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Translation of Sindbis Virus mRNA: Effects of Sequences Downstream of the Initiating Codon

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One incentive for developing the alphavirus Sindbis virus as a vector for the expression of heterologous proteins is the very high level of viral structural proteins that accumulates in infected cells. Although replacement of the structural protein genes by a heterologous gene should lead to an equivalent accumulation of the heterologous protein, the Sindbis virus capsid protein is produced at a level 10- to 20-fold higher than that of any foreign protein. Chimeric mRNAs which contain the first 275 nucleotides of the Sindbis virus 26S mRNA fused to the *lacZ* gene are also translated at the higher level. The enhancing sequences, located downstream of the AUG codon that initiates translation of the capsid protein, have a predicted hairpin-like structure; deletions in this region destroy the activity. These sequences enhance translation in infected cells but have the opposite effect in uninfected cells. Furthermore, translation of this RNA in infected cells is suppressed by a second viral RNA lacking the hairpin-like structure, but translation of the latter RNA is not affected. We propose that the hairpin-like structure presents a barrier to the movement of the ribosomes during translation of mRNA. In infected cells, under conditions in which this mRNA is essentially the only RNA being translated, a slowdown in the transit of the ribosomes gives factors present at low concentrations a chance to bind to the translation complex and permits a high level of functional complexes to be formed. In uninfected cells and in infected cells translating two different viral subgenomic mRNAs, a pause in the movement of the ribosomes along the RNA is no longer an advantage, because the required factors are now usurped by other translation complexes.

Sindbis virus is the prototype of the *Alphavirus* genus of the *Togaviridae* family (22, 25). These viruses have a nonsegmented positive-strand RNA genome of approximately 1.2×10^4 nucleotides (nt). The organization of the genome is depicted in Fig. 1A. The first two-thirds of the RNA codes for the proteins required for transcription and replication of the RNA. Only this region of the genomic RNA is translated in infected cells; three stop codons following the coding sequence for the nonstructural proteins prevent its continued translation. The 3′ one-third of the genome contains the genetic information for the viral structural proteins which are translated from a subgenomic RNA (26S RNA) identical in sequence to this region of the genome. The viral subgenomic RNA is transcribed from the genome-length complementary (minus) strand from an internal promoter.

Alphavirus replicons in which the viral structural protein genes are replaced by a reporter gene provide a valuable tool for studying the expression of heterologous proteins and for analysis of the virus (3, 17, 21, 32). We recently showed that only the expression of the Sindbis virus nonstructural protein genes is required for inhibition of host cell protein synthesis in infected cells (6). Inhibition of host cell protein synthesis occurs at the same rate in BHK cells whether or not the viral structural proteins are synthesized and under conditions in which the level of the viral subgenomic RNA is too low to be detected. However, significant differences between Sindbis virus and replicons and between replicons expressing different proteins exist. Cells infected with Sindbis virus or with SINrep/capsid (which lacks the genes for the viral membrane glycoproteins) produce considerably more capsid protein than is produced in cells infected with replicons expressing a variety of other heterologous proteins. This finding led us to the unexpected discovery that sequences downstream of the initiating codon for the capsid protein enhanced translation of the mRNA in infected cells but had the opposite effect in uninfected cells.

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MATERIALS AND METHODS

Sindbis virus cDNAs and replicons. The Sindbis virus replicons used in this study were derived from the cDNA pSINrep5 (4). They are all identical from the 5′ terminus of the Sindbis virus genome up to nt 7646 which includes the nonstructural protein genes and the promoter for transcription of the subgenomic RNA. They all terminate with the same 310 nt of the Sindbis virus genome plus 37 A residues. The replicons differ in the coding sequences of the region that is transcribed into the subgenomic RNA. These differences are diagrammed in Fig. 1B and C. The subgenomic RNAs in Fig. 1B numbered 2 to 6 and 8 contain the complete LacZ-coding sequences fused in frame to different lengths of the capsid protein gene. The first diagram, 49LacZ, is identical to SINrep/LacZ (4) and contains the first 49 nt of the Sindbis virus subgenomic 26S RNA followed by the *lacZ* gene. The seventh diagram, 404LacZ, was transcribed from a cDNA in which the *lacZ* gene retained only the 798 carboxy-terminal amino acids of β-galactosidase. (The intact β-galactosidase polypeptide contains 1,003 amino acids.) The in-frame deletions indicated in diagrams 9 to 12 of Fig. 1B and diagrams 2 and 3 of Fig. 1C were obtained from previous constructions which contained in-frame deletions in the capsid protein gene (9). We also mutated nt 7667 to 7771 from CATGC to TAAAT with a

