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Adenovirus Early Region 4 Is Essential for Normal Stability of Late Nuclear RNAs

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H2*dl*808 is a deletion mutant of adenovirus type 2 lacking most of transcriptional early region E4. In most normal adenovirus host cells this virus displayed a complex mutant phenotype that included a dramatic reduction in the level of cytoplasmic late RNA, a corresponding defect in late protein synthesis, and a 5- to 10-fold defect in viral DNA accumulation. H5*dl*1004 is a deletion mutant of adenovirus type 5 that also lacks a portion of E4. It exhibited a reduction in levels of cytoplasmic late RNAs that was somewhat less severe than that of H2*dl*808 and a corresponding late protein synthetic defect but no defect in the production of viral DNA. In addition to the defect in the accumulation of late cytoplasmic mRNAs, HeLa cells infected by either H2*dl*808 or H5*dl*1004 showed substantially reduced levels of viral RNAs in their nuclei at late times after infection. Both mature mRNAs and apparent mRNA precursors were affected. The late transcription rates of the deletion mutant viruses were similar to that of wild-type virus. These results suggest that the underaccumulation of RNA in H2*dl*808- and H5*dl*1004-infected cells is caused by a reduction in the stability of viral RNA in the nucleus, and they implicate E4 products in a novel aspect of the regulation of viral gene expression.

The routine outcome of human adenovirus infection of a sensitive cell is the release of about 10^4 progeny virus particles (10). The efficiency of viral replication is assured by careful regulation of viral gene expression, which results ultimately in the production of a large amount of viral DNA and the properly timed synthesis of appropriate amounts of the proteins necessary for assembly of the viral capsid. Most of these proteins (the late proteins) are translated from mRNAs produced by the processing, at alternative splicing and polyadenylation sites, of a single species of precursor (reviewed in reference 19). This precursor, the principal viral transcript late in infection, covers the right-hand 83% of the viral genome and contains sequences that encode about 15 proteins. As might be expected, much of the regulatory apparatus of the virus participates in events necessary for the orderly expression of the late proteins. The synthesis of late mRNA is dependent upon viral DNA replication in a way that is not understood (19, 29) and also upon several viral gene products synthesized before the onset of DNA replication, during the early phase of infection. These products include at least some of the products of early region 1a (E1a), E1b, and E4 (1, 2, 5, 12, 15, 20, 21, 28). The molecular details of the activities of these proteins are of great interest, and progress has been made in understanding the mechanism of action of several of them. For example, E1a produces a product necessary for the rapid transcription of the viral genome and acts by modifying the quantity or activity of cellular transcription factors (16), whereas an E1b product participates in a posttranscriptional step in the accumulation of late viral mRNAs in the cytoplasm of infected cells (2, 20).

We and others have reported the isolation and characterization of adenovirus type 2 (Ad2) deletion mutants that lack most of E4 (6, 12, 28). These mutants are defective for growth on normal adenovirus host cells and must be propagated on the E4-complementing cell line W162 (27). In infections of normal host cells, they share a complicated mutant phenotype that includes the production of dramatically reduced amounts of viral late mRNAs and of the corresponding viral late proteins. In this paper we present an analysis of viral late RNA production in cells infected by one of these mutants, H2*dl*808, and by H5*dl*1004, a recently isolated E4 deletion mutant of adenovirus type 5 (Ad5) that exhibits a similar defect in late protein synthesis (5a). Our results indicate that a reduction in the stability of viral late RNAs in the nuclei of mutant-infected cells is responsible for the failure of late messages to accumulate in the cytoplasm. The involvement of an E4 product(s) in assuring the normal stability of nuclear RNAs in infected cells defines a new role for eucaryotic regulatory genes and suggests that host proteins with a similar function may act in the regulation of gene expression in normal cells.

MATERIALS AND METHODS

Cells and viruses. The cells used in these experiments were maintained in autoclavable Eagle minimum essential medium (Flow Laboratories) containing 10% calf serum (MEM10C) (GIBCO Laboratories). HeLa cells were obtained from J. Williams. The W162 cell line is a Vero cell derivative which contains and expresses the E4 region of Ad5 and supports the growth of adenovirus E4 deletion mutants. Its construction has been described (27).

Wild-type Ad2 was originally from A. Lewis. Wild-type Ad5 was originally from J. Williams. H2*dl*808 is an Ad2 mutant carrying a 2,008-base-pair (bp) deletion mutation with endpoints that lie in tandem repititions between positions 2946 and 2942 on the left and positions 927 and 932 on the right, numbered from the right end of the genome (13, 22). H5*dl*1004 is an Ad5 deletion mutant lacking nucleotides 2845 to 981; its construction and characterization will be presented elsewhere (5a). The positions and extents of the deletions carried by H2*dl*808 and H5*dl*1004 and the positions of E4 coding regions are indicated in Fig. 1.

