Translational Control by Influenza Virus

SELECTIVE TRANSLATION IS MEDIATED BY SEQUENCES WITHIN THE VIRAL mRNA 5'-UNTRANSLATED REGION* (Received for publication, August 6, 1993)

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In cells infected by influenza virus type A, host cell protein synthesis declines rapidly and dramatically, while influenza viral protein synthesis occurs efficiently throughout infection. Previously, we had shown that the selective translation of influenza viral mRNAs in infected cells occurred in a cap-dependent manner and was due at least in part to structures inherent in the mRNAs. Using chimeras containing the noncoding and coding regions of cellular and viral mRNAs, we can now report that the selective translation is mediated by sequences within the 5'-untranslated regions (UTR) of the viral mRNAs. Polysome analysis confirmed that a 45-nucleotide sequence contained in the 5'-UTR of the influenza viral nucleocapsid protein was necessary and sufficient to allow the host cell translational machinery to discriminate between viral and cellular mRNAs. In reciprocal experiments in which the 5'-UTR of the cellular mRNA-secreted embryonic alkaline phosphatase replaced the nucleocapsid protein 5'-UTR, viral protein synthesis was inhibited in virus-infected cells, resembling host protein synthesis. Finally, we demonstrated that the 5'-UTR of another influenza viral mRNA, that encoding the nonstructural protein, also conferred resistance to the shutoff of protein synthesis in influenza virus-infected cells.

Cells infected by influenza virus type A undergo a rapid and dramatic shutoff of host cell protein synthesis while at the same time viral protein synthesis occurs efficiently and selectively (for review, see Katze and Krug (1990) and Garfinkel and Katze (1993)). We have been studying this regulation of protein synthesis as a model system to begin to decipher the molecular mechanisms that allow the discrimination of viral from cellular mRNAs in infected cells. Influenza virus, like many other eukaryotic viruses, utilizes multiple strategies to ensure the efficient and selective translation of its mRNAs during infection. (i) It encodes mechanisms to down-regulate the interferon-induced, dsRNA-activated protein kinase, PKR, during infection. The biochemical basis of the inhibition of PKR activity in influenza virus-infected cells has been determined and is mediated by a cellular protein called P68 based on its molecular weight of 58,000 (Lee et al., 1990; 1992). (ii) It prevents newly synthesized cellular mRNAs from reaching the cytoplasm of influenza virus-infected cells (Katze and Krug, 1984). (iii) It subjects otherwise stable and functional mRNAs already in the cytoplasm to block at both the initiation and elongation steps of translation (Katze et al., 1986). (iv) It encodes mRNAs with structural features that promote selective translation (Alonso-Caplen et al., 1988; Garfinkel and Katze, 1992). To begin to define the molecular mechanisms underlying the selective translation of viral mRNAs in influenza virus-infected cells, we developed an in vivo transfection/infection assay using cDNAs encoding representative viral and cellular genes (Garfinkel and Katze, 1992). Using this assay we determined that during influenza virus infection, protein synthesis from exogenously introduced cellular genes was indeed subject to the host cell shutoff. In marked contrast, an exogenous influenza viral gene (an internally shortened version of the nucleocapsid protein (NP) called NP-S) was not subject to the host cell shutoff and continued to be translated just as the bona fide viral mRNAs. These experiments provided definitive evidence that mass competition due to abundance of viral mRNAs was not the cause of the host cell shutoff, as has been defined for some viral systems (Schneider and Shenk, 1987). Further, this was a direct demonstration of the importance of viral mRNA structure as all the mRNAs were expressed from identical transfection vectors; the only differences between the exogenous viral and cellular genes were their non-coding and coding regions. We then determined that, unlike the selective translation mechanisms invoked by poliovirus and adenovirus, which occur cap independently (Pelleier and Sonenberg, 1988; Dolph et al., 1990), influenza viral mRNAs, like most cellular mRNAs, were translated in a cap-dependent manner. To define exactly which structures of the viral mRNAs were critical to allow discrimination between the viral and the cellular mRNAs during influenza virus infection, we constructed chimeras between the coding and noncoding regions of viral and cellular genes. We can now report that the 5'-UTRs of influenza viral mRNAs contain the necessary sequence information that allow those mRNAs to escape the blocks to cellular protein synthesis normally found in the virus-infected cell.

MATERIALS AND METHODS

Cells and Virus Infections—COS-1 cells were grown in monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The WSN strain of influenza A virus was grown in Madin-Darby canine kidney cells (Eckind and Krug, 1975). Monolayers of COS cells were infected with influenza virus at a multiplicity of infection of 50 plaque-forming units/cell.