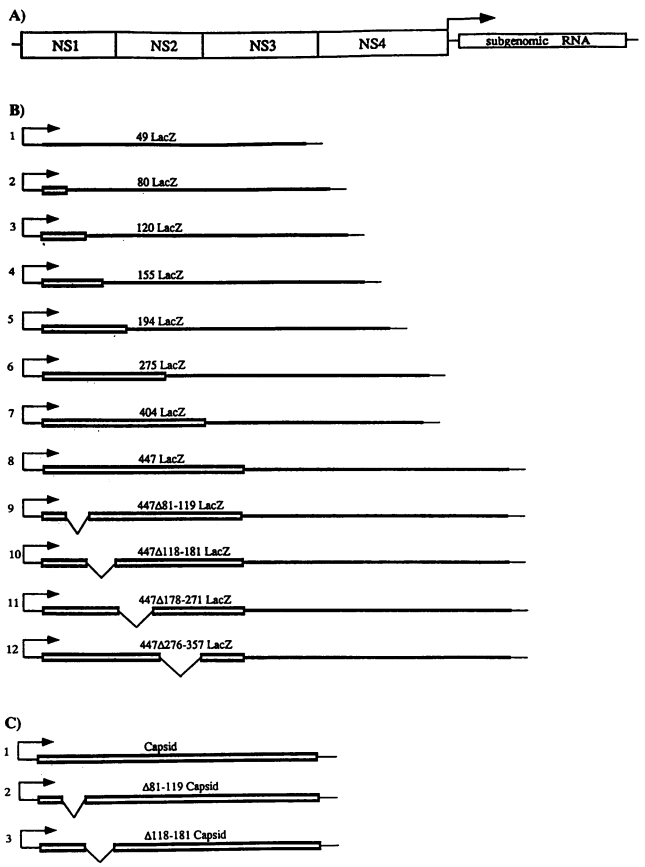

FIG. 1. Diagrams of the genome of Sindbis virus and the regions encompassing the subgenomes of Sindbis virus replicons used in these studies. (A) Sindbis virus genomic RNA. The open boxes represent coding sequences; NS1 to NS4 are the four nonstructural protein genes. The horizontal arrow indicates the location of the promoter for transcription of the subgenomic RNA and its start point. The thin black horizontal lines represent the noncoding sequences at the 5′ and 3′ ends and the region between the NS genes and the structural protein genes. (B) Subgenomic RNAs of replicons that express the *lacZ* gene (diagram 1) or genes that are in-frame fusions between 5′-terminal sequences of the Sindbis virus capsid protein gene and the *lacZ* gene. The number in front of LacZ indicates the number of nucleotides at the 5′ terminus derived from the subgenomic RNA of Sindbis virus. The open box represents capsid sequences, and the thick black line represents *lacZ* sequences. The numbers following the Δ indicate the sequences deleted in the subgenomic RNAs. (C) Subgenomic RNAs of replicons that express the capsid gene.

primer for PCR that introduced these changes into the cDNA plasmid of pSINrep/capsid.

The plasmid containing the cytomegalovirus (CMV) promoter was a gift from John Majors. Sequences corresponding to the subgenomic RNAs of 80LacZ and 447LacZ (Fig. 1B diagrams 2 and 8) were put under the control of this promoter. The region of the SINrep cDNAs closed downstream of the CMV promoter extended from an *Ava*II site 4 nt upstream of the start of the subgenomic RNA promoter to the *Xho*I site following the poly(A) stretch.

All plasmid DNAs were isolated by the alkaline lysis method followed by isopycnic centrifugation in CsCl.

Transcription, transfection, and packaging of the replicons. The transcription, transfection, and packaging methods have been described elsewhere (4, 6). The DNAs were linearized for transcription at the *Xho*I site following the poly(A) stretch (4). Transcriptions were carried out in the presence of the 5′ cap analog 7^{m}G5′ppp5′G (New England Biolabs). The RNAs (2.5 μg each) were introduced into cells by electroporation as described by Liljeström et al. (18). The SINrep RNAs were packaged with the DH-BB(5′SIN) helper RNA (4). Titers of packaged replicons were determined by the cytopathic effect assay on chicken embryo fibroblasts as described previously (6). Replicon titers ranged from 4×10^8 to 8×10^8 infectious units per ml.

Infections and analysis of protein in infected and transfected cells. BHK cells (3×10^5 cells) were seeded onto 35-mm-diameter dishes. They were infected 4 to 6 h later with Sindbis virus replicons at a multiplicity of infection of 20 infectious units per cell in 0.5 ml of minimal essential medium containing 2% fetal calf serum. The dishes were incubated at 8°C for 2 h with continuous gentle shaking. An additional milliliter of the same medium (at 37°C) was added to each well, and the dishes were placed in a CO_2 incubator at 37°C. This procedure for attachment of the virus to cells was adopted to give reproducible synchronous infections of BHK cells (6). At the times indicated in the different figure legends, the cells were harvested for analysis of protein. The medium was removed from the well, the cells were washed three times with phosphate-buffered saline (PBS) and were scraped from the dish into PBS, pelleted by centrifugation and dissolved in loading buffer (60 mM Tris-HCl [pH 6.7], 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 5% glycerol, 0.05% bromophenol blue). Samples were incubated for 5 min in boiling H_2O, and an amount equivalent to 2.5×10^4 cells was analyzed on the gel. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R. The amount of β-galactosidase or chimeric proteins was determined with a computing laser densitometer (Molecular Dynamics, Sunnyvale, CA), using purified β-galactosidase (GibcoBRL, Gaithersburg, Md.) as a standard. When the cells were labeled with [^{35}S]methionine, they were first washed three times with PBS and then incubated for 15 min at 37°C in medium lacking methionine. This medium was then replaced with 0.7 ml of the same medium containing [^{35}S]methionine. After 20 min, 1 ml of complete medium was added to each well, and incubation was continued for another 10 min. This dilution of the [^{35}S]methionine was sufficient to inhibit further incorporation of radioactive label into protein and significantly decreased the background of free [^{35}S]methionine in the polyacrylamide gels.

