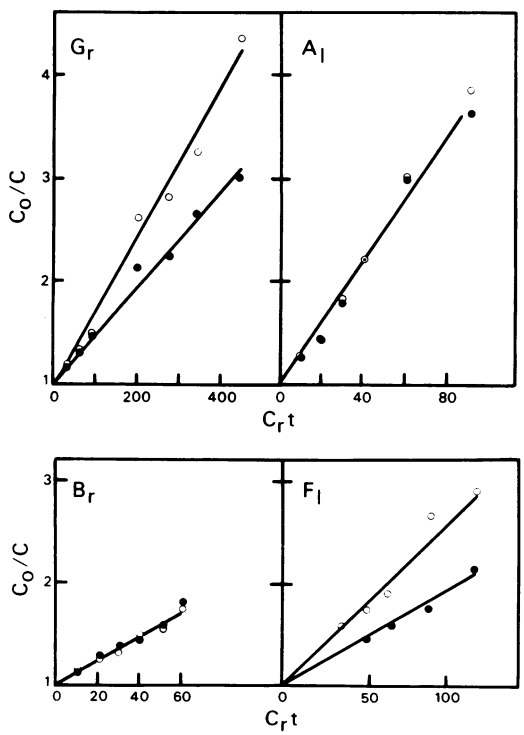


FIG. 4. *Hybridization kinetics of cytoplasmic RNA from cultures infected at 32°C. Duplicate cultures of KB cells were infected with ts125 or WT as described in the legend to Fig. 3, except that incubation was for 24 h at 32°C. WT (●) and ts125 (○) cytoplasmic RNAs were hybridized to the indicated single strands of HindIII fragments.*

TABLE 3. *Relative abundances of Ad5 early cytoplasmic RNA*

Fragment	Strand	Expt	Relative abundances[a]					
			32°C			40.5°C		
			WT	*ts*125	*ts*125/WT	WT	*ts*125	*ts*125/WT
A	*l*	1	0.336	0.313	0.9	0.163	0.763	4.7
		2	0.582	0.522	0.9	0.124	0.762	6.1
		3	0.400	0.313	0.8			
B	*r*	1	0.119	0.125	1.1	0.128	0.808	6.3
		2	0.348	0.364	1.0	0.124	0.349	2.8
F	*l*	1	0.061	0.097	1.6	0.021	0.141	6.7
		2	0.201	0.135	0.7			
		3	0.052	0.095	1.8			
G	*r*	1	0.046	0.073	1.6	0.063	0.202	3.2
		2	0.198	0.202	1.0	0.045	0.331	7.4
		3	0.063	0.060	1.0			

[a] Relative abundances are expressed as $(C_0/C \cdot C_rt) \times 10$.

rise during the experiment, until by 15 h, more than twice as much label was incorporated in *ts*125 as in WT infection.

DISCUSSION

The results of experiments described in this communication implicate an Ad5 early gene product, the 72K DNA-binding protein, in the regulation of early virus gene expression as follows. (i) Conditions under which the 72K protein does not function normally lead to an increased amount of cytoplasmic virus RNA from all early regions of the genome, relative to the amount present in WT infection. (ii) The capacity to synthesize an early gene product, the 72K protein, at a temperature restrictive for *ts*125 replication increased continuously in cells infected by the mutant, relative to synthetic capacity in WT-infected cells at the same temperature.

The extent of early virus transcription (measured by the maximum percentage of each restriction fragment hybridized) in cells infected by either *ts*125 or WT at 32°C in the presence of ara-C agreed reasonably well with published data for Ad5 early transcription (Table 2). At 40.5°C, however, the extent of DNA sequences from the F fragment represented in cytoplasmic RNA was consistently greater than that found at 32°C, for both WT and *ts*125. Thus, although the high temperature alone may have introduced some abnormality in the production of virus RNA, this was not dependent upon the particular allele at the 72K protein locus.

Measurement of the relative abundances of early virus RNA in the cytoplasm of cells infected by *ts*125 and WT at 40.5°C showed that *ts*125 caused from three to seven times as much virus RNA from each early region to accumulate as did WT virus (Fig. 3 and Table 3). No pattern could be discerned relating the portion of the genome to the extent of increased accumulation. Hybridization kinetics (Fig. 3) did not detect more than one component hybridizing to any of the fragments tested (i.e., the plots of C_0/C versus C_rt did not deviate grossly from linearity). Because multiple cytoplasmic RNA species are produced from a single region of the virus genome (4, 6, 7, 11), our results are consistent with the idea that the RNA species from a given region are present in equal abundances.

