In cells infected by influenza virus type A, host protein synthesis undergoes a rapid and dramatic shutoff. To define the molecular mechanisms underlying this selective translation, a transfection/infection protocol was developed utilizing viral and cellular DNA clones. When COS-1 cells were transfected with cDNAs encoding nonviral genes and subsequently infected with influenza virus, protein expression from the exogenous genes was diminished, similar to the endogenous cellular genes. However, when cells were transfected with a truncated influenza viral nucleocapsid protein (NP-S) gene, the NP-S protein was made as efficiently in influenza virus infected cells as in uninfected cells, showing that the NP-S mRNA, although expressed independently of the influenza virus replication machinery, was still recognized as a viral and not a cellular mRNA. Northern blot analysis demonstrated that the selective blocks to nonviral protein synthesis were at the level of translation. Moreover, polysome experiments revealed that the translational blocks occurred at both the initiation and elongation stages of cellular protein synthesis. Finally, we utilized this transfection/infection system as well as double infection experiments to demonstrate that the translation of influenza viral mRNAs probably occurred in a cap-dependent manner as poliovirus infection inhibited influenza viral mRNA translation.

After infection by many cytopathic eukaryotic viruses, host cell mRNA translation is selectively inhibited during which time viral mRNAs are efficiently translated (for reviews, see Kozak, 1986a; Schneider and Shenk, 1987; Sonenberg, 1990). Despite intensive study, the mechanisms underlying this protein synthesis regulation are only now starting to be defined. The best characterized, regarding the host cell shutoff, are the poliovirus and adenovirus systems. The inhibition of cellular protein synthesis in poliovirus infected cells is correlated with the degradation of the 220,000-dalton protein, P220, a component of the cap binding protein complex, eukaryotic initiation factor 4F (eIF-4F; Etchison et al., 1982).

Interestingly it was recently shown that dephosphorylation of another component of the cap binding protein complex, eIF-4E, may play a role in the host shutoff in adenovirus-infected cells (Huang and Schneider, 1991). In support of this it was demonstrated that adenovirus mRNAs, containing tripartite leader sequences, are translated in a cap-independent manner (Dolph et al., 1988). Thus both these viruses induce the modifying and resultant inactivation of components of the cap binding protein complex which then results in the selective cap-independent translation of viral mRNAs over cellular mRNAs. As possible additional strategies to restrict host protein synthesis, poliovirus appears to limit the amounts of functional eIF-2 during infection (Black et al., 1989; O'Neill and Racaniello, 1989), while adenovirus imposes blocks on the transport of cellular mRNAs (Babich et al., 1983) and the initiation and elongation steps of cellular protein synthesis (Katze et al., 1986a).

Like adenovirus and poliovirus, influenza virus establishes a translational control system such that during infection there is a dramatic inhibition of host cell protein synthesis, while viral proteins are synthesized selectively and efficiently (Lazarowitz et al., 1971; Skehel, 1972; Katze and Krug, 1984). We have previously shown that newly synthesized cellular mRNAs never reach the cytoplasm of infected cells (Katze and Krug, 1984) and that pre-existing cytoplasmic cellular mRNAs are blocked at the initiation and elongation stages of mRNA translation (Katze et al., 1986a). In addition we have demonstrated that influenza mRNAs were selectively translated in adenovirus-infected cells (Katze et al., 1984, 1986a, 1986b). In other experiments we have shown that an influenza viral NP protein expressed by a recombinant adenovirus escaped the adenovirus imposed blocks on translation and was synthesized as efficiently as in cells doubly infected with adenovirus and influenza virus (Alonso-Caplen et al., 1988). From these data we concluded that the structure alone of influenza viral mRNAs may confer a translational advantage. Other translational controls encoded for by influenza virus include the down-regulation of the P68 protein kinase to ensure that overall protein synthetic rates remain high during infection (Katze et al., 1988; Lee et al., 1990).

We undertook the present study to begin to define the molecular mechanisms responsible for efficient and selective viral mRNA translation using a transfection/infection assay with both viral and cellular cDNAs. We show that an exogenously introduced influenza viral nucleocapsid (NP-S) protein gene is not subjected to the translational blocks imposed on exogenously introduced cellular genes. This demonstrates that, despite the fact the NP-S gene is expressed independently of the influenza virus replication machinery, it is recognized as viral and not cellular regarding mRNA translation. Furthermore, we unexpectedly found that, in contrast to both adenovirus and poliovirus, influenza virus mRNA translation likely occurred in a cap-dependent manner even though influ-
enzymes were found to be translated in adenovirus-infected cells (Katze et al., 1984). These data taken together confirm the importance of RNA structure in the regulation of protein synthesis in influenza virus-infected cells, but show that the host shutoff mechanisms are distinct from these other virus groups. 