For the experiments in which the proteins were to be immunoprecipitated, each radioactively labeled cell pellet was dissolved in 200 μl of 0.5% sodium dodecyl sulfate. Each sample was then diluted to 1 ml by the addition of 800 μl of buffer (50 mM Tris-HCl [pH7.5], 200 mM NaCl, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and was passed through a syringe needle several times to mix the detergents and to shear the DNA. Five microliters of rabbit anti-β-galactosidase serum (gift of J. Sanes) was added, and each sample was incubated for 4 h at 4°C with gentle agitation. A 50-μl aliquot of a 1:10 (wt/vol) suspension of washed Pansorbin cells (Calbiochem-Novabiochem Corp., San Diego, Calif.) was added to each sample, and incubation was continued for 4 h at 4°C. The Pansorbin was then pelleted by centrifugation and washed three times by resuspension in 500 μl of buffer (50 mM Tris-HCl [pH 7.5], 0.1% sodium dodecyl sulfate, 1 M NaCl) and repelleting. After a final wash with TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), the samples were suspended in loading buffer as described above, incubated for 5 min in boiling H_2O, and centrifuged for 1 min. The supernatant fluids were analyzed by polyacrylamide gel electrophoresis.

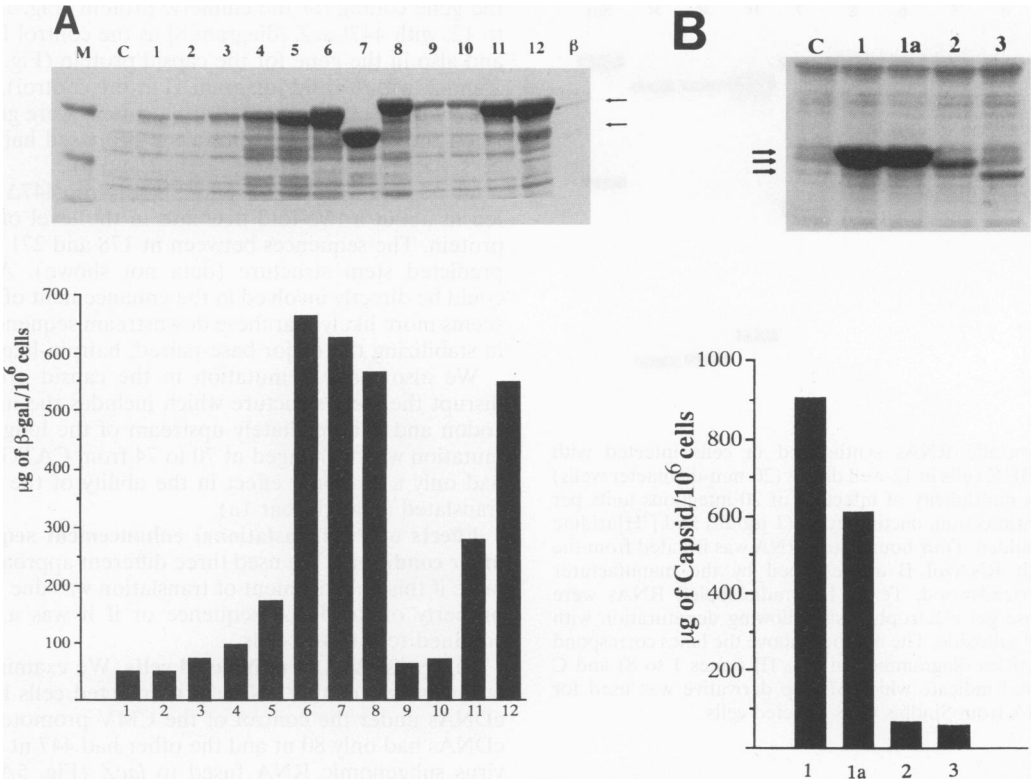

FIG. 2. Accumulation of β-galactosidase, capsid–β-galactosidase fusion proteins, or capsid protein in infected cells. BHK cells in 35-mm-diameter dishes were infected with SINrep particles at a multiplicity of infection of 20 infectious units per cell. At 18 h postinfection, the cells were harvested (see Materials and Methods). Samples equivalent to 2.5×10^4 cells were loaded onto the gel. The numbers above the lanes of the stained gels and on the abscissa of the plots indicate the SINrep derivative in Fig. 1B or C that was used for infection. (A) Levels of β-galactosidase and capsid–β-galactosidase chimeric proteins determined by Coomassie blue staining. The upper panel shows only the region of the gel where β-galactosidase migrates; the lower panel shows the results obtained from quantitation of the stained gel (β-gal., β-galactosidase.) The arrows indicate the migration of β-galactosidase and the chimeric proteins. The chimeric protein in lane 6 migrates faster than the others, because amino-terminal amino acids of the β-galactosidase protein were deleted (see Materials and Methods). (B) Levels of capsid proteins determined by Coomassie blue staining. The upper panel shows only the region of the gel where the capsid protein migrates; the lower panel shows the results obtained from quantitation of the stained gel. The arrows indicate the migration of the capsid protein and its deleted forms. In both panels, 1a refers to samples from cells infected with SINrep/capsid containing mutations in nt 70 to 74 to destabilize the first stem-loop structure which includes the initiating AUG codon (see Materials and Methods and Fig. 4). M, molecular weight markers; C, uninfected control.

RESULTS