Our data for the relative abundances of cytoplasmic RNA from each of the early regions of Ad5 do not correspond to those reported for Ad2 (12). We calculate from the data in Table 3 that in WT-infected cells at 32°C the ratios of the abundances of A, B, and F RNA, relative to G RNA, were 1.3, 2.4, and 1.1, respectively; at 40.5°C the ratios were 2.6, 2.3, and 0.4. The ratios of abundances of early RNA calculated from the data of Flint and Sharp (12) are 0.4, 1.4, and 0.7. The differences may be due to a number of factors, among them the use of different temperatures, different adenovirus serotypes, different methods of quantitation, or the use of ara-C in our experiments. It is clear, in addition, that the asymmetric distribution of thymidine residues along the adenovirus genome (8, 15) will cause a systematic bias in experiments such as ours that employ [^{3}H]thymidine to label the hybridization probe. Nevertheless, the reconstruction experiment (Fig. 2) satisfies us that it is possible to compare the relative amounts of RNA from a given region of the adenovirus genome by the method that we have used. Because all of the observed rates of hybridization fell within the range shown on the

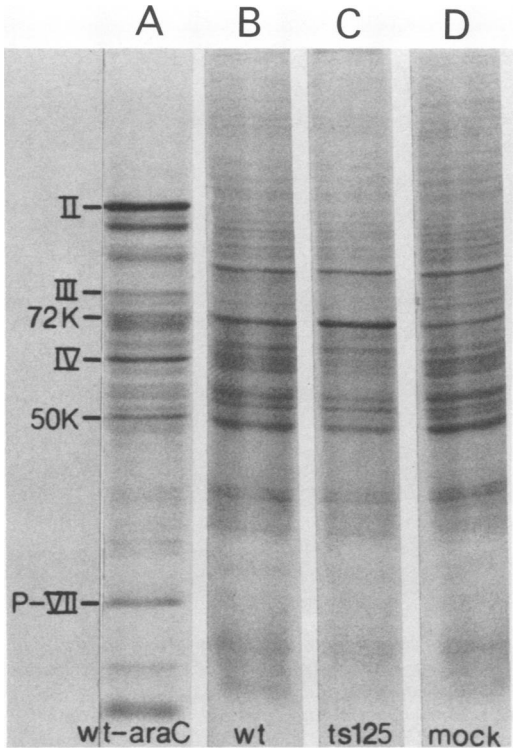
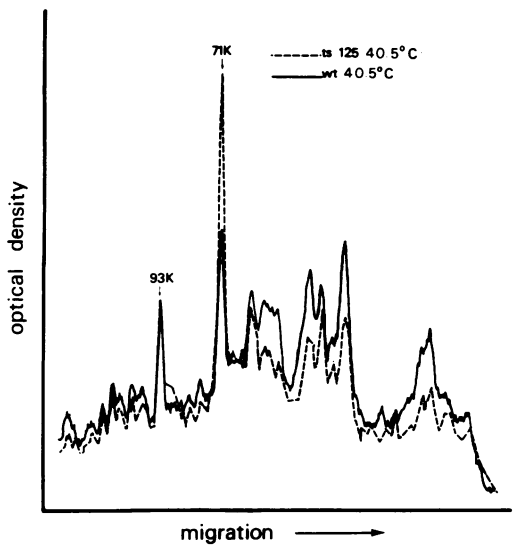


FIG. 5. *SDS-polyacrylamide gel electrophoresis of infected-cell polypeptides. HeLa cell monolayer cultures were mock infected or infected with 40 PFU of WT or ts125 per cell for 15 h at 40.5°C in the presence or absence of 25 μg of ara-C per ml. The cells were then pulse-labeled for 30 min with [^{35}S]methionine. A WT-infected culture was incubated without ara-C and shifted to 32°C before labeling to provide virus polypeptide markers. Whole-cell polypeptides were analyzed by gel electrophoresis and autoradiography as described in the text. Several virus-specific polypeptides were identified by comparison of their mobilities to those of protein standards and are labeled in the first lane of the composite autoradiograph.*

reconstruction, the fact that the highest concentration of RNA gave a twofold underestimate of the true abundance would tend to minimize the differences in abundance of early cytoplasmic RNA that we have observed in *ts*125- and WT-infected cells.

Quantitation of virus RNA in the cytoplasm of cells infected by WT or *ts*125 at 32°C in the presence of ara-C, in contrast to infection at 40.5°C, showed that the amounts of RNA from each of the four early regions were similar, whether the cells had been infected with *ts*125 or WT (Fig. 4 and Table 3). This result implies that part of the temperature-sensitive phenotype of *ts*125 is to allow the accumulation of excess virus mRNA. The hypothesis that an early adenovirus protein negatively regulates early virus RNA accumulation is consistent with our data and provides a simple explanation for the observed enhancement of early virus gene expression by inhibitors of protein synthesis (5, 31). The abundances calculated from the data at 32°C were generally higher than those in cells infected with WT virus at 40.5°C (Table 3), an effect similar to that seen with simian virus 40 (33).