MATERIALS AND METHODS

Cells and Virus Infections—COS-1 cells were grown in monolayers in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. The WSN strain of influenza A virus was grown in Madin-Darby bovine kidney cells and titrated by plaque assay in Madin-Darby bovine kidney cells (Katze and Krug, 1975). Poliovirus type 1 (Mahoney strain; a gift of Nahum Sonenberg and Jerry Pelletier, McGill University, Montreal) was prepared from infected cells as previously described (Lee and Sonenberg, 1982), and the titer was determined by plaque assay on HeLa cells. Monolayers of COS-1 cells were infected with influenza virus or poliovirus type 1 at a multiplicity of infection of approximately 50 plaque-forming units per cell.

Construction of Vectors.—The transfection vectors containing cellular genes, pBc12/CMV/IL-2 and pBc12/CMV/SEAP, were obtained from Bryan Cullen and have been previously described (Cullen, 1986; Berger et al., 1988). To obtain an influenza viral gene that could be distinguished from the viral genes expressed during infection, the cDNA for the influenza viral nucleocapsid protein (NP, PR strain), which contained the complete untranslated region of the NP gene except for the host cell sequences, was used by the removal of an EcoRI fragment following by religation. This resulted in an in-frame deletion of 255 nucleotides (85 amino acids) from the coding region of NP, yielding a cDNA referred to as NP-S. NP-S was then subcloned to the transfection vector pBc12/CMV. pBc12/CMV/IL-2 was digested with HindIII and SmI to remove the complete IL-2 cassette. pBc12/CMV and NP-S were then recombined by blunt-end ligation, resulting in the transfection vector pBc12/CMV/NP-S. Each vector thus contains the same nucleocapsid gene translated region (75 nucleotides from the CMV promoter) in addition to the gene-specific sequences. These vectors are represented graphically (see Fig. 1B).

Transfection Protocol.—COS-1 cells were transfected using the DEAE-dextran/chloroquine method (Cullen, 1987). Monolayers of COS-1 cells were washed once with prewarmed, serum-free DMEM. DNA was added to the cells in serum-free DMEM in the presence of 250 μg/ml DEAE-dextran. The cells were incubated for 2 h, after which chloroquine was added to a final concentration of 80 μM. After another 2-h incubation, the transfection mixture was removed and a solution of 20% glycerol in HEPES-buffered saline was added at room temperature for 2 min. The cells were then washed twice with two times of Hank’s balanced salt solution (HBSS), then incubated in DMEM containing 10% fetal bovine serum at 37°C.

Analysis of Cellular and Viral Protein Synthesis—Cells were labeled on the times indicated with [35S]methionine (1200 μCi/ml) in methionine-free DMEM. After labeling, cells were washed with ice-cold HBSS and lysed in disruption buffer (10 mM Tris hydrochloride, pH 7.5, 50 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfuoride, 10 units/ml aprotinin, 1% Triton X-100). For immunoprecipitation analysis, the clarified extract was diluted with an equal volume of 2X electrophoresis buffer and analysed by SDS-PAGE. IL-2 protein was immunoprecipitated with a purified monoclonal antibody (Genzyme). Protein (and RNA) levels were quantitated by laser densitometry; values given reflect the relative area under the respective peaks.

In order to unequivocally identify the NP-S protein after influenza infection, it was first necessary to preclude the lysates of the majority of the viral NP protein. This was accomplished by first practicing protein G-agarose with monoclonal antibody 4F5 (a generous gift of J. Yewdell, National Institutes of Health). The epitopes recognized on NP by this monoclonal antibody have been determined, and we confirmed that indeed the monoclonal antibody did not recognize NP-S in our immunoprecipitation assays. Cell lysates, including the mock infected and mock transfected controls, were reacted twice with protein G-agarose 4F5 for 30 min each time. The cleared lysates were then reacted with influenza virus-infected cells, which had been prereacted with pooled monoclonal antibody against NP (kindly provided by R. Webster, St. Jude Children’s Research Hospital) which recognizes both the NP and NP-S protein products. The precipitated products were analyzed on an 8% polyacrylamide, 0.35% bis, 4% urea gel. NP-S was further separated from NP-S using a monoclonal antibody which chloroquine was added to a final concentration of 80 μM. After another 2-h incubation, the transfection mixture was removed and a fresh hybridization buffer as previously described (Katze and Krug, 1984). The filters were then hybridized with 32P-laabeled probes for 8 h at 37°C.

Preparation and Analysis of Viral and Cellular mRNAs—Total cytoplasmic RNA was isolated as described previously (Katze et al., 1984). Following transfection and viral infection by influenza virus or poliovirus, transfected cell monolayers were washed with HBBS and disrupted in a solution of 140 mM NaCl, 1.5 mM MgCl2, 10 mM Tris hydrochloride, pH 7.5, 0.5% Nonidet P-40, and 5 mM vanadylribonucleoside complexes. After incubation on ice for 10 min, nuclei and cell debris were removed by centrifugation at 500 × g for 5 min. The RNA was precipitated with chloroform and the supernatant was used for hybridization. Another 2-h incubation, the transfection mixture was removed and a fresh hybridization buffer as previously described (Katze et al., 1984). The filters were then hybridized with 32P-laabeled probes for 8 h at 37°C.

