JOURNAL OF VIROLOGY, Feb. 1979, p. 458-465
0022-538X/79/02-0458/08$02.00/0

Vol. 29, No. 2

Autoregulation of Adenovirus Type 5 Early Gene Expression
III. Transcription Studies in Isolated Nuclei

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Received for publication 25 July 1978

The rate of adenovirus RNA synthesis was compared in nuclei isolated from cells infected at 40.5°C in the presence of 1-β-D-arabinofuranosylcytosine with adenovirus 5 or an early temperature-sensitive mutant of adenovirus type 5, H5*ts*125 (*ts*125). In nuclei isolated at various times after infection, the maximum amount of virus RNA synthesis occurred at 6 h after infection, after which time virus RNA synthesis declined in nuclei from wild-type infections but remained high in nuclei from *ts*125 infections. At 12 h after infection, the amount of virus RNA synthesis was 8- to 11-fold higher in nuclei from *ts*125 infections than in nuclei from wild-type infections. However, the kinetics of virus RNA synthesis in nuclei isolated from both infections were similar. When a *ts*125-infected culture was shifted to 32°C for 3 h (12 to 15 h after infection) before nucleus isolation, the amount of virus RNA synthesis in the isolated nuclei was reduced to nearly wild-type levels. A pulse-chase experiment showed little difference in degradation rates of virus RNA in isolated nuclei from wild-type and *ts*125 infections. Hybridization of RNA synthesized in vitro to restriction fragments of adenovirus type 5 DNA was consistent with early virus RNA. These results support the idea that the 72,000-dalton DNA-binding protein encoded by the mutant gene in *ts*125 can regulate early adenovirus gene expression by inhibiting initiation of transcription of the adenovirus genome.

One of the virus genes expressed early in the lytic cycle of adenovirus type 5 (Ad5) codes for a 72,000-dalton (72K) protein, which binds to single-stranded DNA (14, 23), is required for the initiation of virus DNA synthesis (12, 25), and maps at about 60 to 65 map units within the *Hin*dIII restriction endonuclease A fragment (15) (Fig. 1). H5*ts*125 (*ts*125) is a temperature-sensitive mutant of Ad5 (9) that has a lesion in the structural gene for this protein, resulting in a thermolabile 72K protein (13, 24).

At a restrictive temperature, early virus RNA accumulation in the nuclei and cytoplasm of infected cells is two- to sevenfold higher in *ts*125 infections than in wild-type (WT) infections at 12 h after infection in the presence of a DNA synthesis inhibitor, 1-β-D-arabinofuranosylcytosine (ara-C) (4). To analyze the mechanism of this overproduction of early virus RNA by *ts*125 at a restrictive temperature, we studied virus RNA synthesis in isolated nuclei from infected cells. Analysis of RNA synthesis in isolated nuclei permits a more direct measurement of the rate of transcription than in vivo labeling procedures because very short labeling periods with tritiated UTP in an RNA-synthesizing reaction result in virus RNA with a high enough specific activity to be quantitated conveniently by using filter hybridization techniques.

We show that the rate of virus RNA synthesis in nuclei from WT infections decreases markedly after 6 h, whereas the rate of virus RNA synthesis in *ts*125 infections remains at the high levels seen at 6 h. The apparent failure to shut off virus RNA synthesis in isolated nuclei occurs only in nuclei isolated from *ts*125 infections continuously incubated at a restrictive temperature, indicating that functional 72K protein is required to shut off virus transcription.

(Preliminary reports on phases of this work were presented at the 78th Annual Meeting of the American Society for Microbiology, Las Vegas, Nev., 7-14 May 1978, and at the 1978 Tumor Virus Meeting on SV40, Polyoma and Adenoviruses, Cold Spring Harbor, N.Y., 26-30 July 1978.)

