Cellular mRNA Translation Is Blocked at Both Initiation and Elongation after Infection by Influenza Virus or Adenovirus

MICHAEL G. KATZE,* DOUGLAS DECORATO, AND ROBERT M. KRUG

Graduate Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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During influenza virus infection, protein synthesis is maintained at high levels and a dramatic switch from cellular to viral protein synthesis occurs despite the presence of high levels of functional cellular mRNAs in the cytoplasm of infected cells (M. G. Katze and R. M. Krug, *Mol. Cell. Biol.* 4:2198-2206, 1984). To determine the step at which the block in cellular mRNA translation occurs, we compared the polysome association of several representative cellular mRNAs (actin, glyceraldehyde-3-phosphate dehydrogenase, and pHe7 mRNAs) in infected and uninfected HeLa cells. We showed that most of these cellular mRNAs remained polysome associated after influenza viral infection, indicating that the elongation of the proteins encoded by these cellular mRNAs was severely inhibited. Because the polysomes containing these cellular mRNAs did not increase in size but either remained the same size or decreased in size, the initiation step in cellular protein synthesis must also have been defective. Several control experiments established that the cellular mRNAs sedimenting in the polysome region of sucrose gradients were in fact associated with polyribosomes. Most definitively, puromycin treatment of infected cells caused the dissociation of polysomes and the release of cellular, as well as viral, mRNAs from the polysomes, indicating that the cellular mRNAs were associated with polysomes that were capable of forming at least a single peptide bond. A similar analysis was performed with HeLa cells infected by adenovirus, which also dramatically shuts down cellular protein synthesis. Again, it was found that most of the cellular mRNAs, which were translatable in reticulocyte extracts, remained associated with polysomes and that there was a combined initiation-elongation block to cellular protein synthesis. In cells infected by both adenovirus and influenza virus, influenza viral mRNAs were on larger polysomes than were several late adenoviral mRNAs with comparably sized coding regions. In addition, after influenza virus superinfection of cells infected by the adenovirus mutant *dl331*, a situation in which there is a limitation in the amount of functional initiation factor eIF-2 (M. G. Katze, B. M. Detjen, B. Safer, and R. M. Krug, *Mol. Cell. Biol.* 6:1741-1750, 1986), influenza viral mRNAs, but not late adenoviral mRNAs, were on polysomes. These results indicate that influenza viral mRNAs are better initiators of translation than are late adenoviral mRNAs.

Host cell protein synthesis is effectively shut down after infection by many eucaryotic viruses (for reviews, see references 14, 21, and 27a). Despite intensive study, the mechanisms underlying the inhibition of cellular mRNA translation by viruses are still not well understood. A possible exception is poliovirus infection, in which the inhibition of cellular capped RNAs has been attributed to inactivation of the cap-binding protein complex (initiation factor eIF4F) caused by the virus-induced cleavage of the 220,000-dalton protein in the complex (11).

Adenovirus and influenza virus, each of which efficiently shuts off host cell protein synthesis (1, 2, 5, 6, 24, 31, 51), disrupt host cell mRNA metabolism in similar ways, although adenovirus is a nonenveloped DNA virus and influenza virus is an enveloped RNA virus. In cells infected by either virus, newly synthesized host cell mRNAs fail to appear in the cytoplasm; after adenovirus infection, the transport of cellular mRNAs to the cytoplasm is blocked (2, 13), whereas after influenza virus infection, cellular RNA polymerase II transcripts are degraded in the nucleus (24). In addition, with either virus infection, cytoplasmic cellular mRNAs (predominantly synthesized before infection), which are both stable and functional, as determined by their translatability *in vitro* in reticulocyte extracts, are not translated *in vivo* (2, 24).

The mechanism(s) by which adenoviral or influenza viral mRNAs are selectively translated over cellular mRNAs is not known. Recent work revealed adenoviral and influenza viral processes that are apparently directed at maintaining high levels of overall protein synthesis in infected cells rather than at the selective translation of viral mRNAs. In adenovirus-infected cells at late times, neither viral nor cellular protein synthesis occurs in the absence of VAI RNA, an adenovirus-encoded RNA polymerase III product (53). VAI RNA functions by preventing the phosphorylation of the α subunit of the eucaryotic initiation factor eIF-2 by a protein kinase induced or activated or both during adenovirus infection (26, 39, 46, 48, 50). Similarly, we have shown that influenza virus encodes a gene product which, similar to VAI RNA, prevents the shutdown of overall protein synthesis caused by an eIF-2 α -kinase turned on by viral infection (23).

As the first step in understanding the mechanism of translational selectivity in cells infected by influenza virus or adenovirus, it was necessary to determine whether the block in host cell protein synthesis occurred at initiation or elongation or both. To address this question, we compared the polysome association of specific cellular mRNAs in infected and uninfected cells. We showed that after infection with either influenza virus or adenovirus, most of these cellular mRNAs remained associated with polyribosomes. The polysomal distribution of the cellular mRNAs indicated that

* Corresponding author.

their translation was blocked at both initiation and elongation. We also obtained evidence that influenza viral mRNAs are better initiators of translation than are late adenoviral mRNAs.

MATERIALS AND METHODS

Cells and virus. HeLa cells and the adenovirus type 5-transformed human embryonic kidney cell line 293 (16) were grown in monolayers in Dulbecco modified Eagle medium containing 10% calf serum. Wild-type adenovirus type 2 (Ad2) was purified from infected cells, as previously described (43). The adenovirus type 5 deletion mutant *d*l331 (53) was propagated in 293 cells. The titer of adenovirus stocks was determined by plaque assay on 293 cells (30). The WSN strain of influenza A virus was grown in MDBK cells and titrated by plaque assay in MDCK cells (12).