An alternative explanation for the overproduction of virus RNA in *ts*125-infected cells at 40.5°C is that the virus stock contained defective particles that nevertheless provided DNA templates for early transcription. This possibility



MOI	2		0				50	100
ts125/wt	2.7	1.8	2.4	4.0	2.6	1.4	1.8	1.7

FIG. 6. *Densitometer tracings were used to compare the 72K polypeptide regions of polyacrylamide gels similar to those shown in Fig. 5. Tracings of the WT and ts125 lanes from Fig. 5 are superimposed, using the 93K peak as a standard. Results were quantitated by calculating the areas under the 72K peak and a 93K host-specific peak that showed the least variability in cells infected by either virus. The 72K peak area was normalized to the 93K peak area in each sample, and the small normalized peak area at 72K mock-infected cells was then subtracted from the 72K areas from infected cells. The resulting 72K peak areas were then compared in cells infected by WT and ts125. The inserted table summarizes the ratios of normalized 72K peak areas from different sets of experiments in which replicate cultures were infected with different multiplicities (MOI) of ts125 and WT at 40.5°C and pulse-labeled at 15 h after infection.*

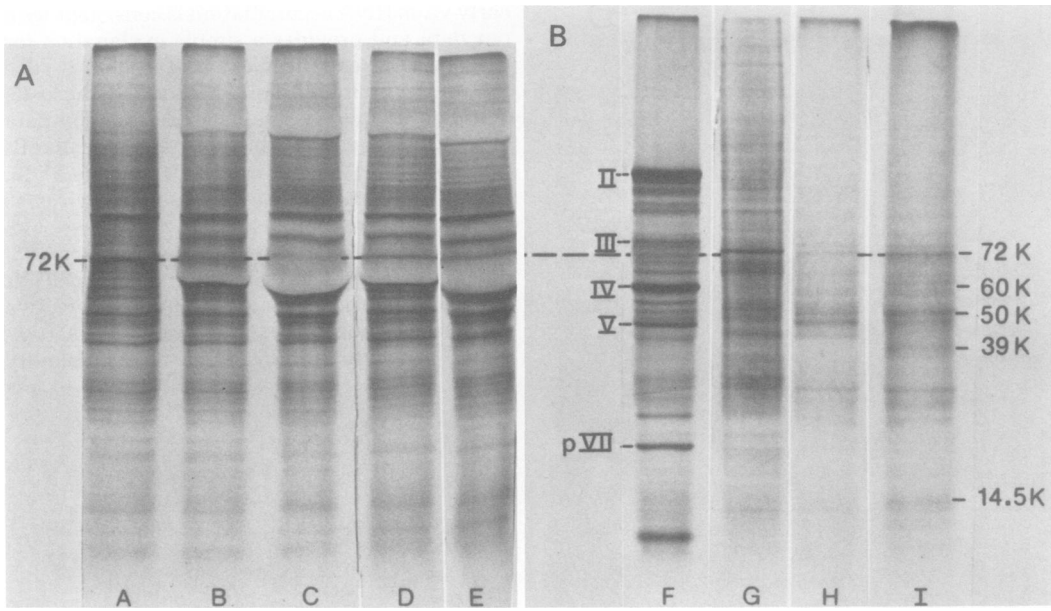


FIG. 7. *Identification of the 72K polypeptide in ts125-infected cells by immunoprecipitation. KB cells in monolayer were infected with 100 PFU of ts125 per cell. After a 1-h adsorption at 37°C, incubation was continued at 40.5°C in the presence of ara-C (25 μg/ml) added at 1 and 12 h postinfection. At 15 h postinfection, the cells were washed with medium without serum or methionine, starved in this medium for 30 min at 32°C, and labeled with 200 μCi of [^{35}S]methionine (20 μCi/ml) for 1 h at 32°C. The cells were then washed three times with cold RSB, scraped off with a rubber policeman, and frozen in RSB. Extracts were prepared and immunoprecipitations were carried out using Staphylococcus A protein (20) as described in the text. (A) Composite SDS-polyacrylamide gel autoradiogram of ts125-infected cell extracts treated with no antiserum (lane A), 2 and 5 μl of hamster antiserum against Ad2-HK-A2325 tumors (lanes B and C), and 2 and 5 μl of control hamster serum (lanes D and E). (B) Polypeptides eluted from immune complexes adsorbed by Staphylococcus A protein are shown in this composite SDS-polyacrylamide gel autoradiogram: Late infected-cell extract (lane F), unadsorbed, for markers; ts125-infected cell extract (lane G), unadsorbed; polypeptides eluted from control serum precipitate of lane E extract (lane H); polypeptides eluted from immune serum precipitate of lane C extract (lane I).*

was effectively ruled out by the results at 32°C (Table 3), unless the unlikely assumption is made that the defective templates can be utilized only at the higher temperature.

