Autoregulation of Adenovirus Type 5 Early Gene Expression

II. Effect of Temperature-Sensitive Early Mutations on Virus RNA Accumulation

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The kinetics of accumulation of early virus RNA in the cytoplasm of KB cells infected at 40.5°C by wild-type (WT) adenovirus type 5 and a temperature-sensitive "early" mutant, H5*ts*125 (*ts*125), were compared by hybridization of unlabeled RNA in solution to the ³H-labeled *l* strand of Ad5 DNA *Hind*III restriction endonuclease fragment A. In the presence of 1-β-D-arabinofuranosylcytosine, A_i RNA accumulated in WT-infected cells for 9 h and then decreased in concentration to 6% of the 9-h concentration by 18 h. In *ts*125-infected cells, A_i RNA accumulated for 12 h and then remained at the same concentration for at least 6 h thereafter. The concentrations of virus RNA from the four early transcription regions of the genome were measured at 15 h in cells infected at 40.5°C in the presence of 1-β-D-arabinofuranosylcytosine by: (i) *ts*125 and WT; (ii) two other *ts* early mutants, *ts*107 and *ts*149; and (iii) a revertant of *ts*125. The revertant and *ts*149, a mutant from a different complementation group than *ts*125, both accumulated all early virus cytoplasmic RNA species in amounts similar to, or less than, WT. However, both *ts*125 and *ts*107, independently isolated mutations in the 72,000-molecular-weight (72K) DNA-binding protein gene, accumulated cytoplasmic early RNA in excess of that found in WT infection. This pattern of RNA accumulation with the mutants and WT virus was the same in the nuclei as in the cytoplasm at 40.5°C. At 32°C, however, the abundance of nuclear virus RNA from all four early regions was the same in cells infected by either *ts*125 or WT. Differences in the relative abundance of nuclear RNA from the four early regions were observed in cells infected at 40.5 and 32°C, but were not dependent upon the infecting virus genotype. These results are consistent with autoregulation of early gene expression by the 72K protein and support the hypothesis that the 72K protein either decreases the rate of early virus transcription or increases the rate of virus RNA degradation in the nucleus.

Early in lytic infection, adenovirus type 5 (Ad5) gene expression gives rise to cytoplasmic mRNA from four separate regions of the genome (Fig. 1), which are located at nearly identical positions on the maps of Ad5 and the closely related serotype Ad2. Through historical accident, most biochemical analyses of adenovirus gene expression have been done with Ad2, whereas most of the genetic studies have employed Ad5. To date, however, no major differences have been found between the two serotypes with respect to their program of gene expression. Early transcripts in the nucleus of cells infected by Ad2 are initiated at four promoters (3) and are larger than the corresponding cytoplasmic RNA molecules (3, 4, 7). Late gene expression gives rise to a different set of tran-

scripts (21, 22, 26), most of which are initiated at different promoters than early RNA (11). Although the extent of late transcription initiated at early promoters is now known precisely, some early RNA is much less abundant in the nuclei and cytoplasm at late times than late RNA (14, 21). Thus, it is possible that regulatory mechanisms at late times both restrict transcription of early genes and promote transcription of late genes.

Temperature-sensitive (*ts*) mutants of Ad5 have been isolated in two early complementation groups (10, 16, 29). Mutants in one group, represented by *ts*125 and *ts*107 (formerly called *ts*307), have a defective 72,000-dalton (72K) single-stranded DNA-binding protein (19, 27, 28) and map near the middle of the genome (20). Mutants in this group transform rodent embryo cells at a higher frequency than the wild type

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FIG. 1. *Hind*III restriction endonuclease cleavage map of Ad5 DNA. Fragment order was determined by C. Mulder (personal communication), and sizes were calculated from electrophoretic mobility on agarose gels (5). The extent of Ad5 genetic sequences present in cytoplasmic RNA is indicated by solid arrows, which also indicate relative direction of transcription (5, 12). Dashed extensions of the arrows represent additional sequences present in nuclear RNA (3, 4, 8, 11). The approximate map locations of the early mutations *ts*149, *ts*125, and *ts*107 are shown by brackets and were determined by genetic (29) and biochemical (20) means.

(WT) (16), and this effect is only observed reproducibly at a restrictive temperature (H. S. Ginsberg, personal communication). The other group, which includes *ts*149 and *ts*36, maps in the left quarter of the genome (29; Fig. 1). Members of both groups are unable to initiate virus DNA replication at restrictive temperatures (16, 17, 28), and under these conditions *ts*125, *ts*149, and *ts*36 synthesize only early virus RNA (2, 6).