Virus infection. Monolayers of HeLa cells were infected with influenza virus at a multiplicity of infection (MOI) of approximately 50 PFU per cell. HeLa cells were infected with Ad2 at an MOI of 50 PFU per cell. For the experiments in 293 cells, *d*l331 and wild-type Ad2 were used at an MOI of 5 to 10 PFU per cell. When indicated, cells infected with wild-type Ad2 or *d*l331 were superinfected at 16 h postinfection with influenza virus at an MOI of 50 PFU per cell. To analyze protein synthesis, cells were labeled at the indicated times for 30 min with [³⁵S]methionine (50 μ Ci/ml) in Dulbecco modified Eagle medium plus 1/10 the normal amount of methionine, and the labeled proteins were subjected to gel electrophoresis on 14% polyacrylamide gels (24).

Preparation of polysomes. Polysomes were prepared by using a modification of the methods of Lenk and Penman (32) and Cervera et al. (10). Cells were collected by centrifugation at $800 \times g$ for 10 min and were washed twice with ice-cold phosphate-buffered saline containing 100 μ g of cycloheximide per ml. Cycloheximide at 100 μ g/ml was included in all buffers to prevent peptide chain elongation and hence polyribosome runoff (15). The cell pellet was suspended in ice-cold buffer containing 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, 1.5 mM MgCl₂, 100 μ g of cycloheximide per ml, and 5 mM vanadyl ribonucleoside complexes. Triton X-100 (0.5%) was then immediately added. After 3 min on ice, 1% Tween 40 and 0.5% sodium deoxycholate were added, and the cells were disrupted by homogenization in a type A Dounce homogenizer. Cell debris was removed by centrifugation at $12,000 \times g$ for 10 min, and the resulting cytoplasmic extract was layered on a 10 to 50% linear sucrose gradient in 10 mM Tris hydrochloride (pH 7.5)–5 mM magnesium acetate–500 mM KCl (buffer A). Polysomes were resolved from ribosomal subunits and other small structures by centrifugation in a Beckman SW28 rotor at 27,000 rpm for 4 h at 4°C. Gradient fractions (1.2 ml) were collected and diluted with an equal volume of a buffer containing 0.2 M Tris hydrochloride (pH 7.5), 25 mM EDTA, 0.3 M NaCl, and 0.2% sodium dodecyl sulfate. Pronase (500 μ g/ml) was added, and the mixture was incubated at 37°C for 30 min. Fractions were pooled as described below, and the RNA was extracted from the pooled samples with phenol-chloroform. The RNA was ethanol precipitated, and poly(A)-containing [poly(A)⁺] RNA was isolated by oligo(dT)-cellulose chromatography.

Metrizamide gradients. Cytoplasmic extracts were prepared as described above, except that β -mercaptoethanol at 0.2% was added to the cell disruption buffer. The extracts were layered on top of metrizamide step gradients containing

(from top to bottom) 0.1 ml of 60% metrizamide, 4.6 ml of 40% metrizamide, and 0.2 ml of 35% metrizamide in buffer A containing 0.2% β -mercaptoethanol (4). The gradients were centrifuged for 65 h at 35,000 rpm in an SW50.1 rotor at 4°C. Gradient fractions (0.5 ml) were collected, and the RNA was obtained from each fraction.

Analysis of RNA. Poly(A)⁺ RNA was translated in micrococcal nuclease-treated rabbit reticulocyte lysates (40) kindly provided by R. Jagus. Translation products labeled with [³⁵S]methionine were analyzed on 14% polyacrylamide gels. For Northern blot analysis, poly(A)⁺ RNA was electrophoresed on a formaldehyde-agarose 1% gel (55) and transferred to a nitrocellulose filter, which was hybridized with the indicated labeled DNAs. For detecting cellular mRNAs, the probes were nick-translated double-stranded cDNAs (47). For detecting influenza viral nucleocapsid protein (NP) mRNA, the virion RNA-sense M13 NP DNA clone (kindly provided by G. Shapiro of this laboratory) was labeled as described by Mason et al. (37). The adenovirus probes were nick-translated pBR322 plasmids, one containing the *Sma*I D fragment (40.5 to 52.5 map units) for the L2 region and the other containing the *Hind*III-*Bam*HI fragment at 50.1 to 59.5 map units for the L3 region. Where indicated, poly(A)⁺ RNA was directly dotted onto nitrocellulose filters which were then hybridized with the labeled DNA probe (2, 59). The resulting labeled dots were cut out and counted in a liquid scintillation counter.

RESULTS

Association of host cell mRNAs with polysomes after influenza virus infection. The shutoff of host cell protein synthesis that occurs after influenza virus infection is shown in Fig. 1A. HeLa cells, either mock infected or infected with influenza virus for 4 h, were labeled for 30 min with [³⁵S]methionine, and the labeled proteins were examined by gel electrophoresis. By 4 h after infection the synthesis of all cellular proteins was drastically inhibited. For example, the synthesis of actin was reduced more than 90%, as determined by densitometer tracing of the autoradiogram (data not shown). Essentially, only viral proteins were synthesized in the infected cell, with NP, the membrane protein (M1), and nonstructural protein 1 (NS1) predominating.