Transfection Vectors and cDNA Constructs—The vector pBC12/CMV (Berger et al., 1988; Cullen, 1986, 1987) was used for all transfections. Details of the construction of the parental pBC12/CMV/NP-S and SEAP vectors are found in Garfinkel and Katze (1992). For the exchange of the SEAP 5'-UTR for the influenza viral 5'-UTR, the cDNA for NP of influenza virus strain A/PB/R/234 was used as a template for PCR-directed synthesis of the 5'-UTR of NP plus the restriction sites EcoRV and SphI, which allowed direct subcloning following deletion of the EcoRV-SphI fragment from pBC12/CMV/SEAP. The primers used were (forward)

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1 The abbreviations used are: NF, nucleocapsid protein; UTR, untranslated region; SEAP, secreted embryonic alkaline phosphatase; PCR, polymerase chain reaction; NS, nonstructural protein.
To distinguish the structural features of influenza viral mRNAs that allowed them to escape the host cell shutoff during infection, we first designed a cDNA chimera such that the mRNA transcript would consist of the 5'-untranslated region of influenza virus, it was found that SEAP enzymatic activity was increased in influenza virus-infected cells. During influenza virus infection, SEAP protein synthesis, as measured both by enzymatic activity (Fig. 2A, left) and corroborated by radiolabeling and immunoprecipitation (not shown), dropped 5–8-fold in several experiments, and as shown earlier (Garfinkel and Katze, 1992). In marked contrast, when cells were transfected with (NP)SEAP and then infected with influenza virus, it was found that SEAP enzymatic activity was undiminished over the course of influenza virus infection (Fig. 2A, right). This result was also confirmed by radiolabeling and immunoprecipitation (not shown). Northern blot analysis indicated that parental SEAP mRNA levels did not decrease over the course of infection nor, as expected, did (NP)SEAP mRNA levels change (Fig. 2B). Polysome analysis indicated that the parental SEAP polysome distribution was consistent with a combined initiation/elongation block, with some of the SEAP mRNA remaining associated with polysomes in samples B and C but with a large percentage of the material displaced to samples D (the material sedimenting with the ribosomal subunits) and E (the top of the gradient) (data not shown; see Fig. 2).

### RESULTS AND DISCUSSION

To distinguish the structural features of influenza viral mRNAs that allowed them to escape the host cell shutoff during infection, we first designed a cDNA chimera such that the mRNA transcript would consist of the 5'-untranslated region of influenza virus, it was found that SEAP enzymatic activity was increased in influenza virus-infected cells. During influenza virus infection, SEAP protein synthesis, as measured both by enzymatic activity (Fig. 2A, left) and corroborated by radiolabeling and immunoprecipitation (not shown), dropped 5–8-fold in several experiments, and as shown earlier (Garfinkel and Katze, 1992). In marked contrast, when cells were transfected with (NP)SEAP and then infected with influenza virus, it was found that SEAP enzymatic activity was undiminished over the course of influenza virus infection (Fig. 2A, right). This result was also confirmed by radiolabeling and immunoprecipitation (not shown). Northern blot analysis indicated that parental SEAP mRNA levels did not decrease over the course of infection nor, as expected, did (NP)SEAP mRNA levels change (Fig. 2B). Polysome analysis indicated that the parental SEAP polysome distribution was consistent with a combined initiation/elongation block, with some of the SEAP mRNA remaining associated with polysomes in samples B and C but with a large percentage of the material displaced to samples D (the material sedimenting with the ribosomal subunits) and E (the top of the gradient) (data not shown; see Fig. 2).

#### 5'-UNTRANSLATED REGION CHIMERAS

- **(NP)SEAP**
  - 5'-GATGCGATGCTGAGAAGCCAGAGCTTAGATAATCG
  - SEAP

- **(NS)SEAP**
  - 5'-GATGCGATGCTGAGAAGCCAGAGCTTAGATAATCG
  - SEAP

- **(SEAP)NP-S**
  - 5'-GATGCGATGCTGAGAAGCCAGAGCTTAGATAATCG
  - NP-S

**Fig. 1. Viral and cellular chimeric cDNA constructs.** The 5'-UTR sequences are shown. The 12-nucleotide sequence shared by NP and NS is underlined. The coding and 3'-noncoding regions of the representative genes are indicated by the open boxes. Details of the construction are given under "Materials and Methods."
the polystosome distribution of (NP)SEAP mRNA with polyribosomes, mostly in samples B and C corresponding to the actively translating polysomes, was essentially the same as the parental NP-S mRNA and as the viral NP mRNA (Garfinkel and Katze, 1992). This mRNA distribution indicated that the 45-nucleotide sequence contained within the NP 5′-UTR was sufficient to allow the translational machinery to discriminate between influenza viral and cellular mRNAs and for the mRNAs to efficiently associate with polyribosomes.