Riboprobes were hybridized at 57°C for 1h. For IL-2 and SEAP, mRNA was detected with random-primed 32P-CTP-labeled DNA probes (Feinberg and Vogelstein, 1984). To detect NP-S mRNA it was necessary to use the heterologous probe pBc12/CMV/IL-2 containing the upstream 75 nucleotides from the CMV immediate early transcript to be able to distinguish NP-S from NP. To detect full-length NP mRNA, a 32P-UTP-labeled riboprobe (Promega) anti-sense to the mRNA was used to distinguish NP mRNA from the virion RNA. Riboprobes were hybridized at 57°C in riboprobe hybridization buffer (50% formamide, 40 mM NaPO4, pH 6.5, 5 X SSC, 0.1% SDS, 1 mM sodium dodecyl sulfate [SDS] Denhardt’s). Hybridization and washes were performed at 37°C in this same buffer. To test the functionality of the SEAP mRNAs, poly(A) RNA was translated in a rabbit reticulocyte extract (Katze et al., 1991) followed by immunoprecipitation with a polyclonal antialkaline phosphatase-specific antibody (Dako).

Polysony Analysis—Polysones were prepared from cells mock or influenza virus infected using a modification of the methods of Lenk and Pennman (1979) as described in Katze et al. (1986a). Briefly, after transfection and infection as described above, the cells were washed in HBBS containing 100 μg/ml cycloheximide. Cycloheximide at 100 μg/ml was included in all buffers to prevent peptide chain elongation and thus polyribosome runoff (Godchax et al., 1967). The cells were then scraped off the monolayer and collected in HBBS. The cells were collected by centrifugation and were resuspended in polysome buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl2, 5 mM vanadylribonucleoside complexes). Triton X-100, 1 mg/ml, was added to a first incubation at 0°C, and the cells then incubated for 3 min on ice followed by addition of 1% Tween 40 and 0.5% sodium deoxycholate. The cells were then disrupted by homogenization in a stainless steel-type Dounce homogenizer. Following centrifugation the resulting supernatant was layered on a 10-50% (w/v) sucrose gradient (in polysome buffer with protein G-agarose; 200 μg/ml sucrose, 0.5 M KCl). Polyribosomes were resolved from monosomes and other smaller structures by centrifugation in a Beckman Ti-41 rotor at 40,000 rpm for 2 h at 4°C. Gradient fractions were collected and diluted with an equal volume of 2X electrophoresis buffer. The fractions were pooled then treated with 500 μg/ml Pronase for 30 min. The RNA was extracted from the pooled fractions with...
phenol/chloroform, then precipitated. For each fraction, cell equivalents of RNA were collected on nitrocellulose filters by vacuum slot blot. The filters were then baked, prehybridized, and hybridized to radiolabeled probes as for Northern blot analysis.

RESULTS

Exogenously Introduced Cellular Gene Expression Is Blocked at the Initiation and Elongation Steps of Protein Synthesis in Influenza Virus-infected Cells—Previous work has demonstrated that influenza virus invoked a translational control mechanism as part of its overall strategy to maximize influenza viral gene expression during infection. To confirm that the structure of viral mRNAs is important for selective influenza viral gene expression, we designed an in vivo assay in which we could follow the translational fate of exogenous cellular and influenza genes during influenza virus infection (Fig. 1A). By transfection analysis, we introduced cellular genes the products of which could be distinguished from endogenous cellular gene products. We analyzed SEAP and IL-2, neither of which are expressed endogenously in COS-1 cells (data not shown). These constructs are represented graphically in Fig. 1B. Since we carried out our transfection/infection assays in COS-1 cells, we first ensured that the host cell shutoff of protein synthesis during influenza virus infection did indeed occur in COS-1 cells (Fig. 2). By 4-h postinfection the host cell shutoff was essentially complete in this cell line which is in general agreement with our earlier results (Katze and Krug, 1984) although the kinetics of the shutoff was slightly slower.

We then analyzed the the pattern of protein expression in cells separately transfected either with cellular SEAP or IL-2 cDNAs and then infected with influenza virus. Levels of newly synthesized SEAP, as measured by enzymatic activity, dropped 5-10-fold over the course of influenza viral infection (Fig. 3A). We found identical reductions in the levels of newly synthesized SEAP protein by pulse-labeling with [35S]methionine and immunoprecipitating with an SEAP-specific antibody (data not shown). A similar shutoff of IL-2 protein synthesis during viral infection was observed by immunoprecipitating the radiolabeled extracts with an IL-2-specific antibody which recognizes both the glycosylated and unglycosylated forms of the protein (Fig. 3B). Quantitation of this decline by laser densitometry scanning revealed that IL-2 synthesis was down over 5-fold during the course of influenza virus infection (data not shown). To confirm that the shutoff of SEAP and IL-2 protein synthesis was at the translational level, we examined the steady state levels of SEAP and IL-2 poly(A)+ mRNAs. Northern blot analysis revealed that little or no decrease in the amounts of SEAP (Fig. 4A) or IL-2 (Fig. 4B) mRNA was evident even 5 h after influenza virus infection. We needed next to determine whether these mRNAs which were not translated in vivo were still functional. Equal amounts of the poly(A)+ mRNA were translated in message-dependent rabbit reticulocyte lysate after which the in vitro-synthesized proteins were immunoprecipitated with SEAP-specific antibody. Essentially the same amount of SEAP protein was synthesized by each poly(A)+ preparation proving that the SEAP mRNAs were indeed functional throughout infection (Fig. 4C). Similar results were observed for the IL-2 mRNAs (data not shown).