Viral infections. Monolayer cell cultures growing in 100-mm petri dishes were infected with mutant or wild-type virus at a multiplicity of infection of 5 PFU per cell, based on virus titers obtained on W162 cells. Viruses were applied to the

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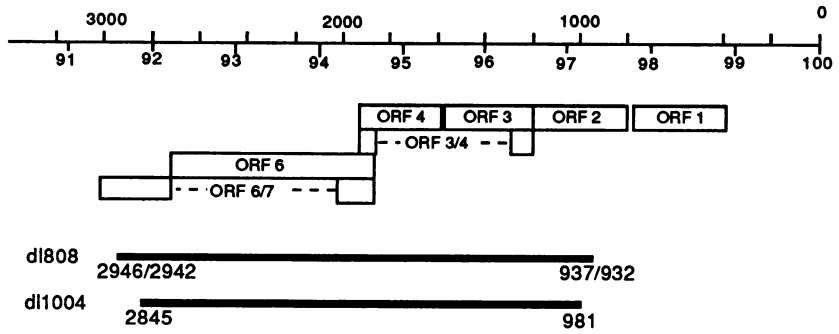

FIG. 1. Map of early region 4. The positions of the deletions carried by H2*dl*808 and H5*dl*1004 and of the open reading frames of E4 are indicated (9, 13, 26). The sequences removed by the deletions are denoted by black bars. The scale is marked in both map units and distance (in nucleotide pairs) from the right-hand end of the viral genome (13).

cells in a total volume of 2.5 ml or less in MEM10C. After 2 h at 37°C, the inoculum was replaced with 10 ml of fresh MEM10C.

Plasmids. The hybridization probes used for detection of adenovirus late region messages in nuclear run-on assays of transcription rates and in Northern (RNA) blot experiments were Ad2 DNA restriction fragments cloned in pBR322 as follows: late region 1 (L1), nucleotides 11555 to 13636 (*Hin*dIII-I); late region 2 (L2), nucleotides 15032 to 18316 (*Hin*dIII-D); late region 3 (L3), nucleotides 18316 (*Hin*dIII) to 21606 (*Bam*HI); late region 4 (L4), nucleotides 25633 to 27372 (*Eco*RI-F); and late region 5 (L5), nucleotides 30046 to 32264 (*Eco*RI-E) (nucleotide numbers are from reference 22).

The probe used in S1 analyses was obtained from pABS7. This plasmid was constructed by digestion of the late region 2 plasmid with *Sph*I, followed by ligation. The resulting plasmid contains 501 bp of adenovirus DNA (positions 17815 to 18316) and can be used to detect either L2 RNAs or the L3 message for pVI, depending on which end of the adenovirus fragment is radiolabeled (see Fig. 2e).

RNA isolation. Cells infected with mutant or wild-type virus were harvested 24 h postinfection and lysed in 0.5% Nonidet P-40 in isotonic buffer (0.14 M NaCl, 10 mM Tris hydrochloride [pH 7.4], 1.5 mM $MgCl_2$). Nuclei were pelleted by centrifugation in a microcentrifuge at 1,000 × *g* for 3 min. Total cytoplasmic RNAs were prepared from the supernatants as previously described (18). Nuclear pellets were suspended once in isotonic buffer without Nonidet P-40, and a portion of each sample was removed for DNA isolation and analysis. The remaining nuclei were pelleted by centrifugation at 7,000 × *g* for 3 min. Pellets were suspended in modified Hirt lysis buffer (20 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.6% sodium dodecyl sulfate) (14), and total nuclear RNAs were prepared by the method of Maniatis et al. (18). Nuclear RNAs to be used for S1 analysis were treated with 100 μg of RNase-free DNase (Cooper Biomedicals) per ml for 45 min at 37°C in the presence of 10 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories, Inc.) and were reextracted with phenol-chloroform (1:1) and chloroform before ethanol precipitation.

S1 nuclease analysis. Plasmid pABS7 was used as a probe for the L3 pVI message and the L2 message family. To assay pVI mRNA, the plasmid was linearized with *Hin*dIII and treated with calf intestinal phosphatase (Pharmacia Fine Chemicals) (18). Dephosphorylated DNA was labeled with ^{32}P at the 5′ end by using T4 polynucleotide kinase (18). The labeled DNA was then digested with *Sph*I to release the 501-bp adenovirus DNA fragment from the pBR322 sequences. To detect L2 messages, pABS7 was digested with *Sph*I. The resulting linear DNA was labeled with ^{32}P at the 3′ end by T4 DNA polymerase (18). Labeled DNA was cut with *Hin*dIII to liberate the adenovirus sequences from the plasmid.