MATERIALS AND METHODS

Cells and virus. KB cells were grown in suspension, and virus stocks were prepared and titrated as previously described (4). The temperature-sensitive

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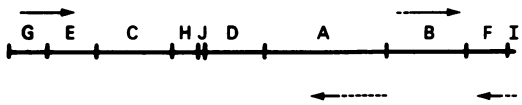

FIG. 1. *HindIII restriction endonuclease map of Ad5 DNA (20). Fragment sizes were calculated from electrophoretic mobility on agarose gels (6). Locations of Ad5 early transcripts were compiled from the literature. Dashed lines denote extent of nuclear transcription (3, 7) of Ad2; solid lines represent mature cytoplasmic mRNA (2, 10) of Ad5.*

mutant *ts*125 was obtained from H. S. Ginsberg, Columbia University.

Preparation of nuclei isolated from infected cells. KB spinner cultures were infected with 200 PFU of WT Ad5 or *ts*125 per cell and incubated at 40.5 or 32°C in a temperature-regulated water bath. ara-C (25 μg/ml) was added to both cultures at the time of infection to prevent DNA synthesis and late gene expression in WT (4). Cell harvest and nucleus preparation procedures were modified from those of Weinmann et al. (29, 30; R. Weinmann, personal communication). Infected cells were harvested by pouring infected cultures over an equal volume of frozen isotonic nucleus buffer (10 mM Tris [pH 7.9], 150 mM NaCl, 10 mM MgCl) and centrifuging for 20 min at 250 × *g* and 0°C. All subsequent steps during isolation were carried out at 0°C. Cell pellets were washed in isotonic nucleus buffer, suspended at a concentration of 1.25×10^7 cells per ml in isotonic nucleus buffer plus 2 mM dithiothreitol, and disrupted by 10 to 12 strokes with a Dounce homogenizer fitted with a tight-fitting B pestle in the presence of 0.14% Triton X-100. Cell disruption was monitored by phase-contrast microscopy. Nuclei were pelleted through a cushion containing 25% glycerol, 10 mM Tris (pH 7.9), 5 mM $MgCl_2$, 1 mM EDTA, and 2 mM dithiothreitol and resuspended in this solution at a concentration of 1.25×10^8 nuclei per ml.

Synthesis and purification of RNA. Nuclear suspensions were diluted into an RNA-synthesizing mixture to give a final concentration of 5×10^7 nuclei per ml in a solution containing 100 mM Tris (pH 7.9), 1 mM $MnSO_4$, 60 mM $(NH_4)_2SO_4$, 6 mM phosphoenolpyruvate, 10 μg of pyruvate kinase per ml, 0.6 mM each ATP, GTP, and CTP, 3×10^{-2} mM UTP, 10% glycerol, 2 mM $MgCl_2$, 0.4 mM EDTA, and 0.8 mM dithiothreitol. [^{3}H]UTP (35 to 42 Ci/mmol) was added to a concentration of 20 μCi/ml, and incubation was for 20 min at 25°C. To label RNA for filter hybridization experiments, cold UTP was omitted from the reaction mixture, and 0.5 mCi of [^{3}H]UTP per ml or 1 mCi of [^{32}P]UTP (248 Ci/mmol) was added. The reactions were stopped by the addition of 0.5% sodium dodecyl sulfate (SDS) and chilling at 0°C. The RNA was purified by extraction with hot phenol (18) saturated with 20 mM sodium acetate (pH 5.5), followed by ethanol precipitation, DNase, SDS, and Pronase treatment, reextraction with phenol, chloroform, and isoamyl alcohol as previously described (4), and, finally, purification over a Sephadex G-50 column in 20 mM sodium acetate (pH 5.5) to remove unreacted [^{3}H]UTP.

Hybridization to DNA on nitrocellulose filters. Ad5 DNA was purified as previously described (4). *Escherichia coli* DNA or whole Ad5 DNA was denatured by treatment with 0.1 M NaOH for 10 min at room temperature, neutralized, and loaded onto nitrocellulose filters in 4× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) by gravity filtration. Filters were air dried and baked for 2 h at 80°C under a vacuum.