Identification of sequences downstream of the initiating AUG codon in the Sindbis virus subgenomic RNA that enhance translation in infected cells. We had reported previously that BHK cells infected with SINrep/LacZ produced 20 to 40 μg of β-galactosidase per 10^6 cells (4). This value is 10- to 20-fold less than the amount of capsid protein produced by cells infected with SINrep/capsid. Coinfection of cells with SINrep/LacZ and SINrep/capsid did not enhance the level of β-galactosidase, demonstrating that the capsid protein itself was not able to affect the level of translation of the subgenomic RNA by some type of *trans*-acting mechanism (data not shown). To study this difference in more detail, we constructed a series of SINrep cDNAs in which the *lacZ* gene was fused in frame to increasing amounts of 5′ coding sequence of the capsid protein gene. SINrep RNAs transcribed from the cDNAs were packaged into particles by cotransfection of BHK cells with a defective helper RNA that packages the SINrep RNA without packaging itself (see Materials and Methods) (4). The subgenomic RNAs transcribed from these SINrep derivatives are diagrammed in Fig. 1B. They all have the same 49 noncoding nt at the 5′ terminus.

To compare the amounts of protein translated from these subgenomic RNAs, we infected cells with the different SINrep particles and analyzed the proteins 18 h postinfection. (No cytopathic effect is seen at this time postinfection in BHK cells infected with SINrep particles [6].) The proteins were separated by electrophoresis on acrylamide gels and were detected by staining with Coomassie blue (Fig. 2A, upper panel). The amounts of β-galactosidase and chimeric proteins were quantitated with a laser densitometer (Fig. 2A, lower panel). We could not rely on enzyme assays, because the chimeric proteins had different specific activities. Only small incremental differences in the amount of the protein were detected as the number of nucleotides from the capsid gene increased from 31 (SINrep/80LacZ [Fig. 2A, lane 2] to 145 (SINrep/194LacZ [Fig. 2A, lane 5]). The increase to 226 (SINrep/275LacZ [Fig. 2A, lane 6]), however, led to a substantial enhancement of translation and to levels that were comparable to the capsid protein (Fig. 2B).

The differences shown in Fig. 2A were due to differences in translation and not to differences in the levels of viral RNAs; the amounts of the Sindbis virus-specific mRNAs were similar in cells infected with the different replicons (Fig. 3). In

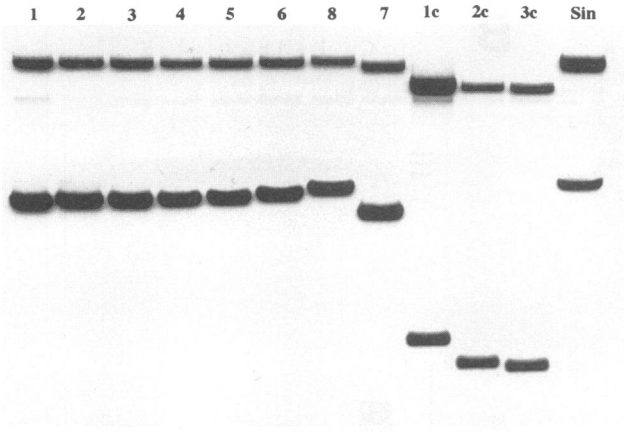

FIG. 3. Virus-specific RNAs synthesized in cells infected with SINrep particles. BHK cells in 12-well dishes (20-mm-diamaeter wells) were infected at a multiplicity of infection of 20 infectious units per cell. One hour postinfection, dactinomycin (1 μg/ml) and [^{3}H]uridine (20 μCi/ml) were added. Four hours later, RNA was isolated from the infected cells with RNAzol B as described by the manufacturer (Tel-Test, Inc., Friendswood, Tex.). The radiolabeled RNAs were analyzed by agarose gel electrophoresis following denaturation with glyoxal in dimethyl sulfoxide. The numbers above the lanes correspond to the SINrep particles diagrammed in Fig. 1B (lanes 1 to 8) and C (lanes 1c to 3c) and indicate which SINrep derivative was used for infection. Sin, RNA from Sindbis virus-infected cells.

addition, no difference in the stability of the proteins could be detected in pulse–2-h chase experiments (data not shown).