Hybridizations were carried out as described previously (18) with 5 × 10^5 cpm of ^{32}P-labeled probe and 0.1 to 30 μg of total nuclear or cytoplasmic RNA. Nuclease S1 digestions were carried out as described previously (18), except that digestions contained 1,000 U of S1 per 0.3-ml reaction. After S1 digestion and precipitation of hybrids with isopropanol, pellets were suspended in 10 μl of 90% formamide–10 mM EDTA (pH 8.0). Samples were boiled for 3 min and quickly cooled on ice. Bromophenol blue dye was added before loading on 5% acrylamide–7 M urea gels. Electrophoresis was carried out at 10 V/cm until the dye began to run off the gel. Gels were dried under vacuum at 80°C for 1 h and exposed to preflashed Kodak XAR-5 X-ray film.

Quantitative estimates of the levels of viral RNAs in mutant-infected cells were made by densitometric comparison of the intensities of bands produced by those RNAs to the intensities of the corresponding bands produced by differing amounts of RNA extracted from cells infected by wild-type virus.

Northern blot analysis. Northern blot analysis of RNA from deletion and wild-type infections was performed as described previously (28), except that electrophoresis was in 1.4% agarose–3% formaldehyde gels. Blots were probed with the Ad2 L3 plasmid radiolabeled with ^{32}P by random hexanucleotide priming by the method of Feinberg and Vogelstein (8).

Nuclear run-on transcription assays. HeLa cells (5.5 × 10^6) were infected with 5 PFU of either Ad2, Ad5, H2*dl*808, or H5*dl*1004 per cell. At 24 h postinfection, the infected cells were washed twice with ice-cold phosphate-buffered saline. The cells were scraped off the dishes into isotonic buffer and lysed in baked glass tubes by the addition of 0.1 volume of 5% Nonidet P-40 in isotonic buffer and incubation on ice for 10 min. Lysed cells were spun in a Sorvall SA600 rotor at 2,300 rpm for 8 min. The nuclear pellet was suspended in isotonic buffer, and approximately 10% was removed for DNA preparation and analysis. The remaining nuclei were pelleted again and suspended in 30 μl of 10 mM $MgCl_2$–20 mM Tris hydrochloride (pH 7.5)–1 M sorbitol–5% Ficoll–0.016% spermidine–2 mM dithiothreitol. Transcription reactions contained 25% glycerol, 40 mM Tris hydrochloride (pH 8.3), 150 mM NH_4Cl, 7.5 mM $MgCl_2$, 1 mg of heparin (Sigma Chemical Co.) per ml, 0.063 mM CTP and GTP, 0.125 mM ATP, 30 U of RNase inhibitor (Bethesda Research Labora-

tories), and 200 μCi of [α-^{32}P]UTP (Dupont, NEN Research Products; 800 Ci/mmol) in a total volume of approximately 100 μl. After 35 min of incubation at 25°C, 15 μl of RNase-free DNase (1 mg/ml) was added to each reaction, and incubation was continued for an additional 15 min at 25°C. One-third volume of 10 mM Tris hydrochloride (pH 7.4)–15 mM EDTA–3% sodium dodecyl sulfate–1 mg of proteinase K per ml–3 mg of heparin per ml was added to each sample, followed by incubation at 42°C for 2 h. Samples were extracted twice with phenol-chloroform (1:1), and the final aqueous phases were applied to 5-ml columns of Sephadex G-50 (Sigma). Fractions were eluted in 10 mM Tris hydrochloride–1 mM EDTA (pH 8.1) (TE), and the fractions containing the excluded peak of radioactivity were precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. The yields of radioactive runoff RNA from equal numbers of wild-type and mutant nuclei in a single experiment generally differed by less than twofold.

Dot hybridization. Viral DNAs were measured by dot hybridization essentially as described previously (18). Isolation of DNA from nuclei was by the sodium dodecyl sulfate-pronase-phenol method (6).

RNAs were suspended in TE and partially degraded by incubation in 0.2 N NaOH on ice for 10 min before hybridization to plasmid DNAs corresponding to the five Ad2 late regions immobilized on nitrocellulose filters. Hybridizations were carried out in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–1× Denhardt solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone)–100 μg of denatured salmon sperm DNA per ml–100 μg of denatured yeast tRNA per ml–5 × 10^6 cpm of run-on RNA. Filters were incubated at 42°C for 2 to 3 days. After hybridization, filters were rinsed in 2× SET (1× SET is 0.15 M NaCl, 5 mM EDTA, and 0.05 M Tris hydrochloride [pH 8.0]) and incubated with 5 μg of RNase A per ml in 100 ml of 2× SET at room temperature for 25 min. Filters were then washed in 0.1× SET–0.1% sodium dodecyl sulfate at 60°C and exposed to Kodak XAR-5 X-ray film. Hybridized RNA was measured by scintillation spectrometry of dots cut from the filter.