Before hybridization, filters were treated with Denhardt solution (8) in 4× SSC for a minimum of 3 h at room temperature. Hybridizations were carried out in Denhardt solution in 4× SSC for 20 h at 68°C in a total volume of 1.5 ml in siliconized scintillation vials. Hybridization solutions were overlaid with mineral oil to prevent evaporation. After hybridization, filters were washed extensively in 2× SSC at room temperature and at 68°C and digested for 2 h at room temperature with 20 μg of boiled pancreatic RNase per ml. The filters were then washed in 2× SSC, dried, and counted in a toluene-based scintillation fluid. All hybridizations were done in duplicate, and the hybridization values reported always represent the average of the duplicates. Filters with an equivalent amount of *E. coli* DNA were incubated in each hybridization vial to determine background.

Hybridization to Ad5 *Hin*dIII restriction fragments. Ad5 DNA was digested with *Hin*dIII, and the resulting restriction fragments were separated on 1% agarose gels as previously described (4). The restriction fragments were denatured, neutralized, and transferred to nitrocellulose sheets exactly as described by Southern (19) except that 6× SSC was substituted for 20× SSC, air dried, and baked under a vacuum at 80°C for 2 h. Filter strips were soaked in Denhardt solution (8), and hybridization and subsequent processing was carried out with [^{32}P]RNA exactly as described above. After hybridization, filter strips were autoradiographed at −70°C by using Kodak X-Omat-R film between DuPont Cronex intensifying screens.

Isotopes and reagents. UTP and CTP were purchased from P-L Biochemicals Inc. ATP and GTP were purchased from Sigma Chemical Co. DNase I, "DPFF" (EC 3.1.4.5), and pancreatic RNase (EC 2.7.7.16) were purchased from Worthington Biochemicals Corp. RNase stock solutions were boiled for 10 min before use to inactivate contaminating DNase. *Hin*dIII restriction endonuclease was purchased from New England Biolabs, and agarose was obtained from Bio-Rad Laboratories. Nitrocellulose filters were B-6 membranes from Schleicher & Schuell Co. All radioisotopes were purchased from New England Nuclear Corp. All other reagents and enzymes were purchased from Sigma Chemical Co.

RESULTS

Incorporation of [^{3}H]UTP into RNA in isolated nuclei. The overall rate of incorporation of [^{3}H]UTP into isolated nuclei was linear throughout the standard incubation period of 20 min at 25°C used for all experiments (Fig. 2). WT- and *ts*125-infected nuclei synthesized approximately the same amount of RNA throughout this time interval. RNA-synthesizing reac-

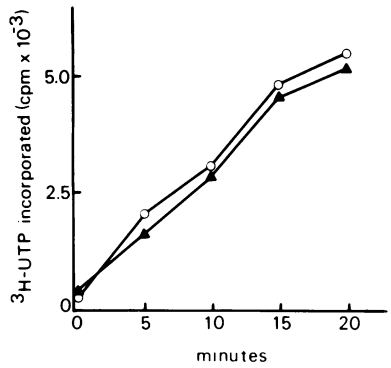

FIG. 2. *Total RNA synthesis in isolated nuclei. KB spinner cultures were infected with 200 PFU of WT (○) or ts125 (▲) per cell and incubated for 12 h at 40.5°C in the presence of 25 μg of ara-C per ml. Nuclei were isolated and incubated as described in the text at 25°C in an RNA-synthesizing mixture containing 20 μCi of [^{3}H]UTP (35 Ci/mmol) per ml. Samples (25 μl) were taken at various times during the incubation and diluted to 1 ml with 20 mM sodium acetate, pH 5.5. SDS was added to 0.5%, the samples were extracted once with hot phenol saturated with 20 mM sodium acetate, pH 5.5, and 100 μl was spotted onto a 1-inch (ca. 2.5-cm) square of DE-81 paper. The DE-81 squares were washed in 0.5 M Na_2HPO_4 and counted in toluene-based scintillation fluid as previously described (4).*

tions (see above) with 5×10^7 nuclei in 1 ml yielded 190 to 375 μg of purified RNA with a specific activity of 2×10^4 to 6×10^4 cpm/μg and a 260/280-nm absorbance ratio between 1.8 and 2.2. Sets of reactions carried out with nuclei isolated in parallel on a given day showed less variation in specific activity and final yield of the resultant purified RNA. Differences in specific activity were generally less than 0.5×10^4 cpm/μg (15%) for any WT-*ts*125 pair of nucleus preparations.