Enhanced translation correlates with a predicted stem structure downstream of the initiating codon. The first 170 nt of the Sindbis virus subgenomic RNA can be folded into an extensively base-paired structure by using the energy minimization program of Jaeger et al. (11). The most striking feature is the very long, G-C rich, hairpin-like structure from nt 77 to 140 (Fig. 4). A very similar structure is seen when this region of the Semliki Forest virus 26S RNA was folded, even though the sequence is not conserved (data not shown). To test the importance of sequences in this region of the Sindbis virus mRNA, we constructed cDNAs which had internal deletions in the gene coding for the chimeric protein (Fig. 1B, diagrams 9 to 12, with 447LacZ [diagram 8] as the control for this series) and also in the gene for the capsid protein (Fig. 1C, diagrams 2 and 3, with Capsid [diagram 1] as the control). The levels of the chimeric proteins and capsid proteins were greatly reduced when the deletions fell within the proposed hairpin (Fig. 2A, bars 9 and 10, and Fig. 2B, bars 2 and 3).

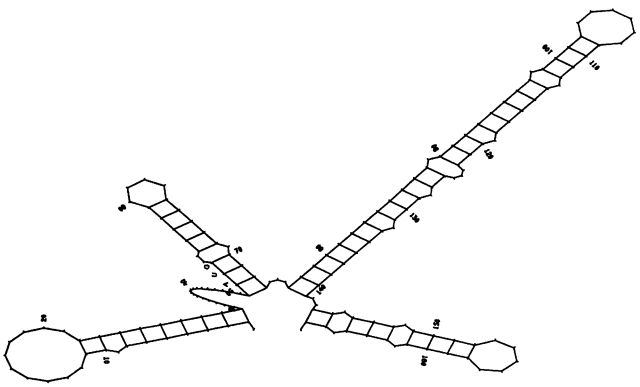

FIG. 4. The optimal secondary structure of the first 169 nt of the Sindbis virus subgenomic RNA predicted by using the energy minimization program of Jaeger et al. (11). The predicted free energy of this structure is −60.6 kcal/mol; the predicted free energy for the sequences from 76 to 142 (the long hairpin-like structure) is −41.3 kcal/mol.

The deletion from nt 178 to 271 (SINrep/447Δ178-271LacZ) led to about a two-fold decrease in the level of the chimeric protein. The sequences between nt 178 and 271 do not have a predicted stem structure (data not shown). Although they could be directly involved in the enhancement of translation, it seems more likely that these downstream sequences play a role in stabilizing the major base-paired, hairpin-like structure.

We also made a mutation in the capsid protein gene to disrupt the stem structure which includes the initiating AUG codon and is immediately upstream of the long hairpin. This mutation which changed nt 70 to 74 from CATGC to TAAAT had only a marginal effect in the ability of the mRNA to be translated (Fig. 2B, bar 1a).

Effects of the translational enhancement sequences under other conditions. We used three different approaches to determine if this enhancement of translation was due to an intrinsic property of the RNA sequence or if it was a phenomenon confined to infected cells.

(i) Translation in uninfected cells. We examined the translation of two of the RNAs in uninfected cells by placing the cDNAs under the control of the CMV promoter. One of the cDNAs had only 80 nt and the other had 447 nt of the Sindbis virus subgenomic RNA fused to *lacZ* (Fig. 5A). BHK cells were transfected with the plasmids, and 40 h later, the cells were labeled with [^{35}S]methionine. In contrast to our previous results, in uninfected cells the protein derived from the 447 LacZ mRNA was present at lower levels than the one translated from the 80LacZ mRNA (Fig. 5B). To establish that this difference was an effect on translation and not on transcription, we compared the levels of mRNAs in the cells 40 h posttransfection by Northern (RNA) blot analysis. The RNAs were detected with a probe specific for the *lacZ* mRNAs. The blots showed that both mRNAs were present at essentially the same concentrations in the two different transfected cells (Fig. 5C, lanes 1 and 2). RNAs isolated from cells infected with SINrep/447LacZ and SINrep/80LacZ were included as controls (lanes 4 and 5).

(ii) Translation at early times postinfection. The results with uninfected cells provided evidence that enhanced translation required infected cells. To obtain further support for this contention, we examined translation of these mRNAs in the context of the Sindbis virus subgenomic RNA as a function of time after infection. At early times after infection, host cell proteins were still being synthesized, and for this reason samples were labeled with [^{35}S]methionine and immunoprecipitated before analyzing them by acrylamide gel electrophoresis (Fig. 6). At the first time point, 1 h postinfection, neither protein was detected. By 2 h postinfection, however, the 80LacZ protein, but not the 447LacZ protein, could be detected. By 4 h postinfection, the amount of the 447LacZ protein had surpassed that of the 80LacZ protein. The effect of these sequences downstream of the initiating AUG at early times after infection was similar to what was seen in uninfected cells—their presence was detrimental to translation.