RESULTS

S1 nuclease analysis. In normal adenovirus hosts, the E4 deletion mutant H2*dl*808 has a profound defect in viral late protein synthesis that results from a failure to accumulate cytoplasmic late mRNAs (28). This mutant also exhibits a modest (five- to sevenfold) reduction in the accumulation of viral DNA. We have recently isolated a second E4 deletion mutant, H5*dl*1004, that exhibits an analogous late protein synthetic defect without an accompanying defect in DNA accumulation (5a). To measure accurately the nuclear and cytoplasmic RNA reductions observed during H2*dl*808 and H5*dl*1004 infections, we employed S1 nuclease analysis. The probe used for these experiments (Fig. 2e) enabled us to detect either the pVI message (a late region 3 transcript) or the late region 2 message family by radiolabeling alternate ends of the probe fragment. The results of an S1 analysis with the 5′-labeled probe of total cytoplasmic RNA from HeLa cells infected with mutant or wild-type virus are shown in Fig. 2a and b. In cells infected by wild-type Ad2 or Ad5, the pVI mRNA should protect a 316-bp fragment of the probe corresponding to the region at the 5′ end of the pVI message between the pVI splice acceptor (nucleotide 18000) and the *Hin*dIII site at nucleotide 18316. This was the predominant species protected by cytoplasmic RNA from such infections. In cells infected by both H2*dl*808 (Fig. 2a) and H5*dl*1004 (Fig. 2b), this band was strikingly reduced in intensity: by 500-fold in the case of H2*dl*808, and by 45-fold in the case of H5*dl*1004 (Table 1).

Nuclear RNA from Ad2- or Ad5-infected cells protected the same 316-bp fragment as does cytoplasmic RNA and also protected a 501-bp DNA fragment corresponding to the complete length of the adenovirus probe (Fig. 2a and b). Protection of the latter fragment was dependent upon viral RNA; the signal vanished completely when the samples were treated with RNase before hybridization (data not shown) and was not observed when RNA from uninfected cells was analyzed (Fig. 2). Late viral mRNAs are derived by processing from a primary transcript that covers the right-hand 83% of the viral genome (4), including the full length of the probe employed here. Thus, protection of the full-length band by nuclear RNA is not surprising. The levels of RNAs that protect both the 316-bp fragment and the 501-bp fragment were reduced by about 60-fold in H2*dl*808-infected cells, compared with those in cells infected with Ad2, whereas the amounts of the two species were reduced by about 50- and 250-fold, respectively, in H5*dl*1004-infected cells when compared with those in cells infected by Ad5 (Table 2).

When the 3′ end-labeled adenovirus probe is used to detect late region 2 RNAs in the cytoplasm, a 154-bp protected fragment should be observed. This fragment corresponds to the region between the *Sph*I site located within the body of L2 mRNAs (nucleotide 17815) and the L2 polyadenylation signal (nucleotide 17969). RNAs from the same infected cells used to measure pVI RNA levels were analyzed for the presence of L2 RNA. The results of an L2 S1 analysis are shown in Fig. 2c and d. Both wild-type and deletion mutant cytoplasmic RNAs yielded the 154-bp protected fragment; however, the deletion mutant-infected cells contained reduced levels of these RNAs compared with those in cells infected by wild-type virus. The magnitude of the reductions in L2 RNA for both deletion mutants were similar to those seen for pVI RNA (Table 1).

Nuclear RNA samples analyzed for the presence of L2 RNAs are expected to protect the 154-bp fragment as well as the full-length 501-bp fragment. Both species were present in cells infected by the wild-type or mutant virus, but their levels in the mutant-infected cells were reduced by about 30-fold for H2*dl*808 and by about 12- and 50-fold, respectively, for H5*dl*1004 (Table 2).

When S1 analyses were carried out on RNAs isolated from H2*dl*808 infections of complimenting W162 cells, the hybridization signals produced by H2*dl*808 RNAs were identical to those of wild-type Ad2 RNA (data not shown).

H2dl808-infected cells generally accumulate less viral DNA during infection than do those infected by wild-type virus or H5*dl*1004 (5a, 28). In addition, DNA accumulation in cells infected by either mutant, relative to that in a parallel wild-type infection, can vary somewhat from experiment to experiment. It is likely that the values measured for the accumulation of late RNAs (or for late transcription rates, see below) are affected by this variation. To attempt to compensate for this effect, we measured the accumulation of viral DNA in infected cells in each experiment, and in each table we include DNA accumulation measurements and values for accumulation or synthesis of RNA normalized for differences in viral DNA accumulation.