To determine the level at which protein synthesis was blocked, we analyzed the polysome distribution of mRNAs encoded by the transfected SEAP gene in uninfected and influenza-virus infected cells. Average polysome size should be proportional to both the length of the coding region and the rate of translational initiation, and inversely proportional to the rate of protein elongation (Lodish, 1976; Lodish and Froshauer, 1977). A block at the initiation step alone would result in displacement of mRNAs from polysomes entirely. A block in elongation would result in polysomes of increased size following infection. A combined initiation-elongation block (which was previously determined to occur on endogenous cellular mRNA translation in influenza virus infected cells; Katze et al., 1986a) would result in a significant proportion of the mRNAs remaining associated with polysomes which probably would not increase in size, and some mRNAs would be on smaller polysomes or displaced from polysomes. Extracts were prepared as described under "Materials and Methods" and applied to sucrose gradients to separate the polysomes from monosomes and ribosomal subunits. To minimize artificial sticking of RNA to polysomes, 500 mM KCl was included in the sucrose gradients; 500 mM KCl also should have caused most 80 S monosomes to dissociate into 60 and 40 S ribosomal subunits (Blobel and Sabatini, 1971). As shown

![Fig. 1. Transfection-infection protocol and viral and cellular cDNA constructs. A, protocol for transfection/infection assay and analysis of RNA and protein. B, transfection vector pBlC12/CMV containing the cDNAs for the representative cellular genes SEAP and IL-2, and the representative influenza viral gene NP-S are shown. Details of the construction of pBlC12/CMV/NP-S are given under "Materials and Methods."](image-url)
the absorbance profile of the sucrose gradient, uninfected and influenza virus-infected cells had similar proportions of polysomes and ribosomal subunits (Fig. 5A). In this particular experiment there was a slightly higher RNA recovery from virus-infected cells but often the reverse was found (e.g. see Fig. 6B). The gradient fractions were pooled into five samples. Samples A though C contained the largest to smallest polysomes respectively, sample D contained ribosomal subunits, and sample E contained the material sedimenting more slowly than the ribosomal subunits.

Total RNA from the five samples was subjected to slot blot analysis and the location of the exogenous SEAP, endogenous actin, and viral NP mRNAs determined by hybridizing the blots with the appropriate 32P-labeled cDNAs and scanning the radioactive signals by laser densitometry (Fig. 5B). In mock infected cells, the majority of SEAP mRNAs were found in polysome fractions B and C (73%) as would be expected for an actively translating mRNA of that size. Following influenza virus infection, much SEAP mRNA remained associated with polysome samples B and C (46%) but an increased fraction of the mRNA was found in ribosomal subunit fraction D (20%) and E (20%), the top of the gradient. Similar result was obtained for the endogenous actin (Fig. 5B, middle; Katze et al., 1986a). The pattern is consistent with that predicted for a combined initiation-elongation block since the mRNAs are still polysome associated but a significant fraction are displaced toward the top of the gradient. An identical analysis was carried out for the exogenous IL-2 RNA, with similar results obtained (data not shown). Finally, the polysome distribution of the viral NP mRNA following influenza virus infection was examined (Fig. 5B, bottom). The polysomes containing the viral NP mRNA were similar in size to those containing the exogenous cellular SEAP mRNA and endogenous actin mRNA in uninfected cells (see Fig. 5B).

An Exogenously Introduced Influenza Gene Is Not Subjected to the Host Cell Shutoff during Influenza Virus Infection—We then followed the fate of an exogenously introduced influenza gene during virus infection. As an exogenous influenza gene we selected the gene encoding the nucleocapsid protein (NP). In order to distinguish the exogenous NP from the infecting viral NP, we utilized a truncated NP cDNA which was short-
FIG. 5. Polysomal distribution of SEAP mRNAs after influenza virus infection. A, sedimentation profile of polyribosomes from pBC12/CMV/SEAP-transfected cells. A cytoplasmic extract from approximately 1 × 10^9 mock infected (M) or influenza virus-infected (FLU) cells was subjected to centrifugation on a 10–50% sucrose gradient, as described under “Materials and Methods.” Fractions were collected, and the A_{260} determined. The gradient fractions were pooled as shown, yielding samples A though E, slot blot analysis of the distribution of exogenous SEAP RNA on polyribosomes (top of B). Total RNA from each gradient sample was denatured and applied to nitrocellulose using a slot-blot apparatus. The autoradiographic signal was quantitated by laser densitometric scanning. The total area of the mock infected samples A-E was 6.40, distributed as follows with the relative percentages included: sample A, 0.7 (11%); sample B, 1.8 (28%); sample C, 2.9 (45%); sample D, 0.5 (8%); sample E, 0.5 (8%). The total area in the influenza infected samples A-E was 11.0. Sample A, 1.5 (14%); sample B, 2.2 (20%); sample C, 2.9 (26%); sample D, 2.2 (20%); sample E, 2.2 (20%). Slot blot analysis of the distribution of endogenous actin RNA on polyribosomes is shown in the middle of B. Total RNA from samples A though E of the gradients represented in A were subjected to slot blot analysis for endogenous actin mRNA as described above. The total area of the mock infected samples A-E was 4.1 distributed as follows: sample A, 0.9 (22%); sample B, 1.4 (34%); sample C, 1.2 (29%); sample D, 0.3 (7%); sample E, 0.3 (7%). The total area in the influenza virus infected samples A-E was 6.6. Sample A, 1.0 (15%); sample B, 1.5 (23%); sample C, 1.7 (26%); sample D, 1.4 (21%); sample E, 1.0 (15%). Analysis of the viral actin RNA is shown on the bottom of B. The total area for the influenza virus-infected samples was 2.8 distributed as follows: sample A, 0.1 (4%); sample B, 1.6 (57%); sample C, 0.9 (32%); sample D, 0.01 (<1%); sample E, 0.1 (4%).