In the first paper of this series, we reported that KB cells infected at 40.5°C by *ts*125 contained more early virus mRNA in their cytoplasm than cells infected by WT Ad5 (5). This appears to be analogous to the situation that occurs with *ts* mutants of simian virus 40 gene A, which are thought to synthesize a T-antigen that is unable to regulate transcription of simian virus 40 early genes normally (1, 24). We have now compared the rate of accumulation of early adenovirus RNA at 40.5°C, in the presence of 1- β -D-arabinofuranosylcytosine (ara-C), at various times after infection of KB cells with *ts*125 and WT, to determine whether the difference observed at 15 h was representative of the early phase of infection. We have also compared the concentrations of early virus RNA in the nucleus and cytoplasm of cells infected by other Ad5 *ts* mutants and a revertant of *ts*125. The results show that continued early RNA accumulation is a specific property of mutations in the 72K protein gene and that virus RNA concentrations in the nucleus are affected in the same way as cytoplasmic RNA.

MATERIALS AND METHODS

Cells and virus. KB cells were grown and virus stocks were prepared and titrated as described previously (5). The *ts* mutants *ts*125, *ts*149, and *ts*107 were obtained from H. S. Ginsberg, Columbia University. A revertant of *ts*125, R7, was isolated by growth of a *ts*125 stock at 38.5°C and two subsequent plaque purifications at 38.5°C with intervening growth at 32°C. The revertant produced large plaques at both 38.5 and 39.5°C (the restrictive temperature first used to isolate *ts*125 [10]) and had a ratio of plaquing efficiencies at 38.5 and 32°C equal to WT Ad5.

Infections. In all experiments, cells from a single pool were subdivided into as many portions as necessary and infected with 100 PFU of each virus stock per cell. Stocks of different virus strains were frozen and stored at -70°C, and a portion of each stock to be used was thawed just before infection. Ara-C was added to all cultures (25 μ g/ml) at 1 h and again at 12 h after infection to prevent virus DNA replication by WT and to control for possible effects of the drug on early RNA metabolism by the mutants.

Cell fractionation. Infected cells were harvested and cytoplasm was prepared by Nonidet P-40 treatment in isotonic buffer as previously described (5). The pellet obtained after low-speed centrifugation of Nonidet P-40-treated cell suspension was used to prepare nuclei by two methods. (i) The pellet was washed twice by resuspension in 10 mM Tris-chloride (pH 7.6), 10 mM NaCl, 1.5 mM MgCl₂, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40 and centrifugation at 250 $\times$ *g* for 10 min at 0°C (7). (ii) The pellet was resuspended in 10 mM Tris-chloride (pH 7.6), 0.14 M NaCl, 1.5 mM MgCl₂, and 0.2% sodium deoxycholate and centrifuged through a cushion of 25% glycerol containing 10 mM Tris-chloride (pH 7.9), 1 mM EDTA, and 5 mM MgCl₂ at 0°C at 250 $\times$ *g* for 10 min. Nuclear pellets from both procedures were examined by phase-contrast microscopy and found to be largely free of visible cytoplasmic contamination.

RNA purification. Cytoplasmic RNA was extracted with phenol, chloroform, and isoamyl alcohol as previously described (5). Nuclei were resuspended in 20 mM sodium acetate (pH 5.5), lysed by addition of sodium dodecyl sulfate to 0.5%, and extracted with hot phenol (21, 25). The aqueous phase was precipitated with ethanol after the hot phenol extractions, treated with DNase, sodium dodecyl sulfate, and Pronase and then reextracted with phenol, chloroform, and isoamyl alcohol in the same way as cytoplasmic RNA (5).

RNA-DNA hybridization. Hybridization kinetic analysis was carried out as previously described with separated strands of *Hind*III restriction endonuclease fragments of ³H-labeled Ad5 DNA (5). Ad5 DNA was labeled in vivo with [³H]thymidine and was purified from virions by phenol extraction (5).