Northern blot analysis of RNA from wild-type-, H2*dl*808-, and H5*dl*1004-infected cells. To confirm the data obtained from S1 experiments, the RNAs produced by the deletion

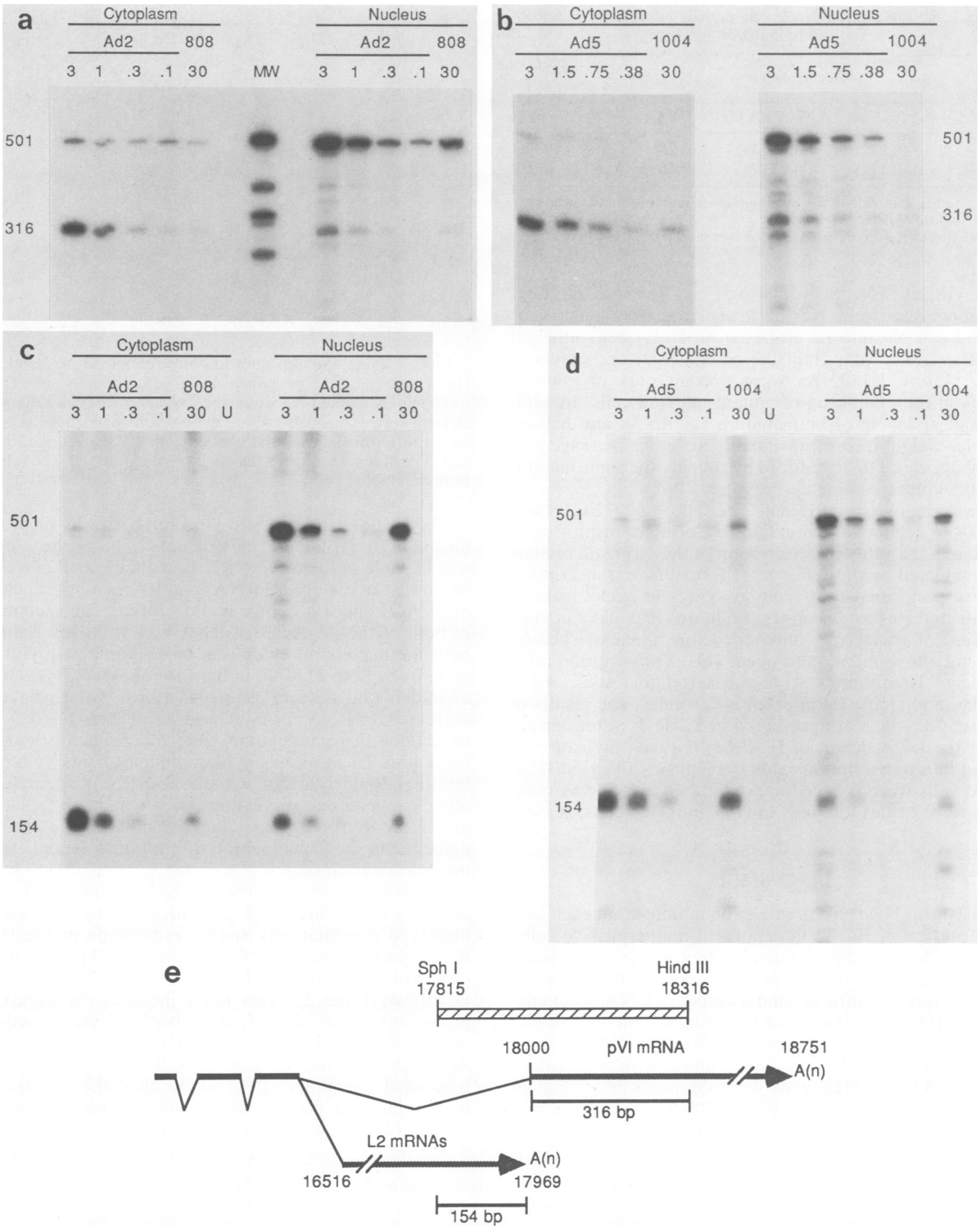

FIG. 2. S1 nuclease analysis of viral late RNAs produced by HeLa cells infected by either Ad2, Ad5, H2*dl*808, or H5*dl*1004. Wild-type and deletion mutant RNAs were hybridized to a ^{32}P-labeled probe for detection of pVI RNA (a and b) or L2 RNA (c and d). After digestion with S1 nuclease, samples were run on 5% polyacrylamide–7 M urea gels, and the gels were dried and autoradiographed. The amount of total cytoplasmic or total nuclear RNA loaded is indicated at the top of each lane. (a) pVI RNA from cells infected by either Ad2 or H2*dl*808. (b) pVI RNA from cells infected by either Ad5 or H5*dl*1004. (c) L2 RNA from cells infected by either Ad2 or H2*dl*808. (d) L2 RNA from cells infected by either Ad5 or H5*dl*1004. U, Uninfected cell RNA. (e) Schematic diagram of the adenovirus probe used for S1 hybridizations. The hatched box indicates the fragment of adenovirus DNA present in pABS7. The three black boxes represent the tripartite leader present at the 5′ end of all adenovirus late mRNAs (4). The arrows indicate the positions of pVI and L2 RNAs. Nucleotide numbers refer to distance from the left-hand end of the viral genome (22). The positions and lengths of the fragments protected from nuclease digestion by the viral RNAs are indicated below the corresponding RNAs.

TABLE 1. Cytoplasmic RNA levels in cells infected by H2*dl*808 and H5*dl*1004

Mutant	Accumulation[a]			Normalized accumulation[b]	
	L2 RNA (*n*)	pVI RNA (*n*)	DNA	L2	pVI
H2*dl*808	0.013 (2)	0.002 (4)	0.24	0.056	0.008
H5*dl*1004	0.052 (2)	0.023 (4)	0.60	0.087	0.038

[a] Expressed as a fraction of the accumulation in cells infected by wild-type virus. *n*, Number of determinations.
[b] Values for RNA accumulation divided by that for DNA accumulation.

mutant viruses were also analyzed by Northern blotting. Autoradiographs from Northern analyses of total nuclear and total cytoplasmic L3 RNAs from HeLa cells infected with either Ad2, Ad5, H2*dl*808, or H5*dl*1004 are shown in Fig. 3. Mature L3 RNAs were present only in reduced amounts in the cytoplasm of mutant-infected cells. In addition, nuclear levels of both mature L3 RNAs and higher-molecular-weight forms (presumably including message precursors) appeared to be reduced by roughly the same amount as are the corresponding cytoplasmic RNAs.