To determine whether the block in cellular mRNA translation was at the initiation or elongation step or both, we analyzed the distribution of cellular mRNAs on the polysomes from uninfected and influenza virus-infected cells. Average polysome size, i.e., the average number of ribosomes per given mRNA molecule, should be proportional to both the length of the mRNA coding region and the rate at which it initiates translation and should be inversely proportional to the rate at which the encoded protein is elongated (33, 34). If the block in host cell mRNA translation was at the initiation step alone, cellular mRNAs would be displaced from polysomes after virus infection. On the other hand, if the block in translation was at the elongation step alone, the polysomes containing host cell mRNAs would increase in size after infection. A combined initiation-elongation block would give an intermediate result: a significant proportion of the host cell mRNAs would remain associated with polysomes, which would probably not increase in size, and in fact, some cellular mRNAs might be on smaller polysomes or displaced from polysomes.

Cytoplasmic extracts were treated with sodium deoxycholate and the nonionic detergent Tween 40 to release the polysomes associated with the cytoskeleton (19, 32, 33), and

FIG. 1. Shutoff of cellular protein synthesis and association of host cell mRNAs with polysomes after influenza virus infection. (A) HeLa cells were either mock infected (M) or infected with influenza virus (F) at 50 PFU per cell. At 4 h postinfection the cells were labeled with [³⁵S]methionine (50 μ Ci/ml) for 30 min. The labeled proteins were analyzed on a 14% gel. The positions of representative influenza virus-specific proteins are shown on the right, and the position of actin is shown on the left. (B) Sedimentation profile of polyribosomes from mock-infected and influenza virus-infected (FLU) cells. A cytoplasmic extract from approximately 2×10^8 cells was subjected to centrifugation on a 10 to 50% sucrose gradient, as described in Materials and Methods. Fractions were collected, and the A_{260} was determined. The gradient fractions were pooled as shown, yielding samples A through G. (C) Northern blot analysis of cell-equivalent amounts of poly(A)⁺ RNAs in samples A through G prepared from mock-infected and influenza virus-infected (FLU) cells, using as probes ³²P-labeled actin, GAPD, and pHe7 DNA clones. The positions of 18S and 28S rRNA markers are shown on the right. (D) Dot blot analysis of distribution of actin mRNA sequences. Poly(A)⁺ RNA from each gradient sample was denatured and dotted onto nitrocellulose, which was hybridized with ³²P-labeled actin DNA. The labeled dots were cut out and counted in a liquid scintillation counter. The total counts of actin mRNA sequences in the mock-infected and influenza virus-infected (FLU) samples were 9,606 and 7,433 cpm, respectively.

500 mM KCl was included in the sucrose gradients to minimize any artifactual sticking of RNA to polysomes. The presence of 500 mM KCl should have caused most 80S monosomes to dissociate into 60S and 40S ribosomal subunits (7). As shown by the absorbance profile of the sucrose gradient analysis (Fig. 1B), uninfected and infected cells had similar proportions of polysomes and ribosomal subunits and the sizes of uninfected- and infected-cell polysomes were similar. The gradient fractions were pooled into seven samples: samples A through E, five polysome

samples, with A containing the largest polysomes and E containing the smallest along with any residual monosomes; sample F, ribosomal subunits; and sample G, the material sedimenting more slowly than the ribosomal subunits. The poly(A)⁺ RNA from the seven samples was analyzed by Northern blotting. The RNA was resolved by electrophoresis on agarose gels, which were blotted onto nitrocellulose filters and then probed with labeled cloned DNAs specific for the cellular mRNAs encoding the following proteins: (i) β -actin (molecular weight, 45,000) (17), (ii) glyceraldehyde-

FIG. 2. Functional viral and cellular mRNAs on polysomes of influenza virus-infected and mock-infected cells. (A) Northern blot analysis of poly(A)⁺ RNA prepared from gradient samples of influenza virus-infected cells shown in Fig. 1B, using a ³²P-labeled probe specific for NP mRNA prepared as described in Materials and Methods. (B) In vitro translation of poly(A)⁺ RNA from the gradient samples of influenza virus-infected cells. Cell-equivalent amounts of poly(A)⁺ RNA were used to program rabbit reticulocyte extracts in the presence of [³⁵S]methionine. The translation products were electrophoresed on a 14% gel. The positions of representative influenza virus-specific proteins and actin are shown on the right. (C) In vitro translation of poly(A)⁺ RNA from the gradient samples of mock-infected cells shown in Fig. 1B. The -RNA lane contained translation products made in the absence of added RNA.

3-phosphate dehydrogenase (GAPD) (molecular weight, 36,000) (44), and (iii) pHe7 (molecular weight, 25,000) (24), encoded by an mRNA discovered in a cDNA library of HeLa cell poly(A)⁺ mRNA (2).

In mock-infected cells, most of the β -actin and GAPD mRNAs were associated with polysome samples B, C, and D, and pHe7 mRNA, a smaller mRNA, was located mainly in polysome samples C and D (Fig. 1C). After influenza virus infection for 4 h, most of the actin and GAPD mRNAs remained associated with polysome samples B, C, and D, but an increased fraction of these two mRNAs was found in polysome-monomer sample E and ribosomal subunit sample F. With the smaller pHe7 mRNA, the association with polysome samples C and D also continued in the infected cells, although there was a larger increase in the amount of this mRNA that became associated with slower-sedimenting material (samples E, F, and G). To obtain better quantitation of the distribution of the actin mRNA in the gradient samples, a portion of the poly(A)⁺ RNA in each sample was denatured and dotted directly onto nitrocellulose, which was hybridized to the labeled actin DNA probe, and the labeled dots were counted. Most of the actin mRNA sequences were polysome associated (Fig. 1D, samples A through E) in both mock-infected and influenza virus-infected cells. In the mock-infected cells, 84% of these sequences were on the polysomes, predominantly in samples B, C, and D. In the infected cells, 72% of these sequences were on the polysomes, but the relative amounts in samples B and C decreased, with a concomitant increase in the relative amounts in the smallest polysomes (sample E) and the top of the gradient (sample G). The approximately 20% decrease in the overall level of actin mRNA sequences after infection shown in Fig. 1D was documented previously (24) and presumably reflects at least in part the turnover of actin mRNAs in the cytoplasm in the absence of new synthesis. These results verify both the persistence of most of the actin mRNAs on the polysomes and the shift of some of these mRNAs from larger polysomes to slower-sedimenting struc-