Incorporation of [^{3}H]UTP into virus-specific RNA in isolated nuclei. To detect virus-specific RNA, 10^6 cpm of RNA synthesized in vitro was hybridized to whole Ad5 DNA immobilized on nitrocellulose filters. When RNA synthesized in nuclei isolated from cells infected and incubated in the presence of ara-C at 40.5°C for 12 h was analyzed by this method, 8 to 11 times more RNA was detected as hybrid from *ts*125-infected cells than from WT-infected cells (Table 1). Hybridization with filters containing 5 and 10 μg of Ad5 DNA showed only small differences in the amount of RNA bound, demonstrating that the hybridizations were carried out under conditions approaching DNA excess. Subsequent hybridizations were also carried out with both amounts of Ad5 DNA and always gave similar results (data not shown).

Shutoff of virus transcription in WT-infected nuclei. To further characterize the increased production of RNA seen at 12 h after infection in nuclei isolated from *ts*125-infected cells, the following experiment was carried out. Pairs of spinner cultures were infected with WT or *ts*125 in the presence of ara-C and incubated at 40.5°C for various times before harvest and nucleus preparation. The results of these experiments are summarized in Fig. 3. In nuclei isolated at 3 or 6 h after infection, the amount of virus RNA synthesis was approximately the same in WT and *ts*125 infections. At subsequent times after infection, WT virus-specific RNA synthesis decreased and remained at low levels

TABLE 1. *Hybridization of in vitro nuclear RNA to Ad5 DNA filters*

RNA source	Amt of Ad5 DNA on filter (μg)	Amt of hybridization (cpm)		
		Expt 1[a]	Expt 2	Expt 3
WT	5	124[b]	81	156
	10	131	115	256
*ts*125	5	959	960	1,173
	10	1,411	1,185	1,414

[a] Each experiment involved nucleus preparations prepared on different days.

[b] Numbers represent the average of duplicate hybridization reactions with background subtracted.

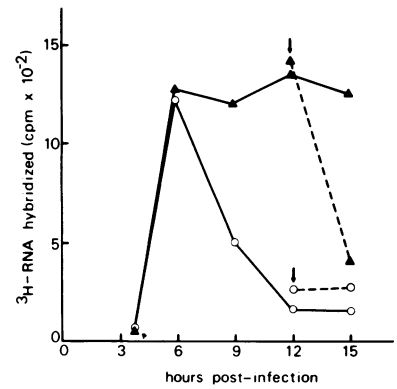

FIG. 3. *Virus RNA synthesis in isolated nuclei. KB spinner cultures were infected with 200 PFU of WT (○) or ts125 (▲) per cell and incubated at 40.5°C in the presence of ara-C. At various times after infection, 250-ml cultures (5×10^7 cells) were harvested, and nuclei were prepared. Isolated nuclei were incubated in an RNA-synthesizing mixture containing 0.5 mCi of [^{3}H]UTP per ml. Purified RNA (10^6 cpm) was hybridized to nitrocellulose filters containing 10 μg of whole Ad5 DNA. In one experiment a WT-ts125 pair of cultures was harvested at 12 h and a second pair was shifted to 32°C (denoted by vertical arrows) for 3 h before harvest.*

for up to 15 h after infection, whereas *ts*125-specific RNA production remained at high levels for up to 15 h.

To test whether the overproduction of virus RNA in *ts*125-infected nuclei at 40.5°C was a result of the temperature-sensitive lesion present in the mutant, a temperature shift experiment was performed. Duplicate pairs of spinner cultures were infected with WT or *ts*125 in the presence of ara-C and incubated for 12 h at 40.5°C. One WT-*ts*125 pair of infected cultures was harvested, and the nuclei were isolated, while the remaining pair of infected cultures was shifted to 32°C for 3 h before harvest and nucleus preparation. Figure 3 shows that when *ts*125-infected cells were shifted to 32°C, virus RNA production in isolated nuclei dropped to a value close to that seen in WT.