Nuclear run-on transcription. The failure of H2*dl*808 and H5*dl*1004 to accumulate viral late RNAs might result from a reduction in the rate of transcription of the viral late regions in cells infected by these mutants. To estimate transcription rates, we performed transcription assays in nuclei isolated from infected HeLa cells under conditions that allow in vitro elongation of transcripts initiated before nuclear isolation but do not allow initiation to occur (11). Transcription rates of the five late regions were measured for Ad2-, Ad5-, H2*dl*808-, and H5*dl*1004-infected HeLa cells. The results of these run-on assays are presented in Table 3. After correction for the DNA defect of H2*dl*808, the data indicate that the viral late transcription rates in H2dl808- and H5*dl*1004-infected cells were reduced approximately twofold as compared with the rates for Ad2- and Ad5-infected cells, respectively.

DISCUSSION

H2*dl*808 and H5*dl*1004 are deletion mutants that each lack a large portion of E4. In infections of nonpermissive cells, H2*dl*808 suffers from a profound defect in the cytoplasmic accumulation of late viral mRNAs, a corresponding defect in late viral protein synthesis, and a defect in DNA accumulation (28). H5*dl*1004 exhibits a pronounced but less severe protein synthetic defect, while producing roughly normal amounts of viral DNA. To examine more closely the nature of the defect in viral late gene expression during E4 mutant infections, we analyzed the late viral RNAs produced in wild type- and E4 mutant-infected HeLa cells.

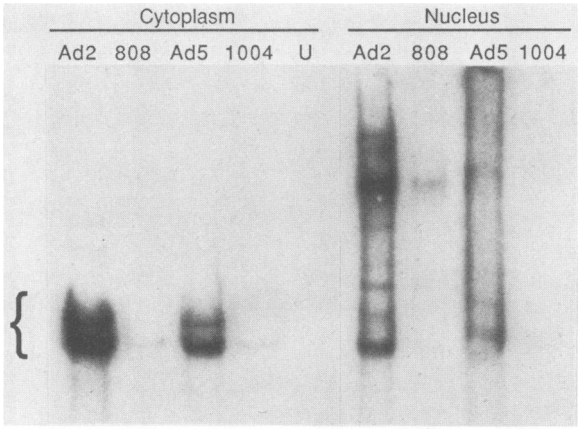

FIG. 3. Northern blot analysis of late region 3 RNA produced by HeLa cells infected by either Ad2, Ad5, H2*dl*808, or H5*dl*1004. Each lane contained 2.5 μg of the indicated RNA, fractionated by electrophoresis through 1.4% agarose–3% formaldehyde gels, transferred to nitrocellulose, and probed with ^{32}P-labeled Ad2 L3 plasmid. The bracket indicates the position of mature L3 RNA. U, RNA from uninfected cells.