In reciprocal experiments, we replaced the viral 5′-UTR with the SEAP 5′-UTR on the influenza viral NP-S mRNA. We reasoned that if the viral 5′-UTR was critical to allow the viral mRNA to escape the host cell shutoff of translation, a viral mRNA lacking this region might be recognized not as a viral mRNA but as a cellular mRNA and would be subject to this shutoff. This chimera consisted of the SEAP 5′-UTR appended to the coding and 3′-noncoding sequences of NP-S, the truncated nucleocapsid protein. As a control, cells were transfected with the parental NP-S and then infected with influenza virus. NP-S protein synthesis remained high throughout influenza virus infection (Fig. 3A, left). However, the pattern of protein synthesis of NP-S directed by the (SEAP)NP-S chimera following influenza virus infection differed markedly. NP-S expression was no longer selectively maintained following infection but was subjected to the host cell shutoff to the same extent (about a 7-fold decrease as quantitated by laser densitometry scan) (Fig. 3A, right) as were cellular mRNAs (e.g., Fig. 2A). Northern blot analysis confirmed that (SEAP)NP-S mRNA levels did not change, even by 5 h postinfluenza virus infection (Fig. 3B). We then proceeded to examine the polysome association of the (SEAP)NP-S chimeric mRNAs in uninfected and influenza virus-infected cells (Fig. 3C). In mock-infected cells, most of the (SEAP)NP-S mRNA was found in polysome samples B and C (70%) as would be expected for actively translating mRNAs of its size. However, following influenza virus infection, while some of the (SEAP)NP-S mRNA did remain polyosome-associated in samples B and C (45%) an increased fraction of the mRNA was found in ribosomal subunit sample D (20%) and in sample E (11%), indicating that this mRNA remains sensitive to the same translational blocks as the parental SEAP mRNA and all other cellular mRNAs (Katze and Krug, 1984; Garfinkel and Katze, 1992). Thus, despite the presence of virtually all of the coding and 3′-UTR sequences found in the influenza viral NP mRNA, NP-S failed to escape the translational block exerted on cellular mRNAs during infection when its cognate 5′-UTR was substituted with a cellular 5′-UTR.

Thus far we demonstrated that the NP 5′-UTR contained critical sequences that can direct the selective translation of viral mRNAs in the influenza virus-infected cell. However, there are seven additional viral genes encoded by influenza virus; we wanted to determine whether the nucleotide sequences contained in another influenza viral 5′-UTR were also necessary and sufficient to allow discrimination by the translational machinery. We therefore appended the 5′-UTR of the nonstructural protein (NS) to the SEAP coding region and 3′-UTR. A cDNA was constructed, as described under “Materials and Methods,” which when transcribed contained the 28-nucleotide 5′-UTR of the NS mRNA and the coding and 3′-noncoding regions of SEAP, represented graphically in Fig. 1. Analysis of protein synthetic activity directed by (NS)SEAP during influenza virus infection or mock infection is presented in Fig. 4. Again, as for the parental NP-S and for the (NP)SEAP

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**Fig. 3.** Influenza viral nucleocapsid protein mRNA translation is subject to the host cell shutoff when the viral 5′-UTR is substituted by a cellular 5′-UTR. A, NP-S protein synthesis following infection by influenza virus. Following mock (M) or influenza virus (FLU) infection, radiolabeled extracts from parental NP-S (left) or chimeric (SEAP)NP-S (right) transfected cells (or cells transfected with vector (V) alone) were subjected to immunoprecipitation analysis as indicated under “Materials and Methods.” B, Northern blot analysis of influenza virus mRNA following mock infection (M) or influenza virus infection (F). Poly(A)+ mRNAs from (SEAP)NP-S transfected cells or cells transfected by vector alone (VECTOR) were prepared at the times indicated postinfection, electrophoresed, and blotted. The blots were hybridized to a 32P-labeled probe specific to NP-S. C, slot blot analysis of the distribution of exogenous (SEAP)NP-S RNA on polysomes. The sedimentation profile is shown on the left of the panel; the samples were prepared as described under “Materials and Methods” and in the legend to Fig. 2. Slot blot analysis of the mock-infected and influenza virus-infected cells is shown on the right of the panel. The distribution in the mock-infected cells was: sample A, 20%; sample B, 60%; sample C, 11%, sample D, 10%; and sample E, <0.1%. In the influenza virus-infected cells, the polyribosomal distribution of (SEAP)NP-S was: sample A, 11%; sample B, 20%; sample C, 21%; sample D, 35%; and sample E, 11%.