tures after virus infection. Clearly, the polysome pattern of the three cellular mRNAs is that predicted for a combined elongation-initiation block in translation.

In infected cells, the polysomes containing cellular mRNAs were similar in size to those containing viral mRNAs. As shown by Northern blot analysis (Fig. 2A), the viral NP mRNA, which encodes a protein with a molecular weight of 56,000, was found predominantly in polysome samples B, C, and D, in which the cellular actin and GAPD mRNAs were also located. To localize the other viral mRNAs, the poly(A)⁺ RNA from the gradient samples was translated in reticulocyte extracts (Fig. 2B). The viral mRNAs were found almost entirely in polysome samples B through E, with the larger mRNAs (e.g., the mRNA encoding NP) located predominantly in samples B through D, whereas the smaller mRNAs (e.g., the mRNA encoding NSI) were also located in large amounts in sample E. The other translation products programmed by the poly(A)⁺ RNA from polysome samples B through E almost certainly represented cellular proteins encoded by the cellular mRNAs which, as shown in Fig. 1, were present on these polysomes. The actin band was particularly evident. The other bands comigrated with proteins programmed by the poly(A)⁺ RNA from the polysome samples of mock-infected cells (Fig. 2C). The predominance of the viral proteins in the translation products programmed by the infected-cell polysomal RNAs (Fig. 2B) was probably due to the higher copy number of the influenza viral mRNAs or to the more effective translation of influenza viral mRNAs in reticulocyte extracts (24) or to both of these factors. When much smaller amounts of polysomal poly(A)⁺ RNA were added to the reticulocyte extracts, the ratio of the cellular to viral translation products programmed by the infected-cell polysomal RNAs increased and the amounts of cellular translation products programmed by the infected- and uninfected-cell polysomal RNAs were comparable (data not shown).

Host cell mRNAs are associated with authentic polysomes in influenza virus-infected cells. We performed several control

experiments to prove that the host cell mRNAs that sedimented in the polysome region in extracts from influenza virus-infected cells were actually associated with polysomes and not some other structures. First, analysis of extracts on metrizamide density gradients (Fig. 3A) showed that the actin and GAPD mRNAs in infected-cell extracts, as well as in mock-infected-cell extracts, banded at the 1.30 to 1.35 g/cm³ density characteristic of monosomes and polysomes (4, 9). No cellular mRNAs in the infected cells were detected at a density of about 1.21 g/cm³, at which messenger ribonucleoproteins band (4, 9). The viral NP mRNA was also located almost exclusively in the 1.30 to 1.35 g/cm³ polysome and monosome region (Fig. 3B).

The second approach was to dissociate the polysomes of infected cells into ribosomal subunits by EDTA treatment, which should release all polysome-associated mRNAs (Fig. 4). In this experiment, in contrast to the other experiments in this study, there were five, rather than seven, pooled samples: samples A, B, and C, containing the polysomes; sample D, the ribosomal subunits; and sample E, the top of the gradient. EDTA treatment effectively dissociated infected-cell polysomes (Fig. 4A) and released the actin, GAPD, and pHe7 mRNAs to the top of the gradient (Fig. 4B). Influenza viral mRNAs were similarly displaced from polysome fractions A through C (data not shown).

The most definitive evidence was obtained by using puromycin, which disrupts polysomes by virtue of their activity in protein synthesis. Puromycin, an analog of aminoacyl-tRNA, acts as an acceptor for the growing peptide chains, which are then released from the polysomes. This causes the polysomes to break down and release their mRNAs (7, 38). Only a single peptide bond has to be formed for puromycin to cause the release of polysomal mRNA. Consequently, unless the block in elongation of the chains encoded by cellular mRNAs was absolute, puromycin would be expected to release cellular, as well as viral, mRNAs from the polysomes of infected cells. We treated infected cells at 4 h postinfection with 200 μ M puromycin for 45 min, which resulted in greater than 99% inhibition of protein synthesis. Gradient analysis of cytoplasmic extracts showed that this drug treatment caused the breakdown of polysomes (Fig. 5A). As shown by Northern blot analysis, cellular mRNAs, i.e., the actin, GAPD, and pHe7 mRNAs (Fig. 5B), as well as the viral NP mRNA (Fig. 5C), were displaced from the polysomes and sedimented toward the top of the gradient. Consequently, the cellular mRNAs were associated with polysomes that were capable of forming at least a single peptide bond.