The accumulation of late viral mRNAs was measured in nuclease S1 protection experiments. The probe employed was capable of identifying RNAs with a 3′ end corresponding to that of mature L2 RNAs and RNAs in which cleavage of the pVI splice acceptor had occurred. The accumulation of both of these species of RNA was strikingly reduced in both the nuclei and cytoplasm of cells infected with either mutant: by 6- to 125-fold in the case of H2*dl*808 and by 7- to 27-fold in the case of H5*dl*1004 (these values have been corrected for DNA accumulation; see Tables 1 and 2). Large reductions in accumulation were independently confirmed for mature L3 messages in both compartments by Northern blotting. Reductions in cytoplasmic RNAs extend to the other viral late regions (28), and we presume that reductions in nuclear RNAs do also. Thus, E4 mutants are profoundly defective in the accumulation of both nuclear and cytoplasmic late viral messages.

The nuclei of mutant-infected cells accumulated reduced amounts of mature viral late mRNAs. In addition, they displayed quantitatively similar reductions in the level of RNA that protected the full length of the S1 probes (normalized values of 6- to 78-fold for H2*dl*808 and 29- to 142-fold for H5*dl*1004; Table 2). This RNA includes the unprocessed precursors of the L2, pVI, and other late viral messages and presumably some by-products of late message processing. Although it is not certain what fraction of this RNA is functional precursor, it is likely that the failure of E4 mutant-infected cells to accumulate normal levels of late viral RNAs extends to message precursors in the nucleus.

TABLE 2. Nuclear RNA levels in cells infected by H2*dl*808 and H5*dl*1004

Mutant	Accumulation[a]					Normalized accumulation[b]			
	L2 RNA[c]		pVI RNA[d]		DNA	L2 RNA		pVI RNA	
	Precursor	Mature	Precursor	Mature		Precursor	Mature	Precursor	Mature
H2*dl*808	0.040	0.044	0.017	0.017	0.24	0.17	0.18	0.071	0.071
H5*dl*1004	0.021	0.082	0.004	0.022	0.60	0.035	0.136	0.007	0.037

[a] Expressed as a fraction of accumulation in cells infected by wild type virus.
[b] RNA accumulation divided by DNA accumulation.
[c] Averages of two determinations.
[d] Averages of four determinations.

TABLE 3. Transcription rates in H2*dl*808- and H5*dl*1004-infected cells

Mutant	Transcription rate,[a,b] mean ratio ± SD (*n*)					DNA accumulation (*n*)[a]	Normalized transcription rate[c]
	L1	L2	L3	L4	L5		
H2*dl*808	0.04 ± 0.005 (3)	0.05 ± 0.01 (3)	0.05 ± 0.02 (3)	0.07 ± 0.01 (3)	0.05 ± 0.02 (3)	0.09 ± 0.06 (3)	0.58
H5*dl*1004	0.14 ± 0.03 (7)	0.11 ± 0.03 (6)	0.10 ± 0.02 (2)	0.20 (1)	0.26 (1)	0.31 ± 0.12 (6)	0.52

[a] Expressed as a fraction of the value measured in cells infected by wild-type virus. *n*, Number of experiments.
[b] The ranges of hybridization signals obtained, in counts per minute above background, are as follows. For wild-type virus (Ad2 or Ad5): L1, 804 to 4,643; L2, 499 to 6,706; L3, 982 to 1,753; L4, 386 to 648; L5, 581 to 1159. For H2*dl*808: L1, 31 to 70; L2, 28 to 70; L3, 51 to 68; L4, 28 to 43; L5, 16 to 61. For H5*dl*1004: L1, 123 to 588; L2, 153 to 878; L3, 20 to 134; L4, 103; L5, 242.
[c] Average transcription rate divided by DNA accumulation.

A reduction in the rate of transcription of viral DNA could be responsible for the underaccumulation of viral RNA during H2*dl*808 and H5*dl*1004 infections. Therefore, viral late transcription rates were measured by nuclear run-on assays in mutant- and wild type-infected cells. After correction for DNA accumulation, the mutants displayed transcription rates about twofold lower than that of the wild type. These differences in transcription rate do not seem likely to account for the much larger reduction in nuclear RNA accumulation observed in these cells (8- to 25-fold, after normalization; Table 4). The failure of mutant-infected cells to accumulate normal nuclear RNA levels must therefore be the result of abnormally rapid destruction or removal from the nucleus of the RNA produced. Since neither late mRNAs nor their precursors accumulate in the cytoplasm, the simplest interpretation of these data is that nuclear RNA is rapidly degraded. We conclude that E4 products act directly or indirectly to stabilize viral late RNAs in the nuclei of infected cells.