RESULTS

Kinetics of early viral RNA accumulation at 40.5°C. A culture of KB cells was divided

into equal parts and infected with *ts125* or WT at 40.5°C. Ara-C was added to both cultures 1 and 12 h after infection, and portions of each culture were harvested at indicated times. Cytoplasmic RNA was prepared and hybridized in solution to the *I* strand of ³H-labeled *Hind*III restriction endonuclease fragment A, which encodes the early virus RNA for the 72K polypeptide (5, 12, 20). Abundances were calculated from the kinetics of hybridization, by determining the slope of the least-square fit of C_0/C versus $C \cdot t$, where C_0 is the total concentration of [³H]DNA, C is the concentration of DNA remaining single stranded at time t , and C_r is the total RNA concentration. The validity and limitations of this method have been discussed previously (5). Here, we use the term "abundance" to denote the slope of the hybridization kinetics, plotted in the above manner, and "relative abundance" to denote a comparison of the abundance of different RNA sequences in the same preparation. The abundance of A_I RNA increased in a similar manner for both WT- and *ts125*-infected cells during the first 9 h of infection; thereafter, the amount of virus RNA in WT-infected cells decreased steadily, whereas the amount of virus RNA remained constant in cells infected by *ts125* (Fig. 2). By 15 h, A_I RNA was three to six times more abundant in the cytoplasm of cells infected by *ts125* as in cells infected by WT, in agreement with data reported previously (5).

Cytoplasmic virus RNA abundance in cells infected by *ts* mutants at 40.5°C. The maintenance of a constant amount of early virus RNA in the cytoplasm of cells infected by *ts125* under restrictive conditions could be a result of either a specific genetic lesion or a general abnormality of *ts* early mutants. Two other *ts* early mutants, *ts107* and *ts149*, and R7 were therefore examined for this abnormality. *ts107* is an independently isolated mutant in the same complementation group as *ts125* (16, 17), and *ts149* is in a different complementation group (10). KB cells were infected in parallel by the mutants, WT, and R7 at 40.5°C. Ara-C was added to all cultures at 1 and 12 h after infection, and the cells were harvested at 15 h. Cytoplasmic RNA from each culture was hybridized in solution to each of four separated strands of ³H-labeled *Hind*III restriction endonuclease Ad5 DNA fragments: A_I , B_r , F_I , and G_r . These probes contain the genetic sequences that encode the majority of early virus RNA (5; Fig. 1). For each experiment, the same batch of cells was divided into smaller cultures which were infected, harvested, and extracted at the same time. All hybridizations of RNA from each experiment to a given fragment

FIG. 2. Time course of cytoplasmic A_I RNA accumulations at 40.5°C. KB cells were infected with 100 PFU of Ad5 WT (●, ○) or *ts125* (▲, △) per cell at 40.5°C. Ara-C (25 µg/ml) was added to all cultures 1 and 12 h after infection. At indicated times, 500- to 1,000-ml samples were taken, and cytoplasm was immediately prepared from the harvested cells (5) and frozen until completion of the experiment. RNA was purified from all cytoplasm preparations in each experiment at the same time. A 500-µg amount of each RNA preparation per ml was hybridized in solution to ³H-labeled *I*-strand of Ad5 *Hind*III fragment A (100-ng equivalents per ml; specific activity 2.9×10^6 cpm/µg). Samples were removed from the hybridizations at various times and digested with endonuclease S_1 , and radioactivity was counted on Whatman DE-81 ion-exchange paper as described previously (5). Virus RNA abundance was calculated from the plot of C_0/C versus $C \cdot t$ (5) and is expressed as the slope, $C_0/(C \cdot C \cdot t)$. Open and closed symbols refer to two separate experiments.

of DNA were done at the same time with the same batch of probe. Figure 3A shows the kinetics of hybridization to A_I DNA, and the results of three experiments with the four DNA fragments are summarized in Table 1. The amount of cytoplasmic virus RNA from each of the four early regions was the same in cells infected by *ts149* as in cells infected by WT. However, these cells contained only 16 to 50% as much early virus RNA as cells infected by either *ts125* or *ts107*. Cells infected by revertant R7 contained even less early cytoplasmic RNA than WT-infected cells at 25 h after infection.

Nuclear virus RNA abundance. To determine whether the increase in RNA levels observed for *ts125* and *ts107* relative to WT and *ts149* was specific for cytoplasmic RNA, hybridizations were carried out with RNA purified from the nuclei of cells used in two of the three experiments shown in Table 1. The nuclei were purified by centrifugation through hypertonic glycerol (experiment 1) or by extensive detergent

FIG. 3. Kinetics of hybridization of RNA from KB cells infected at 40.5°C by *ts* mutants of Ad5 to A_1 DNA. Cells were infected in the presence of ara-C for 15 h as described in the legend to Fig. 2. Nuclear and cytoplasmic fractions were prepared, RNA was extracted as described in the text, and hybridizations were carried out as described in the legend to Fig. 2. Symbols: ●, *ts107*; ▲, *ts125*; ■, □, *ts149*; ○, WT. Open and closed symbols denote two separate experiments. (A) Cytoplasmic RNA; (B) nuclear RNA.