FIG. 6. Protein synthesis from an exogenous influenza gene is not subjected to the host cell shutoff of protein synthesis following influenza virus infection. A, NP-S protein synthesis following infection with influenza virus infection. Following mock (M) or influenza virus (FLU) infection, radiolabeled extracts from pBC12/CMV/NP-S-transfected cells (or cells transfected with vector, V-alone) were subjected to successive rounds of immunoprecipitation as described under “Materials and Methods.” The synthesis of NP-S was further quantitated by laser densitometric (bottom of panel) with the following values obtained. M, 1.5; F3, 1.6; F5, 1.7. B, sedimentation profile of NP-S-transfected and influenza virus-infected cells. Samples were prepared as described in Fig. 5A. C, slot blot analysis of the distribution of exogenous NP-S RNA on polysomes with quantitation. The total area of the mock infected samples A-E was 1.5 distributed as follows. Sample A, 0.3 (20%); sample B, 0.9 (60%); sample C, 0.2 (13%); sample D, 0.1 (7%); sample E, 0.01 (1%). The total area of the virus-infected samples A-E was 1.8 distributed as follows. Sample A, 0.3 (17%); sample B, 1.0 (56%); sample C, 0.3 (17%); sample D, 0.1 (6%); sample E, 0.07 (4%).

cells (Fig. 6C). Moreover, the profile of NP-S RNA closely resembled that of the native influenza virus NP mRNA (see Fig. 5B). We conclude that the transfected NP-S is recognized, not as a foreign cellular gene, but as an influenza gene, and thus is not subjected to the host-cell shutoff. The evidence further suggests that the structure of the viral mRNA confers these properties since the cellular genes, when introduced exogenously in the same vector and in an identical manner into the COS-1 cells, were not expressed after viral infection.

Influenza NP-S Viral mRNA and Other Viral mRNAs Are Translated in a Cap-dependent Manner—As earlier described both adenovirus and poliovirus mRNAs appear to be translated in a cap-independent mechanism (Etchison et al., 1982; Pelletier and Sonenberg, 1988; Dolph et al., 1990; Huang and Schneider, 1991) presumably as a result of inactivation of the cap binding protein complex components and the structure of the viral mRNAs. It was of interest therefore to test whether influenza viral mRNAs could also be translated in this manner particularly because of our earlier results which showed that influenza viral mRNAs were efficiently translated in late adenovirus-infected cells (Katz et al., 1984, 1986a). We transfected IL-2 and NP-S cDNAs individually into COS-1 cells and analyzed the rate of protein synthesis at various times after poliovirus infection (Fig. 7A). In contrast to our results in influenza virus-infected cells, NP-S synthesis was dramatically inhibited after poliovirus infection, to essentially the same extent as the cellular IL-2 synthesis (approximately 8–10-fold). Northern blot analysis confirmed that the block was at the level of NP-S mRNA translation since little decrease in NP-S mRNA was observed over the course of poliovirus infection (Fig. 7B). Finally, to provide additional evidence
that influenza viral mRNA translation was indeed cap-dependent, we performed double infection experiments. As predicted from our transfection analysis, when cells were previously infected with influenza (for 2 h) and then subsequently infected with poliovirus (for an additional 4 h), essentially all influenza viral protein synthesis was blocked (Fig. 8A, compare lanes F and F/P). Only NP protein synthesis was detectable at approximately 10% the levels in cells infected by influenza virus alone. Similar results were obtained if cells were first infected with poliovirus and then infected with influenza virus (data not shown). To ensure that the decreases in protein synthesis were not due to declines in viral mRNA levels, we examined the steady state level of NP mRNA after poliovirus superinfection. Northern blot analysis revealed that viral RNA levels did not appreciably decline at times post-poliovirus infection when viral protein synthesis was dramatically diminished (Fig. 8B). Thus despite the fact that influenza viral mRNAs can escape the translational blocks both in adenovirus and influenza virus infected cells, this is not the case in poliovirus-infected cells.