The mutant *ts*125 has an increased ability to transform rodent embryo cells at either permissive or restrictive temperatures, but the effect is more pronounced at the restrictive temperature (16). Indirect evidence that normal function of the 72K DNA-binding protein inhibits or restricts transformation has come from analysis of virus DNA sequences integrated in cell lines transformed at permissive and restrictive temperatures by *ts*125: lines transformed and maintained at the restrictive temperature consistently contain a greater portion of the adenovirus genome than do lines transformed at the permissive temperature (30). At 40.5°C, *ts*125 contains an increased concentration of cytoplasmic RNA from *Hin*dIII fragment G (Table 3), the region of the adenovirus genome thought to encode the information responsible for cell transformation. It is therefore possible that the rate of transformation is at least partially governed by the amount of mRNA transcribed or accumulated from this region of the genome. An alternative possibility, that virus integration and early transcription are functionally and therefore quantitatively linked, is a less likely explanation for the data, because the amount of *ts*125 DNA integration during infection at restrictive or permissive temperatures appears to be similar to that of WT (34).

Measurement of the capacity of infected cells to synthesize a 72K protein (Fig. 6) suggested to us that at least some of the increased amount of fragment A RNA in the cytoplasm of cells infected by *ts*125 at 40.5°C was probably functional mRNA. The 72K polypeptide was tentatively identified as the adenovirus-coded, DNA-binding protein by immunoprecipitation. The 72K band was markedly reduced by adsorption of the labeled cell proteins with antiserum specific for Ad5 early proteins, but not with non-

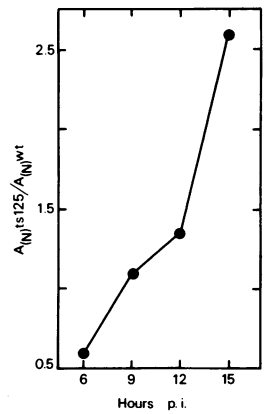


FIG. 8. *Relative rates of synthesis of the 72K polypeptide at different times in cells infected at 40.5°C with ts125 and WT. KB monolayers were infected with 20 PFU of WT Ad5 or ts125 per cell. At indicated times, the cells were labeled with [^{35}S]methionine for 30 min, and the amount of label entering the 72K polypeptide was analyzed by SDS-polyacrylamide gel electrophoresis as described in the text. Relative rates of synthesis are expressed as the normalized area, $A_{(N)}$, under the 72K peak from ts125-infected cells divided by the $A_{(N)}$ in the same region from WT-infected cells.*

immune serum (Fig. 7A). A 72K polypeptide appeared in the immune precipitate along with polypeptides (Fig. 7B), some of which may be Ad5 early gene products. Quantitative comparisons between polypeptides in the immune precipitates of cells infected by WT and *ts*125 were not attempted, however, because the efficiency of precipitation of different polypeptides by this antiserum under our conditions is not known.

In a time course experiment, the capacity to synthesize the 72K polypeptide at 40.5°C was initially lower in *ts*125-infected cells than in WT-infected cells (Fig. 8). From 6 to 15 h after infection, the rate of synthesis in a WT infection decreased steadily, while the rate of synthesis in cells infected by *ts*125 increased (data not shown). By 15 h the rate of synthesis in *ts*125 was 2.5 times that in WT and was greater than the WT rate at 6 h. It is tempting to speculate that the decreasing capacity to synthesize the 72K protein in WT infection results from autoregulation of mRNA production by the 72K protein. Autoregulation of early virus genes has been demonstrated recently for simian virus 40 (21, 33) and for bacteriophage T4 (22). In both cases, a temperature-sensitive DNA-binding protein was shown to cause increased amounts of virus mRNA to be produced under restrictive conditions. Because virus DNA replication does not occur in the presence of ara-C, free DNA-binding protein could accumulate in Ad5-infected cells and decrease the rate of early transcription. The lack of this regulatory function in *ts*125 would result in continued accumulation of virus mRNA, until an abnormally high steady-state RNA concentration was reached.

Whereas our data are consistent with the idea that the 72K protein normally modulates the amount of early virus RNA in the cytoplasm, some of which is functional mRNA, they do not distinguish among alternative models for the mechanism underlying this regulatory function. Possible mechanisms, which include action of the 72K protein during transcription, processing, transport, or degradation of virus RNA, are currently being investigated.

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