(iii) Coinfection and competition. The third approach we used was to determine what happened when the different Sindbis virus subgenomic mRNAs were translated in the same cell. As shown in Fig. 7 when BHK cells were coinfected with SINrep/80LacZ and SINrep/447LacZ, the level of the 447LacZ

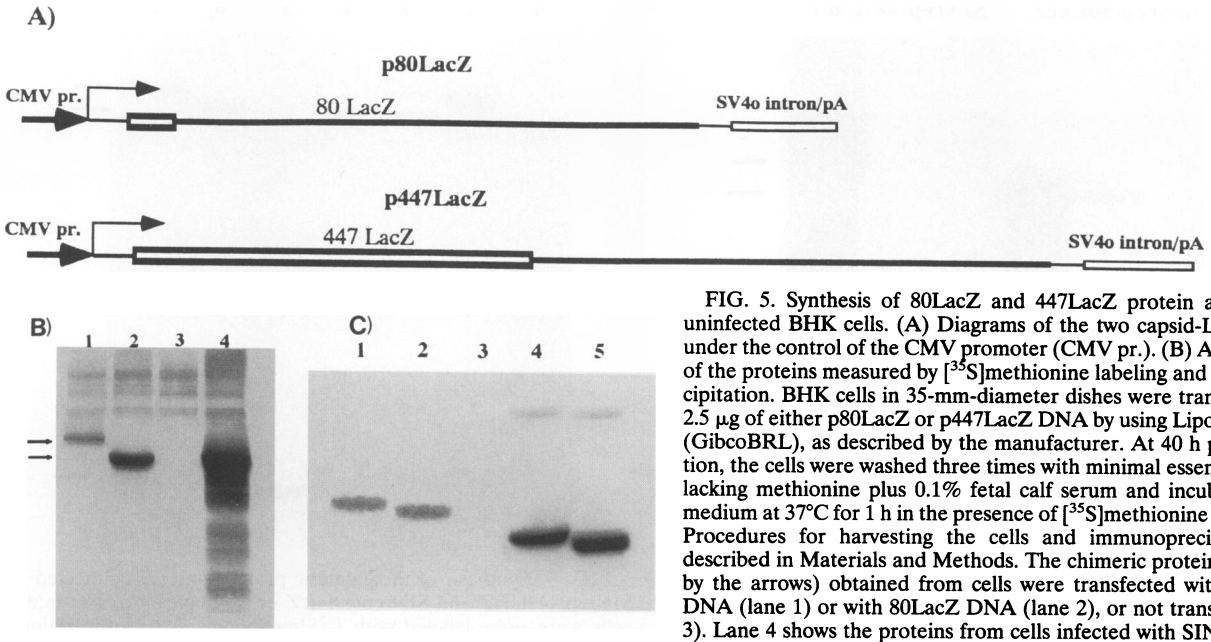

FIG. 5. Synthesis of 80LacZ and 447LacZ protein and RNA in uninfected BHK cells. (A) Diagrams of the two capsid-LacZ cDNAs under the control of the CMV promoter (CMV pr.). (B) Accumulation of the proteins measured by [^{35}S]methionine labeling and immunoprecipitation. BHK cells in 35-mm-diameter dishes were transfected with 2.5 μg of either p80LacZ or p447LacZ DNA by using LipofectAMINE (GibcoBRL), as described by the manufacturer. At 40 h posttransfection, the cells were washed three times with minimal essential medium lacking methionine plus 0.1% fetal calf serum and incubated in this medium at 37°C for 1 h in the presence of [^{35}S]methionine (60 μCi/ml). Procedures for harvesting the cells and immunoprecipitation are described in Materials and Methods. The chimeric proteins (indicated by the arrows) obtained from cells were transfected with p447LacZ DNA (lane 1) or with 80LacZ DNA (lane 2), or not transfected (lane 3). Lane 4 shows the proteins from cells infected with SINrep/80LacZ incubated for 1 h with [^{35}S]methionine 4 h postinfection. (C) Northern blot analysis of RNA isolated from cells transfected with the two capsid-*LacZ* cDNAs under the control of the CMV promoter. The procedures were identical to those described above. RNA was isolated with RNAzol B (see the legend to Fig. 3). RNAs were obtained from cells transfected with p447LacZ DNA (lane 1) or with 80LacZ DNA (lane 2) or not transfected (lane 3). The RNAs transcribed from the CMV promoter are larger than the subgenomic RNAs synthesized in cells infected with SINrep/447LacZ (lane 4) or SINrep/80LacZ (lane 5) because of additional sequences at the 3′ terminus. Exposure of the film for lanes 1, 2, and 3 was 12 times longer than for lanes 4 and 5.

protein was considerably lower than in cells infected only with SINrep/447LacZ (compare lanes 2 and 3). In contrast, the level of the 80LacZ protein was not affected by coinfection (compare lanes 3 and 4). The results obtained when the coinfecting particle was SINrep/capsid were similar, although perhaps not quite so dramatic. The level of the capsid protein, but not the SINrep/80LacZ protein, was diminished when cells were coinfected with SINrep/capsid and SINrep/80LacZ (compare lanes 5 and 6). In cells coinfected with SINrep/447LacZ and SINrep/capsid, both proteins were affected, but the effect was small and probably equal.