**DISCUSSION**

Influenza virus utilizes multiple strategies to ensure the selective and efficient translation of its viral mRNAs (for a recent review, see Katze and Krug, 1991). We propose that the structure of influenza viral mRNAs plays a major role in this regulation and allows the viral mRNAs to escape the translational initiation and elongation blocks placed on cellular mRNAs (compare Fig. 5 with 6). Several lines of evidence support this hypothesis. First and most convincing, in the present report we showed that a transfected viral gene, but not cellular gene, can escape the influenza virus-imposed shutoff of protein synthesis. This not only provides a way for us to now define the critical sequences, but also shows that influenza virus-encoded replication strategies including viral mRNA transcription and transport are not required to make a viral mRNA resistant to the translational restrictions. Second, we have previously demonstrated that in cells doubly infected with influenza virus and adenovirus, influenza viral mRNAs were translated more efficiently than adenoviral mRNAs and in some cases selectively over both adenoviral and cellular mRNAs (Katze et al., 1986a). Polysome analysis revealed that several late adenoviral mRNAs were on smaller polysomes than influenza viral mRNAs with similar or smaller coding regions. Furthermore, as mentioned above, we constructed a recombinant adenovirus that could express the influenza viral NP in the absence of other influenza virus gene products. The NP gene was inserted into the adenovirus genome under control of the adenovirus major late promoter with either none or varying portions of the tripartite leader appended to the 5' end of NP mRNA (Alonso-Caplen et al., 1988). We found that the adenovirus tripartite leader had no effect on the translation of NP mRNA, but most importantly, the size of the polysomes containing the NP sequences with no tripartite leader sequences was similar to the size of polysomes found for the NP mRNA synthesized in cells infected with adenovirus and influenza virus.

It may be argued that influenza viral mRNAs have a competitive advantage merely because they are present in much larger amounts than cellular RNAs. This is unlikely for the following reasons: (i) the host shutoff precedes the maximal accumulation of viral mRNAs (Katze and Krug, 1984); (ii) after infection, at the nonpermissive temperature by certain influenza temperature-sensitive mutants which synthesize only 10–20% of the wild-type levels of the viral mRNAs, host protein synthesis is still inhibited; and (iii) perhaps the best

4 G. Shapiro and R. Krug, unpublished observations.
Evidence is presented in the present report utilizing the transcription/translation assay. Even though the exogenously introduced viral NP gene is expressed at approximately the same levels as the exogenous cellular genes, the mRNA encoded by truncated NP is efficiently translated in influenza virus-infected cells (Fig. 6).

We have demonstrated that influenza viral mRNA translation likely occurred in a cap-dependent manner (Figs. 7 and 8), although one cannot absolutely rule out that influenza utilizes alternative cap-independent mechanisms. Our results are not surprising as influenza viral mRNAs contain the cap structure and first 10–13 nucleotides of cellular RNAs as a result of cap snatching (Krug et al., 1989). Thus influenza virus regulates selective translation differently than the picornaviruses (e.g., poliovirus and encephalomyocarditis virus; Pelletier and Sonenberg, 1988; Jang et al., 1989; Kaminski et al., 1990) and adenovirus (Huang and Schneider, 1991), both of which do not require a functional cap binding protein complex for efficient translation. The mechanisms underlying the host shutoff are more clear cut for the picornaviruses since the viral mRNAs are uncapped, thus providing them a selective advantage during infection. Adenovirus mRNAs, however, are capped but may contain elements within its tripartite leader which allow translation in a cap-independent manner (i.e. in poliovirus-infected cells) and in the absence of functional eIF-4E (Dolph et al., 1988, 1990). Since influenza viral mRNAs do not contain the full tripartite leader sequences (although a proportion do contain the first 10–13 nucleotides of the leader; Katze et al., 1984), it still remains to be determined how influenza viral mRNAs, which are translated in a cap-dependent manner, are translated in late adenovirus-infected cells (Katze et al., 1984). Nonetheless, these data do reveal that the mechanisms regulating selective mRNA translation are distinct in these systems.

A plethora of alternative mechanisms have been suggested to account for the host shutoff in other virus systems and are worth noting: (i) Carrasco and colleagues have long suggested that changes in the permeability of virus-infected cells to monovalent cations may regulate selective mRNA translation (Lodish and Porter, 1980) infected cells; (iii) there may be a competition for limiting factors on the basis of simple mass balance account for the shutoff in reovirus (Walden et al., 1981) and vesicular stomatitis virus (Lodish and Porter, 1980) infected cells; (iii) there may be a specific inhibitor of mRNA translation in mengovirus-infected cells (Penseiro and Lucas-Lenard, 1985) while a specific enhancer of viral mRNA translation may exist in frog virus-infected cells (Raghow and Granoff, 1983); (iv) Bablanian and colleagues have suggested roles of poly(A) and initiation factors in the selective translation of vaccinia viral mRNAs (Bablanian et al., 1991); and (v) there is a suggestion but not direct proof that increased eIF-2 alpha-phosphorylation may result in the selective shutoff of certain cellular mRNAs (De Benedetti and Baglioni, 1983, 1984; Kaufman et al., 1989; O'Malley et al., 1989).