Association of host cell mRNAs with polysomes after adenovirus infection. Adenovirus infection also resulted in a dramatic shutoff of host cell protein synthesis (Fig. 6A). At 20 h postinfection, essentially only viral proteins were synthesized (e.g., polypeptides II [hexon], III [penton base], and IV [fiber]). As with influenza virus infection, this shutoff occurs even though functional cellular mRNAs are present in the cytoplasm of adenovirus-infected cells (2). To identify the step at which cellular mRNA translation was blocked, we analyzed the polysomes of mock- and adenovirus-infected cells for the presence of cellular mRNAs. As shown by the absorbance profile of the sucrose gradient analysis (Fig. 6B), there was no significant difference between the polysome profiles of the mock- and adenovirus-infected cells. The poly(A)⁺ RNA from the seven gradient samples (A through G) were analyzed for host mRNAs by Northern blot analysis (Fig. 6C). Twenty hours after adenovirus infection, most of the β -actin, GAPD, and pHe7 mRNAs

FIG. 3. Metrizamide gradient analysis of extracts from mock- and influenza virus-infected cells. (A) Cytoplasmic extracts from mock-infected and influenza virus-infected (FLU) cells were subjected to centrifugation in metrizamide gradients, as described in Materials and Methods. The gradients were divided into 10 equal fractions (shown at the top). The refractive index of each fraction was read, yielding the density at 4°C (shown at the bottom). The RNA from each fraction was analyzed by a Northern blot probed with ³²P-labeled actin and GAPD DNA clones. (B) RNAs from the metrizamide gradient fractions from influenza virus-infected cells analyzed by a Northern blot probed with ³²P-labeled DNA specific for influenza viral NP mRNA.

remained associated with the same polysome samples with which they were associated in mock-infected cells, although an increased fraction of these cellular mRNAs in infected cells was found in the polysome-monomosome sample E, in the ribosomal subunit sample F, and in the material at the top of

FIG. 4. Release by EDTA of cellular and viral mRNAs from polysomes of influenza virus-infected cells. (A) Sucrose density gradient analysis of untreated and EDTA-treated extracts from influenza virus-infected cells. (B) Northern blot analysis of poly(A)⁺ RNAs from samples A through E of the -EDTA and +EDTA gradients shown in panel A, using as probes ³²P-labeled actin, GAPD, and pHe7 DNAs. The positions of 18S and 28S rRNAs are shown on the right.

the gradient (sample G). Dot blot analysis showed that 75% of the β -actin sequences were associated with polysome samples A through E, as compared with 92% in the mock-infected cells (data not shown). Thus, the polysome pattern of these cellular mRNAs was that predicted for a combined elongation-initiation block in translation. Puromycin treatment of adenovirus-infected cells verified that these cellular mRNAs were associated with polysomes that were capable of forming at least a single peptide bond. The drug caused disruption of the polysomes of infected cells (Fig. 6B) and the release of cellular mRNAs from the polysomes to the top of the gradient (Fig. 6D).

The location of adenoviral mRNAs was monitored by using cloned DNA probes specific for (i) the major late L3 mRNA encoding the hexon protein (protein II) with a molecular weight of 120,000 and (ii) three L2 mRNAs encoding the penton base protein (protein III; molecular weight, 85,000), polypeptide V (molecular weight, 48,500), and polypeptide VII (molecular weight, 20,500). In our gel system, the major L3 mRNA and the largest L2 mRNA were not separated from each other. A portion of each of these viral mRNAs was located in polysome samples B through D, in which the cellular mRNAs was found, but a larger fraction was present in the polysome-monomosome fraction E and in the ribosome subunit fraction F (Fig. 7A). For example, most of the intermediate-size L2 mRNA was located in samples E and F. As shown by the analysis of the RNA in the gradient samples by translation in reticulocyte extracts (Fig. 7B), many of the adenoviral mRNAs, including the larger mRNAs which code for the structural proteins II, III, and IV, were skewed to the smaller polysomes (samples C through E) and the ribosomal subunit area of the gradient (sample F). Proteins encoded by cellular mRNAs were also evident in the translation products programmed by the poly(A)⁺ RNAs in the polysome samples, although the

adenoviral mRNAs were most likely present in higher copy numbers than were the cellular mRNAs. In particular, there was essentially as much functional actin mRNA in the polysome samples from the infected cells (Fig. 7B) as in the same samples from the mock-infected cells (Fig. 7C).

Comparison of polysome association of influenza viral and adenoviral mRNAs in doubly infected cells. Comparison of the polysomes of cells infected by influenza virus to the polysomes of cells infected by adenovirus indicated that influenza viral mRNAs were on larger polysomes than were several late adenoviral mRNAs with comparably sized coding regions (compare Fig. 2 and 7). This suggests two possibilities: (i) all protein synthesis initiation is reduced in late adenovirus-infected cells (42) or (ii) adenoviral mRNAs in general are poorer initiators of protein synthesis than are influenza viral mRNAs. To distinguish between these possibilities, we directly compared influenza viral and adenoviral polysomes within the same cell, since in cells infected by the two viruses both influenza viral and adenoviral proteins are efficiently synthesized (22). HeLa cells were infected with adenovirus for 16 h and then superinfected with influenza virus for an additional 4 h. Cytoplasmic extracts were prepared and were analyzed on sucrose density gradients (Fig. 8A). The poly(A)⁺ RNA from the seven gradient samples (A through G) was analyzed by Northern blotting for the presence of influenza viral NP mRNA (Fig. 8B) and the adenoviral L2 and L3 mRNAs (Fig. 8C). The NP and the larger L3/L2 adenoviral mRNAs were located in the same polysome samples (B through D), even though the coding region of the NP mRNA is much smaller than those of the L3 mRNA and the largest L2 mRNA. The intermediate-size L2 mRNA, which has a coding region similar to that of the NP mRNA, was found in the greatest amount in polysome sample D and was thus on smaller polysomes than the NP mRNA was. These results provide evidence that the influ-