TABLE 1. Relative abundance of early virus cytoplasmic RNA at 40.5°C 15 h after infection

Expt	Virus	Relative abundance ^a of RNA hybridizing to:			
		G_r	A_r	B_r	F_r
1	<i>ts107</i>	0.035 (5.8) ^b	0.194 (2.4)	0.131 (2.8)	0.066 (9.4)
	<i>ts125</i>	0.021 (3.5)	0.144 (1.8)	0.104 (2.3)	0.043 (6.1)
	<i>ts149</i>	0.006 (1.0)	0.082 (1.0)	0.046 (1.0)	0.007 (1.0)
2	<i>ts107</i>	0.068 (5.2)	0.303 (3.6)	0.335 (2.8)	0.037 (24.7)
	<i>ts125</i>	0.040 (3.1)	0.276 (3.3)	0.494 (4.1)	0.0350 (23.3)
	<i>ts149</i>	0.018 (1.4)	0.080 (1.0)	0.144 (1.2)	0.0055 (3.7)
	WT	0.013 (1.0)	0.084 (1.0)	0.120 (1.0)	0.0015 (1.0)
	R7	0.011 (0.8)	0.024 (0.3)	0.048 (0.4)	0.0021 (1.4)
3	<i>ts125</i>		0.102 (6.8)	0.074 (3.0)	
	WT		0.015 (1.0)	0.025 (1.0)	

^a Abundance is expressed as $C_0/(C \cdot C_r t)$.

^b The numbers in parentheses are relative abundances normalized to *ts149* (experiment 1) or WT (experiments 2 and 3).

washing in hypotonic buffer (experiment 3). Figure 3B shows the kinetics of hybridization of nuclear RNA to A_1 DNA and the calculated abundance of virus nuclear RNA from each early region is shown in Table 2. As with cytoplasmic RNA at 40.5°C, all nuclear virus RNA was present in greater amounts in cells infected by either *ts125* or *ts107* than in cells infected by *ts149* or WT. A difference was consistently observed in the relative amounts of virus RNA from the four regions in nuclei compared to cytoplasm. Regardless of the infecting virus genotype, the ratio of A_r RNA to B_r , F_r , and G_r RNA in the nuclei was generally about 1/10 of that found in cytoplasmic RNA.

At 32°C, the amount of virus nuclear RNA from each of the four early regions was not significantly different in cells infected for 24 h by *ts125* compared with cells infected by WT (Table 3). In this experiment, ara-C was added at 1 h and again at 12 h after infection to both cultures. As with cytoplasmic RNA at 40.5°C (5), the concentrations of nuclear RNA from each region relative to total nuclear RNA (the slopes of the hybridization curves) were greater at 32 than at 40.5°C.

When the total percentage of each DNA fragment hybridized was determined by kinetic analysis (5) and by hybridization for 24 to 48 h, saturation values were approximately the same

TABLE 2. *Relative abundance of early virus RNA in the nucleus of cells infected at 40.5°C 15 h after infection*

Expt	Virus	Relative abundance ^a of RNA hybridizing to:			
		G _r	A _i	B _r	F _i
1	<i>ts107</i>	0.080 (8.0)	0.083 (4.9)	0.802 (3.3)	0.058 (4.8)
	<i>ts125</i>	0.044 (4.4)	0.046 (2.7)	0.550 (2.3)	0.043 (3.6)
	<i>ts149</i>	0.010 (1.0)	0.017 (1.0)	0.240 (1.0)	0.012 (1.0)
3	<i>ts125</i>		0.140 (8.8)	0.275 (2.1)	
	WT		0.016 (1.0)	0.130 (1.0)	

^a The data are expressed as in Table 1.

TABLE 3. *Relative abundance of early virus nuclear RNA at 32°C, 24 h after infection*

Virus	Relative abundance ^a of RNA hybridizing to:			
	G _r	A _i	B _r	F _i
WT	0.14	0.28	0.47	0.11
<i>ts125</i>	0.12	0.28	0.47	0.07

^a The data are expressed as in Table 1.

for nuclear RNA and cytoplasmic RNA from the G_r, B_r, and F_i regions. However, the amount of A_i DNA hybridized was consistently greater than 50% (55 to 67%) with all nuclear RNA preparations, compared with 22 to 24% for cytoplasmic RNA.