Finally, what then are the possible regions of the influenza viral mRNAs which are critical for translation? The 5′-untranslated region of both viral and cellular mRNAs has often been found to contain critical sequences which regulate mRNA translation (Hinnebusch, 1984; Kozak, 1986b, 1986c; Gallie et al., 1987; Katz et al., 1986; Falcone and Andrews, 1991). At first examination there is nothing remarkable about the primary/secondary structure or length of the influenza viral NP mRNA 5′-untranslated region. It is again important to note that the 5′ ends of influenza viral RNAs are derived from host cell polymerase II transcripts (Krug et al., 1989).

Furthermore, at least in vitro, the virus prefers the 5′ ends of capped mRNAs with reduced secondary structure (Krug et al., 1980). Since less secondary structure is associated with enhanced translational efficiency (Kozak, 1986c), it may be contended that influenza steals the caps of the best translated cellular mRNAs (Kozak, 1986a). A convincing argument against this hypothesis is provided by the fact that the NP cDNA used in this report was prepared from virus RNA and as such did not contain host cell sequences, but yet was still translated in virally infected cells. The influenza viral RNAs also contain a common 12-nucleotide sequence (Skehel and Hay, 1978) downstream from the stolen 5′ ends that might be involved with translational regulation. On this basis, one would predict that all influenza viral mRNAs would be translated with equal efficiencies in vivo but it appears that this is not the case (Katze et al., 1986b). Nevertheless, we are currently deleting this sequence from our truncated NP gene and are also transferring these sequences to cellular genes to test whether it imparts the observed translational advantage. In addition we are constructing chimeras between cellular and viral genes to test whether in addition to the 5′ end, the coding region or even the 3′ ends of the viral genes (Vassalli et al., 1989) are important for translational efficiency.

Acknowledgments—We thank Don Cleveland, Larry Kedes, and Bryan Cullen for gifts of plasmaids; Jonathan Yewdell and Robert Webster for monoclonal antibodies to NP; and Firelli Alonso-Caplen for initial construction of NF-S and for continuing expert advice. We also thank Marlene Wambach for technical assistance with RNA isolation and Marjorie Domencik for figure preparation.

REFERENCES

Alonso-Caplen, F. V., Katze, M. G., and Krug, R. M. (1988) J. Virol. 62, 1606–1616.

Babich, A., Feldman, L., Nevins, J., Darnell, J. E., and Weinberger, C. (1983) Mol. Cell. Biol. 3, 1212–1221.

Bablanian, R., Goswami, S. K., Esteban, M., Banerjee, A. K., and Merrick, W. C. (1991) J. Virol. 65, 4440–4460.

Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. R. (1988) Gene (Amst.) 61, 1–20.

Black, T., Safer, B., Hovanessian, A., and Katze, M. G. (1989) J. Virol. 63, 2244–2251.

Blobel, G., and Sabatini, D. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 390–394.

Carasco, L. (1977) FEBS Lett. 76, 11–15.

Carrasco, L., and Local, J. C. (1983) Pharmacol. Ther. 23, 109–145.

Cullen, B. R. (1986) Cell 46, 973–982.

Cullen, B. R. (1987) Methods Enzymol. 152, 684–704.

De Benedetti, A., and Baglioni, C. (1983) J. Biol. Chem. 258, 14566–14569.

De Benedetti, A., and Baglioni, C. (1984) Nature 313, 73–81.

Dolph, P. J., Racanello, V., Villamarin, A., Palladino, F., and Schneider, R. J. (1988) J. Virol. 62, 2059–2066.

Dolph, P. J., Huang, J., and Schneider, R. J. (1990) J. Virol. 64, 2699–2677.

Etkind, P. R., and Krug, R. M. (1975) J. Virol. 16, 1446–1475.

Falcone, D., and Andrews, D. W. (1991) Mol. Cell. Biol. 11, 256–264.

Feinberg, A. P., and Vogelstein, B. (1984) Anal. Biochem. 132, 6–13.

Gallie, D. R., Sleat, D. E., Watta, J. W., Turner, P. C., and Wilson, T. M. A. (1989) Nucleic Acids Res. 16, 8653–8711.

Godchaux, W., Adamson, S. D., and Herbert, E. (1967) J. Biol. Chem. 27, 57–72.

Hinnebusch, A. G. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6424–6446.

Huang, J., and Schneider, R. J. (1991) Cell 65, 271–280.

Jang, S. K., Davies, M. V., Kaufman, R. J., and Wimmer, E. (1989) J. Virol. 63, 1651–1660.

Kaminski, A., Howell, M. T., and Jackson, R. J. (1990) EMBO J. 9, 3753–3759.

J. Virol. 62, 1606–1616.

Krug, R. M. (1986a) Nature 326, 224–2251.