Kinetics of virus RNA synthesis in isolated nuclei. The difference in amounts of virus-specific transcription observed between WT and *ts*125 in isolated nuclei could be due to differential rates of elongation of the RNA chains in the isolated nuclei or to increased initiation in the intact cell at the time of isolation. To distinguish between these two possibilities, the kinetics of virus RNA synthesis were analyzed in isolated nuclei prepared from 10-h infections at 40.5°C (Fig. 4). These results show

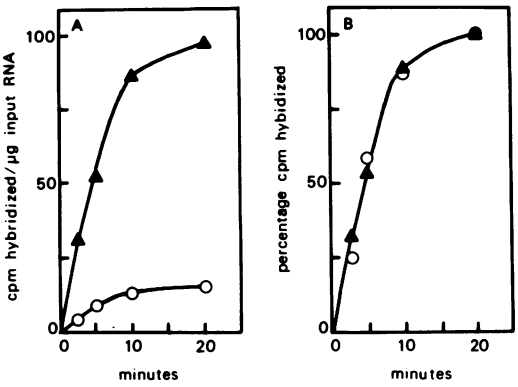

FIG. 4. *Kinetics of virus RNA synthesis in isolated nuclei. KB spinner cultures were infected with 200 PFU of WT (○) or ts125 (▲) per cell and incubated at 40.5°C in the presence of ara-C for 10 h. Nuclei were isolated and incubated in an RNA-synthesizing reaction as described in the text. At various times, RNA synthesis was stopped by the addition of 0.5% SDS and chilling at 0°C. RNA was purified, and 10^6 cpm of total RNA was hybridized to 10 μg of whole Ad5 DNA immobilized on nitrocellulose filters as described in the text. (A) Amount hybridized per microgram of input RNA. (B) Amount hybridized per microgram of input RNA normalized to the 20-min value by dividing the value for each point by the 20-min value for each curve and expressing as a percentage.*

that virus RNA synthesis followed the same kinetics in nuclei prepared from WT and *ts*125 infections. In both nucleus preparations, the rate of virus RNA synthesis was essentially linear throughout the first 10 min of incubation, after which the rate declined in both cultures.

Because host polymerase II, the enzyme responsible for transcription of early virus RNA (16, 28), does not reinitiate in vitro under conditions similar to ours (26), the kinetics of early virus RNA production in WT and *ts*125 most likely parallel the rate of total early virus RNA chain elongation in each of the infections. Therefore, we interpret the similarity of the kinetics of virus RNA synthesis in WT and *ts*125 infections to indicate that initiation of transcription rather than elongation of transcripts is the most likely level of regulation of early virus RNA synthesis by the 72K protein. The reason for the difference in kinetics observed between total RNA synthesis (Fig. 2) and virus RNA synthesis (Fig. 4) in isolated nuclei is unclear, but may reflect the kinetics of the different RNA polymerases involved in virus transcription and cell transcription.

Stability of RNA synthesized in isolated nuclei. To test the possibility that preferential degradation of WT RNA could be responsible for the differences observed in amounts of virus RNA synthesized in isolated nuclei, a pulse-chase experiment was performed. WT- and *ts*125-infected nuclei were prepared from 9-h infections at 40.5°C in the presence of ara-C. This time was chosen because at 9 h the amount of virus RNA produced in nuclei from WT infections in a 20-min reaction was approximately half of the maximum observed at 6 h (Fig. 3), but was still enough to be readily quantitated by filter hybridization. Each nucleus preparation was divided into two samples. One sample of each preparation was incubated under standard RNA-labeling conditions (see above) for 10 min before termination of the reaction with 0.5% SDS at 0°C. The second sample was incubated under standard conditions for 10 min, chilled to 0°C in the presence of a 100-fold excess of cold UTP, centrifuged for 2 min at 300 × *g* to remove [^{3}H]UTP, and suspended and incubated in an RNA-synthesizing mixture containing a 100-fold excess of cold UTP for a second 10-min period before termination with SDS. The results of filter hybridizations with RNA isolated from the above reactions are shown in Table 2. Each hybridization reaction contained 10^6 cpm of purified nuclear RNA and between 30 and 50 μg of total RNA. The amount of hybridizable RNA seen relative to that isolated after 10 min of labeling reveals that (i) synthesis of virus RNA continued upon reincubation after chilling and