A block in the splicing or polyadenylation of message precursors should decrease the ratio of processed nuclear mRNA to precursor. Since, in the nuclei of mutant-infected cells, the levels of pVI and L2 precursors were similar to or lower than levels of the corresponding mRNAs, it is likely that the processing of the precursor RNA that is present occurs efficiently. Similarly, a reduction in the stability of mature mRNA in the nucleus of mutant-infected cells would reduce nuclear mRNA levels relative to those of the precursor. Such a reduction was not observed: the defect in precursor accumulation seems to fully account for the defect in accumulation of the processed species. This suggests that the principal effect of E4 mutations is on the stability of message precursors and that nuclear mRNAs, once formed, are of roughly normal stability.

Studies of H5*dl*338, a deletion mutant of E1B that lacks the 55-kilodalton (kDa) E1b protein, have revealed a two- to threefold defect in the cytoplasmic accumulation of viral late mRNAs in mutant-infected cells, due to a defect in transport of mRNAs from the nucleus or to reduced stability of mRNAs in the cytoplasm (20). Since the 55-kDa protein of E1B interacts physically with the 34-kDa protein encoded by E4 open reading frame (ORF) 6 (23), it has been inferred that the E4 ORF 6 product also acts, as a member of the complex of 55- and 34-kDa proteins, in mRNA transport or stability. E4 ORF 6 mutants therefore might be expected to show a phenotype similar to that of H5*dl*338. Both of the mutants studied here lack ORF 6. In the case of H2*dl*808, a difference of about the right magnitude in the accumulation of late mRNA in the cytoplasm relative to that in the nucleus was consistently observed (Table 4) and might be due to the disruption of the process through which the complex of 55- and 34-kDa proteins acts. However, this reduction does not entirely account for the average 11- to 50-fold reduction in late cytoplasmic RNA accumulation observed in those cells (Table 4). In addition, H5*dl*1004, which also lacks ORF 6, exhibited no greater a reduction in the accumulation of cytoplasmic than in the accumulation of nuclear RNA. Finally, E1b 55-kDa protein mutants do not suffer from a large decrease in the level of viral RNA in the nucleus. It seems clear, therefore, that E4 must have a role in the production of late mRNAs outside of any part played in a 55- and 34-kDa protein transport complex.

TABLE 4. Summary of normalized transcription rate and RNA accumulation data[a]

Mutant	Transcription rate	Nuclear RNA accumulation		Cytoplasmic RNA accumulation	
		L2	pVI	L2	pVI
H2*dl*808	0.58	0.18	0.07	0.06	0.01
H5*dl*1004	0.52	0.09	0.02	0.09	0.04

[a] The normalized values from Tables 1, 2, and 3 are presented. The averages of mature and precursor RNA values are presented for nuclear accumulation.

The data we have presented suggest that one or more products of E4 participate in the maintenance of the normal stability of viral RNAs at late times of infection. Those products might function by any of a variety of mechanisms. For example, an E4 product that persists into the late phase of infection might bind directly to late RNA, protecting it from nuclease degradation. Alternatively, an E4 product might inactivate a cellular nuclease or prevent its production, thus increasing the stability of messages made late after infection. E4 products might also affect RNA stability indirectly, perhaps by participation in a step during mRNA processing whose disruption alters the stability of mRNA precursors in the nucleus. Genetic evidence implicating the products of ORF 3 (an 11-kDa polypeptide [7, 24, 25]) and ORF 6 in the accumulation of late messages has been obtained recently (5a). The isolation and purification of these proteins would clearly be of great value in determining the precise role of E4 during productive adenovirus infection.

It is unclear why novel gene products might be required for the normal stability of nuclear adenovirus RNA. Perhaps, as a mechanism to increase the efficiency of utilization of their own transcripts, adenoviruses induce a general instability in nuclear RNA while specifically protecting their own RNAs through the action of E4 products. This suggestion has some precedent: the destabilization of host RNAs is at least partially responsible for the inhibition of host cell protein synthesis in herpesvirus infections (17), although there is no evidence for the involvement of viral gene products in the stabilization of late messages in that case. A

second possibility is that E4 products may serve to circumvent a host cell defense response designed to destabilize foreign RNAs. The production of host RNA continues in adenovirus-infected cells (1, 3), and it would be of interest to know whether the E4 ORF 3 and ORF 6 products affect the stability of newly made host transcripts.

Whatever the mechanism of action of E4 products, and whether or not their activities extend to cellular RNAs, the results presented here reveal a new aspect of the regulation of expression of the adenoviral genome and may provide the first example of a class of eucaryotic regulatory proteins that act to control the stability of cellular nuclear RNAs.

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