FIG. 5. Release of cellular and viral mRNAs from polysomes of influenza virus-infected cells caused by puromycin. (A) Sucrose density gradient analysis of extracts from influenza virus-infected cells with and without puromycin treatment. Infected cells were treated with 200 μ M puromycin, as described in the text. (B) Northern blot analysis of poly(A)⁺ RNAs from samples A through G of the puromycin-treated and untreated gradients shown in panel A. The blots were hybridized with ³²P-labeled actin, GAPD, and pHe7 DNAs. (C) Northern blot analysis of the same gradient samples, using as a probe ³²P-labeled DNA specific for influenza viral NP mRNA. The positions of 18S and 28S rRNA markers are shown on the right.

enza viral NP mRNA and likely the other influenza viral mRNAs are more effective initiators of protein synthesis than are several adenoviral mRNAs.

Further evidence for this conclusion came from analysis of the polysomes in cells infected by both the adenovirus mutant *dI331* and influenza virus. This adenovirus mutant does not synthesize VAI RNA, resulting in the phosphorylation of the initiation factor eIF-2 by a protein kinase and the consequent shutdown of all protein synthesis (46, 48, 50). Superinfection with influenza virus reduces, but does not eliminate, the activity of this protein kinase, resulting in a limitation in functional eIF-2 (23). Under these conditions, influenza viral mRNAs are selectively translated over adenoviral mRNAs (23). We would therefore expect that in these doubly infected cells influenza viral, but not adenoviral, mRNAs would be associated with polysomes.

The experiments with the adenovirus *dI331* mutant were done in 293 cells, in which the mutant phenotype is most manifest. Few polysomes were formed in 293 cells infected with the *dI331* virus as compared with cells infected with wild-type Ad2 (Fig. 9A). Northern blot analysis of the poly(A)⁺ RNA from the gradient fractions showed that the adenoviral L2 and L3 mRNAs were not associated with polysomes in cells infected by the *dI331* mutant (Fig. 9B). Instead, these adenoviral mRNAs were predominantly in gradient samples F and G. After superinfection of *dI331*-infected cells with influenza virus, some polysomes, principally small ones, reformed (Fig. 9A). Northern blot analysis indicated that high levels of the influenza viral NP mRNA

were located in polysome samples B through E (Fig. 9C). In contrast, the adenoviral L2 and L3 mRNAs remained largely associated with slower-sedimenting material, although there was a small shift of these mRNAs away from the top of the gradient (compare Fig. 9B and C). The polysome association of the influenza viral NP mRNA was not quite as complete as it was in HeLa cells infected with influenza virus alone (compare Fig. 9C and 2A), presumably reflecting the limitation in functional eIF-2 in the doubly infected cells. The results of *in vitro* translation studies of total poly(A)⁺ RNA isolated from these polysome fractions confirmed that other influenza virus mRNAs, especially the NS1 mRNA, were also polysome associated (data not shown).

DISCUSSION

After infection with either influenza virus or adenovirus, cellular protein synthesis is severely inhibited even though high levels of functional cellular mRNAs exist in the cytoplasm of infected cells (2, 24). We investigated this block in host mRNA translation by comparing the polysomal distribution of three cellular mRNAs in uninfected and virus-infected cells. After infection by either virus, most of these cellular mRNAs remained polysome associated, indicating that the elongation of the proteins encoded by these cellular mRNAs must have been inhibited. The polysome association of cellular mRNAs was also seen in cells infected by both viruses (data not shown). Since the polysomes containing these cellular mRNAs did not increase in size but either

FIG. 6. Shutoff of cellular protein synthesis and association of host cell mRNAs with polysomes after adenovirus infection. (A) HeLa cells were either mock infected (M) or infected with adenovirus (AD) at 50 PFU per cell. At 20 h postinfection the cells were labeled with [³⁵S]methionine (50 μ Ci/ml) for 30 min, and the labeled proteins were analyzed on a 14% gel. The positions of representative adenovirus proteins (II [hexon], III [penton base], and IV [fiber]) are shown on the right, and the position of the actin protein is shown on the left. (B) Sucrose density gradient analysis of extracts from mock-infected cells, from AD2-infected cells (20 h postinfection), and from AD2-infected cells treated with 200 μ M puromycin (PURO) for 45 min before harvest. The gradients were divided into seven samples (A through G), and poly(A)⁺ RNA was prepared. (C) Northern blot analysis of cell-equivalent amounts of poly(A)⁺ RNA in gradient samples A through G prepared from mock- and adenovirus-infected cells, using as probes ³²P-labeled actin, GAPD, and pHe7 DNAs. The positions of 18S and 28S rRNA markers are shown on the right. (D) Northern blot analysis of poly(A)⁺ RNAs in gradient samples A through G prepared from puromycin (PURO)-treated, adenovirus-infected cells, using the actin, GAPD, and pHe7 probes.

remained the same size or decreased in size, the initiation of cellular mRNA translation must also have been defective. A similar initiation-elongation block has also been documented in the stress response. After *Drosophila* cells are heat shocked or HeLa cells are treated with amino acid analogs, the translation of the nonstress mRNAs is blocked at both initiation and elongation (4, 54).