These three different studies establish that the sequences downstream of the initiating AUG enhanced translation but only in infected cells. These same sequences had the opposite effect in uninfected cells and during the early stage of infection where their presence decreased the level of translation.

DISCUSSION

The ability of a viral mRNA to be translated in infected cells under conditions in which host cell protein synthesis is inhibited is a well-known phenomenon that has received much attention, but in many respects it remains an enigma (5, 12, 14, 23). In several systems, it has been shown that the concentration of one or more translation initiation factors is decreased after viral infection (13, 24). It has been well documented that p220, the γ-subunit of the eucaryotic initiation factor eIF-4F is destroyed during infection of HeLa cells by poliovirus (23, 24, 31). This factor is required for the translation of capped mRNA. Its inactivation should not affect the translation of poliovirus mRNA which is not capped. eIF-4F is not the only initiation factor altered in poliovirus-infected cells. The activity of eIF-2α is also inhibited (2, 20) and is essential for GTP-dependent $tRNA^{Met}$ binding to 40S ribosomes (10), a step also required for translation of poliovirus mRNA. Infection of cells by many different viruses can activate the p68 protein kinase which phosphorylates eIF-2α, leading to its inactivation. The inactivation is not total, and viral mRNAs must be able to use the limiting concentrations of eIF-2α to be translated. Some viruses have devised ways to modulate the inactivation (13, 19, 23). For example, cells infected with influenza virus induce a cellular protein which is able to interfere with the activity of the protein kinase (16). Under conditions in which this factor is limiting, influenza virus mRNAs are translated more efficiently than cellular mRNAs (7, 8). One mechanism for discriminating between viral and cellular mRNAs is that viral mRNAs have higher affinities either for initiation factors or elongation factors or both and therefore are translated more efficiently when these factors become limiting.

Infection of many different cultured vertebrate cells by alphaviruses results in the inhibition of host cell protein synthesis (12, 29). The shutoff occurs in cells infected with viral replicons that do not synthesize any viral structural proteins, implicating the nonstructural proteins and viral RNAs in the inhibitory mechanism (6, 17). Van Steeg et al. had concluded from their studies that host cell protein synthesis was modified by the level of initiation factors required for translation (27, 28). Using in vitro assays for protein synthesis, they showed that the translation of both host mRNA and Semliki Forest virus genomic RNA was inhibited when the soluble fractions (ribosomal washes) were obtained from virus-infected cells. Translation of the viral 26S subgenomic RNA was not significantly affected. Inhibition was partially relieved by the addition of eIF-4B to the in vitro system (27, 28). More recently, the in vitro translation studies were carried out with chimeric mRNAs containing the chloramphenicol acetyltransferase-coding sequences downstream of the 5′ nontranslated sequences of either the genomic (42S) or 26S RNAs of Semliki Forest virus (1). These more-recent studies provided further

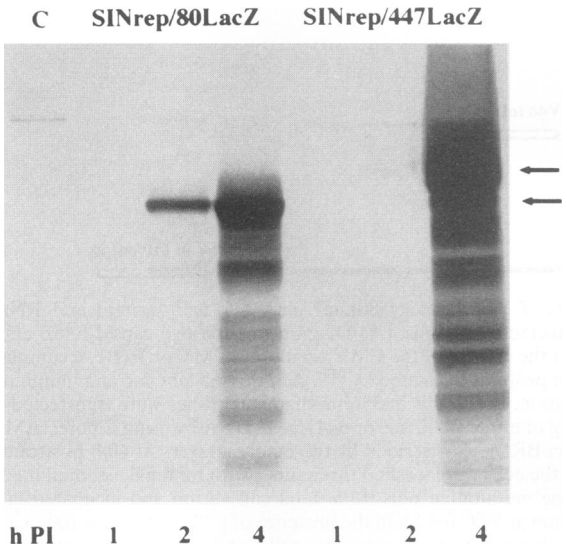

FIG. 6. Appearance of the 80LacZ and 447LacZ proteins at early times after infection. BHK cells infected with either the SINrep/80LacZ or the SINrep/447LacZ packaged replicons were incubated for 30 min with [^{35}S]methionine (50 μCi/ml) at the times indicated below the gel (in hours postinfection [h PI]). The procedures for harvesting the cells and immunoprecipitation are described in Materials and Methods. The lane marked C contains uninfected cells. On the right, the lower arrow identifies the 80LacZ protein, and the upper arrow identifies the 447LacZ protein. The protein bands below the chimeric β-galactosidase bands at the 4-h time point are most likely premature terminations (or degradation products) of β-galactosidase. They are specifically immunoprecipitated with the anti-β-galactosidase serum.