TABLE 2. *Stability of RNA synthesized in isolated nuclei*

RNA source	Total labeling time (min)	Chase period (min)	Amt hybridized (cpm)	RNA hybridized/input RNA (cpm/μg)[a]	Relative hybridization[b]
WT	10		575	15.8	1.00
	10	10	589	14.3	0.90
*ts*125	10		1,499	34.5	1.00
	10	10	1,354	24.0	0.70

[a] A 10^6-cpm (30- to 50-μg) amount of RNA synthesized in vitro was hybridized in each reaction.

[b] Ratio of counts per minute per microgram for input RNA to counts per minute per microgram for input RNA from a sample labeled for 10 min.

centrifugation of the nuclei, and (ii) the amount of hybridizable virus RNA decreased during the chase by 10% in WT and by 30% in *ts*125. Thus, it is unlikely that the differences in rates of virus RNA production between WT and *ts*125 in isolated nuclei are due to increased degradation in WT.

Hybridization of in vitro nuclear RNA to *Hin*dIII restriction fragments. To determine whether the virus RNA synthesized in isolated nuclei was synthesized predominately from the early regions of the genome, [^{32}P]RNA synthesized in vitro from both WT and *ts*125 infections was hybridized to *Hin*dIII restriction fragments A through H of Ad5 DNA immobilized on nitrocellulose filters by the method of Southern (19). Figure 5 shows the pattern of hybridization obtained for RNA synthesized in nuclei isolated at 6.5 h after infection with WT and 12 h after infection with *ts*125, both at 40.5°C in the presence of ara-C. Fragments C, D, and H, the three DNA fragments representing late regions of the Ad5 genome, show only barely detectable levels of hybridization, whereas all early fragments show extensive hybridization. RNA synthesized in nuclei isolated from WT infections at 12 h after infection at 40.5°C in the presence of ara-C gave no detectable hybridization by this method.

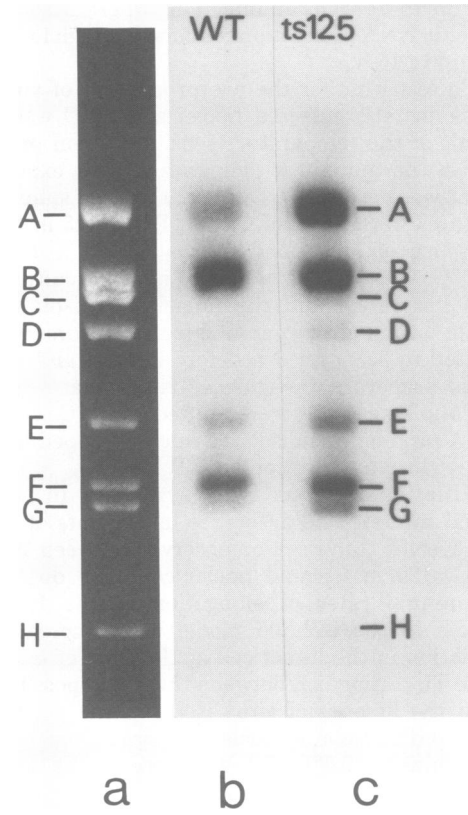

FIG. 5. *Hybridization of virus RNA labeled in vitro with [^{32}P]UTP to HindIII restriction fragments. Cells were infected at 40.5°C in the presence of ara-C with 200 PFU of WT or ts125 per cell. Nuclei were isolated at 6.5 h after infection with WT and 12 h after infection with ts125. RNA synthesis was carried out in isolated nuclei in the presence of 1 mCi of [^{32}P]UTP per ml. A total of 4 × 10^6 cpm of purified RNA was hybridized as described in the text to nitrocellulose filters containing 1.5 μg of Ad5 DNA previously digested with HindIII and subjected to electrophoresis in 1% agarose gels. (a) Ethidium bromide stain of original agarose gel showing Ad5 HindIII fragments A through H. (b and c) Autoradiograms of WT and ts125 hybridizations.*