Several lines of evidence established that the cellular mRNAs that sedimented with the polysomes in infected cells during sucrose density gradient centrifugation were actually associated with polysomes and not with some other structure(s). With influenza virus-infected cells, we showed that the cellular mRNAs were associated with structures that had the density in metrizamide gradients characteristic of monosomes and polysomes and that EDTA, which disrupts

polysomes, released the cellular mRNAs to the top of the sucrose gradient. The most definitive evidence was obtained by using puromycin. With cells infected either by influenza virus or adenovirus, puromycin treatment for 45 min caused both the dissociation of polysomes and the release of the cellular mRNAs from fast-sedimenting material. Because such an effect by puromycin requires the formation of at least a single peptide bond, the cellular mRNAs must have been associated with polysomes which have some capacity to elongate chains. It is clear, however, that this capacity for chain elongation must have been greatly impaired, because the synthesis of completed cellular proteins was severely inhibited.

Previous studies have not examined the step at which cellular mRNA translation is blocked in influenza virus-

FIG. 7. Functional viral and cellular mRNAs on polysomes of adenovirus-infected and mock-infected cells. (A) Northern blot analysis of poly(A)⁺ RNA prepared from the gradient samples of adenovirus-infected cells shown in Fig. 6B, using ³²P-labeled probes specific for the L2 and L3 adenoviral mRNAs. In our gel system, the major L3 mRNA and the largest L2 mRNA were not separated from each other. The positions of 18S and 28S rRNA markers are shown on the right. (B) In vitro translation of poly(A)⁺ RNA from gradient samples of adenovirus-infected cells. Cell-equivalent amounts of poly(A)⁺ RNA were used to program the rabbit reticulocyte extracts in the presence of [³⁵S]methionine. The translation products were electrophoresed on a 14% gel. The positions of representative adenovirus-specific proteins and actin are shown on the right. (C) In vitro translation of poly(A)⁺ RNA from the gradient samples of mock-infected cells shown in Fig. 6B.

infected cells. However, two previous studies performed such an analysis of adenovirus-infected cells. Babich et al. (2) found that the two small HeLa cell mRNAs, pHe7 and pHe28, were shifted to association with fewer ribosomes after adenovirus infection and interpreted this result as indicating that the block in translation of cellular mRNAs was largely at the level of initiation. There are several arguments against this interpretation. First, if a block in initiation alone accounted for the greater than 90% inhibition of cellular mRNA translation that was observed, then the cellular mRNAs would have been completely displaced from polysomes rather than simply shifted to smaller polysomes. This can clearly be seen by examining the situation in *d/331*-infected cells, in which the block in adenoviral mRNA translation was solely at the level of initiation: the adenoviral mRNAs were completely displaced from the polysomes (Fig. 9C; 49). Second, the results with very small mRNAs like pHe7 and pHe28 may be somewhat misleading. Under circumstances in which both initiation and elongation are inhibited, but the inhibition of elongation is not absolute, it is predicted that the initiation defect would be more apparent for smaller mRNAs and the elongation defect would be more apparent for larger mRNAs. This is exactly what was observed. After either adenovirus or influenza virus infection, the amount of pHe7 mRNA that became associated with slower-sedimenting material was greater than that of the larger actin and GAPD mRNAs. Also, Babich et al. (2) noted that a greater shift occurred for the pHe28 mRNA than for the larger pHe7 mRNA. Indeed, a second group of investigators (25) showed that large polysomes in adenovirus-infected cells contained significant levels of actin mRNAs that were functional in reticulocyte extracts. How-

ever, these investigators found that the actin mRNAs that were associated with small polysomes and monosomes were not translatable in reticulocyte extracts. In contrast, we showed that all the actin mRNAs in adenovirus-infected cells, whether on large or small polysomes, were functional in reticulocyte extracts (Fig. 7B).

Our demonstration that influenza virus and adenovirus each block cellular mRNA translation at both initiation and elongation provides another example of the similar strategies by which these two viruses interrupt cellular gene expression. Both viruses also block the appearance of new cellular mRNAs in the cytoplasm, although by different mechanisms (2, 13, 24). It can be argued that the transport and translational blocks are interrelated. Perhaps each of these viruses has a mechanism for inhibiting the elongation of chains on polysomes encoded by preexisting mRNAs, whereas newly synthesized mRNAs, which are viral mRNAs only, can form polysomes that are not inhibited in elongation. This hypothesis implies that newly synthesized mRNAs have access to a different pool of ribosomes than do old mRNAs. A similar hypothesis has been proposed for the stress response (54). In line with this hypothesis, certain adenovirus E1B and E4 mutants allow both the transport of cellular mRNAs and their subsequent translation (3, 18). In addition to using similar strategies for interrupting cellular gene expression, adenovirus and influenza virus each has a gene product that prevents the shutdown of overall protein synthesis caused by an eIF-2 kinase (23, 46, 48).

The molecular mechanisms by which influenza virus and adenovirus impose a selective block against the elongation and initiation of cellular, but not viral, proteins are not known. The only clearly documented specific control of

FIG. 8. Influenza viral mRNAs are on larger polysomes than are adenoviral mRNAs in cells infected by both viruses. (A) Sucrose density gradient analysis of extracts from mock-infected cells and from cells infected with both wild-type adenovirus and influenza virus (AD2-FLU). HeLa cells infected with adenovirus for 16 h were superinfected with influenza virus for an additional 4 h. (B) Northern blot analysis of poly(A)⁺ RNAs from the gradient samples of the doubly infected cells shown in panel A, using as a probe ³²P-labeled DNA specific for influenza viral NP mRNA. (C) Northern blot analysis of the same gradient samples probed with labeled DNAs specific for the adenoviral L2 and L3 mRNAs. The positions of 18S and 28S ribosomal RNA markers are shown on the right in panels B and C.

translation elongation in eucaryotes is that exhibited by the signal recognition particle, which blocks the elongation of secretory proteins containing a signal sequence until the signal recognition particle interacts with a receptor on microsomal membranes (57). One speculative hypothesis is that the virus encodes a gene product that stops all translation elongation, but that a second viral gene product that interacts specifically with viral mRNAs enables the viral mRNAs to escape this elongation block.