DISCUSSION

Our results support the conclusion that synthesis of functional 72K DNA-binding protein in the absence of virus DNA replication leads to either a decreased rate of synthesis of virus RNA or an increased rate of degradation of virus RNA in the nucleus. (i) The abundance of cytoplasmic RNA from one early region of the virus genome decreased during infection by WT at 40.5°C in the presence of ara-C, whereas this RNA remained at high levels in the cytoplasm of cells infected by *ts125* under the same conditions. (ii) The temperature-dependent difference in early RNA abundance at 15 h was specific for cells infected by viruses with *ts* mutations in the 72K protein gene. (iii) The differences in early RNA abundance occurred in nuclear as well as cytoplasmic RNA.

The basis for the greater abundance of virus RNA in the cytoplasm of cells infected by *ts125* at 40.5°C for 15 h compared with cells infected by WT Ad5 appears to be a decrease in virus RNA abundance in WT infection after 9 h. A_i RNA accumulated for 9 h in WT infection, and then decreased in abundance with an apparent half-life of about 3 h, whereas in *ts125* infected cells, A_i RNA accumulated for 12 h and remained at this level for at least 6 h (Fig. 2). We

reported previously that the rate of synthesis of the 72K protein, an early gene product from the A_i region, increased continuously in *ts125*-infected cells relative to its rate of synthesis in WT infection at 40.5°C (5). It therefore appears that this pattern of synthesis reflected changes in the relative concentrations of virus mRNA at different times after infection. Assuming that the structure of A_i RNA is not altered by the mutation in *ts125* in such a way as to increase its chemical stability, then the difference between A_i RNA abundance in WT and *ts125*-infected cells after 9 h must result from the absence of a regulatory mechanism governing virus production in the mutant.

The maintenance of a high level of early virus RNA at a restrictive temperature is a specific property of mutants in the gene for the 72K protein. Neither a revertant of *ts125* nor another *ts* early mutant in a different complementation group (*ts149*) accumulated more cytoplasmic virus RNA than WT at a time after infection when the amount of *ts125* RNA was three to six times that of WT RNA. On the other hand, another *ts* mutant in the 72K protein gene, *ts107*, gave rise to amounts of early virus cytoplasmic RNA similar to *ts125* at 15 h after infection (Table 1). Because *ts149*, like *ts125*, is defective in initiation of virus DNA synthesis at 40.5°C (15, 16; T. Shutzbank, personal communication), failure of *ts149* to accumulate abnormally large amounts of virus RNA at this temperature shows that lack of initiation of virus DNA synthesis, per se, does not cause this accumulation.

The same pattern of virus RNA accumulation at 40.5°C in mutant-infected cells was observed when nuclei, instead of cytoplasm, were the source of virus RNA (Table 2). This pattern was not affected by the method of purification of the nuclei. At 32°C, however, nuclear RNA from cells infected by *ts125* contained similar amounts of all early virus sequences as nuclear RNA from WT-infected cells (Table 3).

The abundance of nuclear RNA hybridizing to a given early region relative to RNA from the

other early regions of Ad5 DNA showed consistent differences in cells infected at 32 and 40.5°C, regardless of the infecting-virus genotype. At the high temperature, A₁ RNA was reduced and B₁ RNA was increased, relative to G₁ and F₁ RNA. These changes may reflect a differential effect of temperature on the processing, degradation, or transport of different virus nuclear RNA species. Nuclear RNA also saturated a greater percentage of A₁ DNA than cytoplasmic RNA. This difference was not dependent on the temperature or the virus genotype and is consistent with the existence of a much longer primary transcript from this region of adenovirus DNA (3, 4, 8, 11).

The fact that the differences in abundance of early RNA in the nuclei of cells infected by the mutants paralleled differences in cytoplasmic RNA abundance indicates that transport of nuclear RNA to the cytoplasm is not affected by *ts* mutations *ts*125 and *ts*107. Mutations in the 72K protein gene must therefore increase the rate of transcription or decrease the rate of degradation of virus RNA in the nucleus at restrictive temperatures. Results of experiments with isolated nuclei support an effect on transcription and are the subject of a separate communication (Blanton and Carter, manuscript in preparation).

In abortive or semipermissive infection of rodent cells by human adenoviruses, little or no virus DNA replication ensues (9), and in abortive infection, only early genes are expressed (23). This situation is analogous to infection of KB cells by *ts* mutants of the 72K protein at a restrictive temperature. In abortive infection a small proportion of cells becomes transformed (15), and the absence of a functional 72K protein correlates positively with increased transformation frequency (16). If the 72K protein also modulates early gene expression in rodent cells, then the absence of functional 72K protein at restrictive temperatures in cells infected by *ts*125 or *ts*107 could prolong the expression of early virus genes, the product or products of which are necessary for oncogenic transformation (13, 18).

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