DISCUSSION

The results reported here further support the model of an autoregulatory control mechanism for early gene expression in Ad5 mediated by the 72K DNA-binding protein (4). By 12 h after infection, at a temperature at which the 72K protein is not functional in *ts*125 infections, the rate of synthesis of virus RNA in nuclei from *ts*125-infected cells was 8 to 11 times that observed in nuclei from WT-infected cells (Table 1). The temperature shift experiment (Fig. 3) demonstrates that the greater rate of synthesis of virus RNA in *ts*125-infected nuclei is temperature dependent and suggests that functional 72K protein may be involved in the shutoff of virus RNA synthesis (4). This conclusion is also supported by our previous finding that only temperature-sensitive mutants with a lesion in the 72K protein (*ts*125 and H5*ts*107) exhibit overproduction of early virus RNA at restrictive temperatures, whereas another early mutant mapping outside the 72K region (H5*ts*149) does not exhibit this overproduction (5).

The results with isolated nuclei support the hypothesis that the abnormally high accumula-

tion of early virus RNA in both the cytoplasm and nucleus of *ts*125-infected cells at restrictive temperatures (4, 5) is due to differences in the rates of virus transcription between WT- and *ts*125-infected cells at 40.5°C in the presence of ara-C. The pulse-chase experiment (Table 2) revealed little difference in the rate of degradation of WT and *ts*125 RNA within the nucleus. Therefore, it is unlikely that an abnormally low rate of degradation in nuclei from *ts*125-infected cells was responsible for the differences in the amount of virus RNA labeled in isolated nuclei from *ts*125- and WT-infected cells. The possibility that a large subpopulation of the virus RNA synthesized in nuclei from WT infections is very rapidly degraded cannot be excluded. However, if such degradation is occurring in WT infections, it must involve a specific subpopulation of virus RNA because the virus RNA detected after 10 min of incubation appears to be relatively stable during a subsequent 10-min chase.

Hybridization of RNA synthesized in nuclei isolated from WT- and *ts*125-infected cells to *Hin*dIII restriction fragments of Ad5 DNA (Fig. 5) gave a result similar to that expected for early Ad5 RNA (Fig. 1) (2, 3, 9). Little hybridization was detected to fragments C, D, and H, which are transcribed only at late times in infection (9). We conclude from these studies that the transcription observed in isolated nuclei at 40.5°C in the presence of ara-C closely resembles normal early Ad5 transcription patterns. Apparently normal transcription patterns have been demonstrated by others in nuclei isolated from Ad2-infected cells for late transcription (26), as well as for the small virus-coded RNAs referred to as VA RNAs (25).

The results of the time course experiment (Fig. 3) show that the amount of RNA synthesis in nuclei isolated from WT and *ts*125 increased to a maximum at 6 h after infection, after which time virus RNA synthesis in nuclei from WT infections decreased, whereas virus RNA synthesis in nuclei from *ts*125-infected cells remained at the level seen at 6 h. This temporal pattern of RNA synthesis in isolated nuclei parallels the pattern of virus RNA accumulation seen in the cytoplasm of cells infected with WT or *ts*125 in the presence of ara-C at 40.5°C, but the peak rate of RNA synthesis in isolated nuclei precedes the peak virus RNA accumulation in the cytoplasm of infected cells by about 3 h (5). These results further support the idea that the increased rate of transcription seen in nuclei isolated from cells infected with *ts*125 at 40.5°C in the presence of ara-C is responsible for the abnormally large accumulation of early virus RNA seen in the cytoplasm of similarly infected cells. These data suggest that in the normal lytic cycle, early virus RNA accumulates until enough functional 72K protein has been synthesized to inhibit, by a negative feedback type of mechanism, early virus RNA synthesis and subsequent accumulation in the cytoplasm.