The selectivity at the initiation step may involve, at least in part, competition between the viral and cellular mRNAs for limiting components of the translational machinery,

presumably mainly initiation factors (8, 20, 35, 56). Several types of competition could play a role. First, as discussed above, the viral mRNAs may have a competitive advantage because they are essentially the only newly synthesized mRNAs in the cytoplasm of infected cells. In addition, the viral mRNAs may have a competitive advantage because they are intrinsically better initiators of translation, or because they are present in much larger amounts than are the cellular mRNAs, or both. It is possible that influenza viral mRNAs are intrinsically superior mRNAs. However, it is not likely that late adenoviral mRNAs are more efficient initiators of translation than cellular mRNAs are. We

FIG. 9. Preferential association of influenza viral mRNAs with polysomes in cells infected with both influenza virus and the adenovirus *dl331* mutant. (A) Sucrose density gradient analysis of extracts from 293 cells infected with wild-type Ad2, with the adenovirus mutant *dl331*, or with both *dl331* and influenza virus (331-FLU). (B) Northern blot analysis of poly(A)⁺ RNAs from the gradient samples of *dl331*-infected cells probed with the DNAs specific for the adenoviral L2 and L3 mRNAs. (C) Northern blot analysis of poly(A)⁺ RNAs from the gradient samples of cells infected by both *dl331* and influenza virus. The blots were first hybridized to the DNAs specific for the adenoviral L2 and L3 mRNAs and then to the DNA specific for the influenza viral NP mRNA. The intense bands seen in the NP/L2 region in polysome samples B through E occurred after the hybridization to the NP mRNA probe.

showed that several late adenoviral mRNAs are on smaller polysomes than are cellular and influenza viral mRNAs with similar or smaller coding regions, indicating that these particular late adenoviral mRNAs are not as effective initiators of translation. It was shown previously that mRNAs encoding adenovirus structural proteins are on smaller polysomes than would be expected (60). Other late adenoviral mRNAs may be better initiators, since it has been shown that different late adenoviral mRNAs vary considerably in their efficiency of translational initiation, with the mRNA encoding polypeptide IX apparently being the most efficient (61). Nevertheless, because at least some late adenoviral mRNAs are inefficient initiators, late adenoviral mRNAs may require a large mass excess for their selective translation. It may be argued that the poor shutoff of host cell protein synthesis by the adenoviral E1 and E4 mutants is attributable to the decreased ratio of viral to cellular mRNAs in the cytoplasm that occurs after infection by these viruses (3, 18, 58). In contrast, after infection at the nonpermissive temperature by certain influenza virus temperature-sensitive mutants which synthesize only 10 to 20% of the wild-type levels of viral mRNAs, host protein synthesis is still inhibited (G. Shapiro and R. Krug, unpublished results). In addition to these various types of mRNA competition, influenza virus or adenovirus might induce changes in initiation factors that might also play a role in the selective initiation by viral mRNAs.

Our results suggest that influenza viral mRNAs are intrinsically better initiators of translation than are late adenoviral mRNAs. In cells infected by both influenza virus and wild-type adenovirus, influenza viral mRNAs were on larger polysomes than were late adenoviral mRNAs with comparably sized coding regions. It is unlikely that this was the result of relative mRNA levels since it has been shown that influenza viral and adenoviral mRNAs accumulate to simi-

larly high levels in doubly infected cells (22; M. G. Katze, unpublished results). In addition, after influenza virus superinfection of cells infected by the adenovirus mutant *dl331*, a situation in which there is a limitation in the amount of functional eIF-2 (23), influenza viral mRNAs, but not late adenoviral mRNAs, were on polysomes (Fig. 9).

Consequently, influenza viral mRNAs may have structural features that enhance their ability to initiate translation. The first 10 to 13 nucleotides at the 5' ends of influenza viral mRNAs are derived from host cell polymerase II transcripts (28, 45). In vitro experiments with the virion-associated transcriptase complex indicate that the virus preferentially utilizes the 5' ends of capped RNAs with reduced secondary structure (29). Because reduced secondary structure, particularly at the 5' end, is associated with increased translational efficiency (27, 41), it is conceivable that in the infected cell influenza virus steals the 5' ends of the most efficiently translated cellular mRNAs (27a). The influenza viral mRNAs also contain a common 12-nucleotide sequence (52) downstream from the stolen 5' ends that might be involved in translational initiation. Most of the late adenoviral mRNAs also contain a common 5' end, the 200-nucleotide tripartite leader, which has been shown to enhance the translation at late times of an adenoviral mRNA that was inserted into the E1B region under the control of the major late promoter (36). However, even with this tripartite leader, several late adenoviral mRNAs are not as efficient at translational initiation in late adenovirus-infected cells as influenza viral mRNAs. This suggests the possibility that the tripartite leader would not enhance the translation of those mRNAs that are already effective initiators of translation, such as the influenza viral mRNAs. To address these issues, we are constructing recombinant adenoviruses that contain influenza virus genes with and without an adenovirus tripartite leader sequence.

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