Kozak, M., et al., 1989) and vesicular stomatitis virus (Lodish and Porter, 1980) infected cells; (iii) there may be a specific inhibitor of mRNA translation in mengovirus-infected cells (Penseiro and Lucas-Lenard, 1985) while a specific enhancer of viral mRNA translation may exist in frog virus-infected cells (Raghow and Granoff, 1983); (iv) Bablanian and colleagues have suggested roles of poly(A) and initiation factors in the selective translation of vaccinia viral mRNAs (Bablanian et al., 1991); and (v) there is a suggestion but not direct proof that increased eIF-2 alpha-phosphorylation may result in the selective shutoff of certain cellular mRNAs (De Benedetti and Baglioni, 1983, 1984; Kaufman et al., 1989; O'Malley et al., 1989).

Finally, what then are the possible regions of the influenza viral mRNAs which are critical for translation? The 5′-untranslated region of both viral and cellular mRNAs has often been found to contain critical sequences which regulate mRNA translation (Hinnebusch, 1984; Kozak, 1986b, 1986c; Gallie et al., 1987; Katz et al., 1986; Falcone and Andrews, 1991). At first examination there is nothing remarkable about the primary/secondary structure or length of the influenza viral NP mRNA 5′-untranslated region. It is again important to note that the 5′ ends of influenza viral RNAs are derived from host cell polymerase II transscripts (Krug et al., 1989).
**Translational Control by Influenza Virus**

Katz, R. A., Cullen, B. R., Malavarca, R., and Skalka, A. M. (1986) *Mol. Cell. Biol.* **6**, 372-379

Katz, M. G., and Krug, R. M. (1984) *Mol. Cell. Biol.* **4**, 2198-2206

Katz, M. G., and Krug, R. M. (1991) in *Translationally Regulated Genes in Higher Eukaryotes* (R. E. Thach, ed) S. Karger AG, Basel/New York, in press

Katze, M. G., Chen, Y. T., and Krug, R. M. (1984) *Cell* **37**, 483-490

Katze, M. G., DeCorato, D., and Krug, R. M. (1986a) *J. Virol.* **60**, in press

Katze, M. G., Detjen, B. M., Safer, B., and Krug, R. M. (1986b) *Mol. Cell. Biol.* **6**, 1741-1750

Katze, M. G., Tomita, J., Black, T., Krug, R. M., Safer, B., and Hovanessian, A. G. (1988) *J. Virol.* **62**, 3710-3717

Katze, M. G., Wambach, M., Wong, M.-L., Garfinkel, M., Meurs, E., Chong, K., Williams, B. R. G., Hovanessian, A. G., and Katze, M. G. (1991) *Mol. Cell. Biol.* **11**, 5497-5505

Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. B. (1989) *Mol. Cell. Biol.* **9**, 946-958

Kozak, M. (1986a) *Adv. Virus Res.* **31**, 229-292

Kozak, M. (1986b) *Cell* **44**, 283-292

Kozak, M. (1986c) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 2850-2854

Krug, R. M., Broni, B. A., Lafiandra, A. J., Morgan, M. A., and Shatkin, A. J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 5874-5878

Krug, R. M., Alonso-Caplen, F., Julkunen, I., and Katze, M. G. (1989) in *The Influenza Viruses* (Krug, R. M., ed) pp. 89-152, Plenum Press, New York

Lazarowitz, S. G., Compana, R. W., and Choppin, P. W. (1971) *Virology* **46**, 830-843

Lee, K. A. W., and Sonenberg, N. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 3447-3451

Lee, T. G., Tomita, J., Hovanessian, A. G., and Katze, M. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6208-6212

Lenk, R., and Pennman, S. (1979) *Cell* **16**, 269-301

Lodish, H. F. (1978) *Annu. Rev. Biochem.* **45**, 39-72

Lodish, H., and Froshauer, S. (1977) *J. Biol. Chem.* **252**, 8804-8811

Lodish, H. F., and Porter, M. (1986) *J. Virol.* **36**, 719-733

O'Malley, R. P., Duncan, R. F., Hershey, J. W. B., and Mathews, M. B. (1989) *Virology* **168**, 112-118

O'Neill, R. E., and Racaniello, V. R. (1989) *J. Virol.* **63**, 5069-5075

Pelletier, J., and Sonenberg, N. (1988) *Nature* **334**, 320-325

Pensiero, M. N., and Lucas-Lenard, J. M. (1985) *J. Virol.* **56**, 161-171

Raghow, R., and Granoff, A. (1983) *J. Biol. Chem.* **258**, 571-578

Schneider, R. J., and Shenk, T. (1987) *Annu. Rev. Biochem.* **56**, 317-332

Skehel, J. J. (1972) *Virology* **49**, 23-36

Skehel, J. J., and Hay, A. J. (1978) *Nucleic Acids Res.* **5**, 1207-1219

Sonenberg, N. (1990) *New Biologist* **2**, 402-409

Vassalli, J.-D., Huarte, J., Belin, D., Gubler, P., Vassalli, A., O'Connell, M. L., Parton, L. A., Rickles, R. J., and Strickland, S. (1989) *Gress & Dev.* **3**, 2163-2171

Walden, W. E., Godefroy-Colburn, T., and Thach, R. E. (1981) *J. Biol. Chem.* **256**, 11739-11746