Others have reported that peak 72K protein synthesis occurs at around 12 to 15 h postinfection even in the presence of ara-C at 37°C (11) and that 72K protein synthesis remains at high levels for long periods of time. Our results indicate an early shutoff of early virus RNA occurring shortly after 6 h after infection with WT, as well as a decrease in *Hin*dIII fragment A early RNA accumulation in WT occurring 9 h after infection at 40.5°C (5). The apparent discrepancy between our results with early RNA and the 72K protein synthesis results reported by others is unclear, but may be due to the different temperatures employed in the different studies.

Recent results by others indicate that multiple mRNA species exist for the 72K polypeptide, with one species predominating early in infection and a second species, initiated at a second promoter, predominating late in infection (L. T. Chow, T. R. Broker, and J. B. Lewis, personal communication). Cycloheximide blocks the transition from early to late species, whereas ara-C does not. It is therefore conceivable that the 72K protein is responsible for the shutoff of RNA synthesis from the early promotor. In light of the failure of ara-C to block the transition to the late 72K mRNA species, it is noteworthy that we observe small but detectable amounts of hybridization to late *Hin*dIII fragments by RNA synthesized in nuclei isolated 12 h after infection with *ts*125 at 40.5°C in the presence of ara-C.

All of our in vitro RNA-synthesizing reactions were carried out at 25°C. Two lines of evidence suggest that the nonfunctional 72K protein synthesized in *ts*125-infected cells at 40.5°C can become functional upon a shift to a permissive temperature: (i) Ginsberg et al. (13) have shown that the 72K protein synthesized at a restrictive temperature is immunoreactive only if the protein is shifted to a permissive temperature; and (ii) in vivo shiftdown experiments in the presence of cycloheximide (Blanton and Carter, unpublished data; T. Shutzbank, personal communication) demonstrate that at least some of the 72K protein made at restrictive temperatures can recover biological activity, in terms of DNA synthesis, when shifted to a permissive temperature. Because all of our in vitro RNA-synthesizing reactions were carried out at the permissive temperature of 25°C, the observed differences in rate of transcription may reflect an event occurring before nucleus isolation, because they were observed in nuclei that must have contained functional 72K protein. How-

ever, we would expect *ts*125-infected nuclei to contain a reduced number of 72K protein molecules at 40.5°C (23), so a direct effect of the 72K protein in vitro cannot be ruled out.

It is likely that at 25°C we are measuring elongation of RNA chains previously initiated at 40.5°C in vivo in our experiments. Vennstrom et al. (26) have shown under similar conditions that no detectable reinitiation by polymerase II occurs in isolated nuclei. If we are in fact measuring synthesis of previously initiated RNA chains, then a likely mechanism for the shutoff of virus RNA synthesis in nuclei from WT-infected cells is that the 72K DNA-binding protein prevents initiation of early virus RNA synthesis.

Although we have attempted no direct test of differential initiation of virus RNA chains, the observation that virus RNA synthesis in nuclei isolated from WT and *ts*125 infections at the restrictive temperature proceeds with the same initial kinetics, coupled with the observation that virus RNA synthesis is almost complete by 10 min in nuclei from both infections (Fig. 4), suggests that a differential rate of elongation does not cause the difference in virus RNA synthesis observed between WT and *ts*125.

A regulatory function for early gene expression similar to the one suggested here for the 72K protein in adenovirus has been reported for T antigen in simian virus 40 (17, 21, 22). It has been proposed that T antigen inhibits early transcription and initiates DNA synthesis by binding to the origin of replication on the simian virus 40 genome (1). As with the simian virus 40 T antigen, the 72K protein in Ad5 shuts off early transcription and is required for initiation of DNA synthesis (12, 25). Although specific binding of the 72K protein to any location on the adenovirus genome remains to be demonstrated, the similarities between the two viruses are striking.

ACKNOWLEDGMENTS

We thank Roberto Weinmann for helpful discussions.

This investigation was supported in part by Public Health Service contract NO1 CP-53516 with the Virus Cancer Program of the National Cancer Institute, by Public Health Service grant CA 18450 from the National Cancer Institute, and by institutional grant 1N-109B from the American Cancer Society awarded to R.A.B.

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