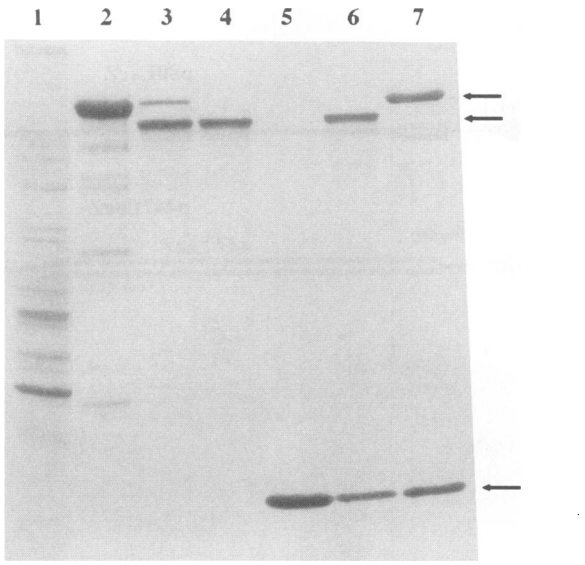

FIG. 7. Synthesis of the chimeric proteins in cells coinfected with SINrep/447LacZ and SINrep/80LacZ or SINrep/capsid. The infected cells were pulse-labeled with [^{35}S]methionine 8 h postinfection as described in Materials and Methods. Lane 1 shows the pattern obtained with uninfected cells. Cells were infected with SINrep/447LacZ (lane 2), SINrep/447LacZ and SINrep/80LacZ (lane 3), SINrep/80LacZ (lane 4), SINrep/capsid (lane 5), SINrep/capsid and SINrep/80LacZ (lane 6), and SINrep/capsid and SINrep/447LacZ (lane 7). The protein bands below the chimeric β-galactosidase band in lane 2 are related to β-galactosidase, since they immunoprecipitated with specific antiserum (Fig. 6).

support for the conclusion that the nontranslated region of the viral subgenomic RNA is responsible for the efficient translation of this RNA. Their data also show that the 26S chimeric RNA interacts much more efficiently than the corresponding chimeric 42S RNA with initiation factors eIF-4E and eIF-4B.

Our studies reported here demonstrate that sequences downstream of the translation-initiating AUG codon can also affect translation: they enhance translation in infected cells but not in uninfected cells. Furthermore, translation of this RNA in infected cells was suppressed by a second viral RNA lacking the hairpin-like structure, but translation of the latter RNA was not affected in this setting. The enhancing sequences are predicted to form a hairpin-like structure with a free energy value of −41.3 kcal/mol.

A plausible explanation for our results derives from Kozak's studies on the effects of a hairpin structure located downstream of an initiating codon on the recognition of that codon (15). Using an in vitro translation system, she analyzed a series of mRNAs in which the first AUG was in a suboptimal context for initiation. A hairpin structure was placed at different distances between this AUG and the second AUG which was in an optimal context. When the hairpin was very close to the first AUG (2 nt) or remote (32 nt), ribosomes frequently initiated at the second AUG. When the hairpin was located 14 nt downstream of the first AUG, almost all of the translation initiated at this codon. Fourteen nucleotides corresponds to the approximate distance between the 3′ leading edge of the ribosome and its AUG recognition center. The interpretation was that the hairpin caused the 40S ribosome to pause at the upstream AUG, giving the ribosome additional time to initiate at that codon.

The observation that a hairpin structure downstream of the initiating AUG codon affects initiation and the evidence that initiation factors are limiting in alphavirus-infected cells serve as the basis for our model which does not, however, clearly distinguish between effects on initiation of translation and on those which would affect elongation. We assume that the hairpin-like structure downstream of the AUG in the Sindbis virus mRNA is a barrier to the movement of the ribosome and that this pause may become the rate-limiting step in translation. In infected cells, under conditions in which this mRNA is present in abundance and is essentially the only RNA being translated, pausing gives those factors present at low concentrations a chance to bind to the translation complex, permitting a high level of functional complexes to be formed. A similar viral mRNA lacking the hairpin-like structure will form fewer functional translation complexes. It will still be translated relatively efficiently because of its 5′ nontranslated sequences (1), and because it is present at a high concentration, a considerable amount of protein is produced (4, 17, 32). In infected cells translating the two different viral mRNAs (Fig. 7), only translation of the mRNA with the hairpin-like structure is suppressed. A slowdown in the movement of the ribosomes along the RNA is no longer an advantage, because some fraction of the limiting factors is now usurped by the other translation complex. An analogous situation must prevail in uninfected cells where there are many other mRNAs being translated. A rate-limiting pause is an advantage for an mRNA only when there are no other RNA molecules with which it has to compete for translational factors.

Our studies provide further evidence that the structure of a mRNA is an important element in regulating the synthesis of a protein (10, 14), but how that structure may affect the rate of

translation has to be evaluated in the context of the cellular environment existing at the time of translation. The structure at the 5′ terminus can play a critical role in controlling the rate of initiation. Several studies have also shown that ribosomes pause during elongation and the structure of the RNA can modify the rate at which ribosomes traverse the RNA (26, 30). We have demonstrated that translation of an alphavirus mRNA depends not only on the 5′ terminus, as has been emphasized previously, but also on downstream sequences. This observation can be exploited in the alphavirus vectors to increase the yields of heterologous proteins. It will be interesting to learn if there are other viral mRNAs that employ this mechanism to enhance expression.

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