**Fig. 4.** Analysis of exogenous (NS)SEAP chimera translation in transfected/infected cells. Following mock infection (dashed bars) or influenza virus infection (solid bars) at the times indicated, medium from (NS)SEAP-transfected cells was subjected to assay for alkaline phosphatase activity as described under “Materials and Methods.”
chimera, translation of the (NS)SEAP chimeric mRNA continued undiminished throughout influenza virus infection, as measured by SEAP enzymatic activity. In the same experiment, parental SEAP protein synthesis decreased 6-fold by 5 h postinfluenza virus infection (data not shown). These data demonstrate that the 5′-UTR encoded by NS also can direct sustained protein synthesis from an mRNA normally subject to the host cell shutoff of translation during influenza virus infection.

In this study we have defined the viral mRNA sequences required for selective translation. The 5′-untranslated regions of two representative influenza virus mRNAs have been shown to contain determinants critical for ensuring selective translation during influenza virus infection. The sequences contained in these 5′-UTRs alone were shown to be necessary to confer selective translation onto a non-viral mRNA and sufficient to eliminate the blocks to translation normally exerted over cellular mRNAs during infection. Further, we have shown that the absence of the small influenza viral 5′-UTR caused an entire viral mRNA (over 1500 nucleotides in NP) to become sensitive to the host cell shutoff of translation. Selective translation mediated by the 5′-UTR has been defined for several viral mRNAs (Pelletier and Sonenberg, 1988; Dolph et al., 1990) and non-viral mRNAs (for example, ferritin and heat shock mRNAs (Klausner et al., 1993; Lindquist and Petersen, 1990). Thus it is not entirely surprising that influenza viral RNA 5′-UTRs would provide some basis for translational discrimination. What is notable is that, unlike the selective translation of polioviral and adenoviral mRNAs, which occur in a cap-independent manner and appear for now to require multiple elements within extended 5′-UTRs, about 750 nucleotides of the polioviral mRNA and 200 nucleotides of adenoviral mRNAs (Pelletier and Sonenberg, 1988; Meerovitch et al., 1989, 1993; Zhang et al., 1989), influenza virus selective translation occurred in a cap-dependent manner, like almost all cellular mRNA translation (Garfinkel and Katze, 1992) (for review, see Kozak, 1991, 1992) and may require as few as 28 virus-specific nucleotides to ensure this regulation.

Which sequences then are responsible for the selective translation of the viral mRNAs during influenza virus infection? The only homology between the NP and NS nucleotide sequences is the 12-nucleotide conserved sequence derived from the 3′-UTR of all of the influenza virus type A virus RNAs (indicated in Fig. 1). However, this sequence has been shown to be required for replication and packaging of the viral RNAs (Luytjes et al., 1989; Seong and Brownlee, 1992). Because influenza virus, like most RNA viruses, has evolved a compact genome (Strauss et al., 1990) it is possible that this conserved sequence could also function in regulating translation. We envisage at least three possible models for the selective translation of influenza viral mRNAs. The first and most likely model, based on observations in other viral (e.g. Meerovitch et al. (1993)) and non-viral (e.g. Klausner et al. (1993)) systems, is that the primary sequence or a specific higher order structure of the influenza viral 5′-UTR provides a competitive advantage possibly through the recruitment or avoidance of trans-acting factors which could be positively acting if they bind to the viral UTRs or negatively acting if they interact with the cellular 5′-UTRs. Given the vast diversity and abundance of cellular mRNAs, most of which are not translated during influenza virus infection (Lazarowitz et al., 1971; Skehel, 1972; Katze and Krug, 1984), the former possibility may be more likely. Second, given the recent report (Feigenblum and Schneider, 1993) that influenza viral mRNAs may be translatable despite moderate reductions of functional eukaryotic protein synthesis initiation factor 4E, the cap-binding protein, it is possible that the sequence alone of the viral, but not the cellular, 5′-UTR provides the necessary signals to the translational machinery. This could result from a specific secondary structure, or lack thereof, which could mediate either a reduced need for, or higher affinity for, functional eIF-4E. Finally, the least likely model is that the viral 5′-UTR is providing a unique signal for internal initiation of translation. This model is least likely, since we have previously shown that influenza viral mRNA translation initiated in a cap-dependent manner (Garfinkel and Katze, 1992), and to our knowledge there is no evidence for cap-dependent internal initiation occurring in eukaryotic cells. The definition of the exact mechanisms underlying the selective translation will allow us not only to understand the life cycle of the virus more profoundly but also will have several practical applications, both experimental and clinical. We propose that a more intimate understanding of the molecular virology of the translational regulation invoked by influenza virus will eventually allow for construction of more successful reassortant viruses used in the reverse genetics systems described by the Palese and Brownlee laboratories (Luytjes et al., 1989; Seong and Brownlee, 1992). Further, this understanding could eventually allow for the creation of a new class of anti-influenza drugs, which could be aimed specifically at the cis-acting elements or trans-acting factors (or both) mediating selective translation of the viral mRNAs during infection.

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