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A Small Yeast RNA Selectively Inhibits Internal Initiation of Translation Programmed by Poliovirus RNA: Specific Interaction with Cellular Proteins That Bind to the Viral 5′-Untranslated Region

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We have purified, sequenced, and prepared a synthetic clone of a small (60-nucleotide) RNA molecule from the yeast *Saccharomyces cerevisiae* that had previously been isolated on the basis of its ability to selectively block the translation of poliovirus mRNA. RNA derived from the clone by transcription with T7 RNA polymerase appears to block translation initiation by internal ribosome entry (cap independent) but does not significantly affect cap-dependent translation. Deletion analysis of the poliovirus 5′-untranslated region (5′-UTR) has shown that yeast inhibitor RNA (I-RNA) requires internal ribosome entry site sequences to inhibit the translation of poliovirus RNA in vitro. Using a bicistronic RNA construct, we show that I-RNA preferentially inhibits translation by internal ribosome entry. Gel retardation and UV cross-linking studies demonstrate that I-RNA specifically binds proteins which interact with RNA secondary structures within the poliovirus 5′-UTR presumably involved in internal initiation. Specifically, purified I-RNA competes with virus RNA structures within the 5′-UTR which bind a cellular protein with an approximate molecular mass of 52 kDa. Finally, when transfected into HeLa cells, I-RNA efficiently inhibits the replication of poliovirus RNA presumably by inhibiting translation of the input virus RNA.

Poliovirus, the prototype member of the *Picornaviridae* family, is a single-stranded, plus-sense RNA virus which multiplies in the cytoplasm of infected cells. The genome RNA is approximately 7,500 nucleotides long and codes for a 250-kDa polyprotein (25, 44). The polyprotein is cleaved by three virus-encoded proteases, resulting in the synthesis of mature virus structural and nonstructural proteins. The unusually long 5′-untranslated region (5′-UTR) of poliovirus RNA (750 nucleotides) is highly structured (1, 48) and contains six to eight upstream AUG codons, none of which appear to be used in translation (36). Translation of the majority of cellular mRNAs proceeds by the binding of ribosomes to the 5′ cap structure followed by the scanning of mRNA until the appropriate AUG is encountered by the ribosome (27). In sharp contrast, the translation of naturally uncapped poliovirus RNA has been shown to be mediated by a mechanism involving the internal entry of ribosomes near the initiator AUG (39). Recent studies have demonstrated that the internal entry of ribosomes requires an element located between nucleotides 320 and 631 within the 5′-UTR of poliovirus RNA (39). This sequence element has been termed the ribosome landing pad or, more generally, the internal ribosome entry site (IRES). Although a number of cellular polypeptides have been implicated in IRES-dependent translation, the precise mechanism(s) of internal initiation of translation remains poorly understood. In addition to poliovirus, many other picornaviruses have been shown to utilize this novel mechanism for the initiation of translation (3–6, 21, 22, 28, 29). The RNA genomes of another picornavirus, hepatitis A, and a flavivirus, hepatitis C, have been shown to utilize internal ribosome entry for translation initiation (16, 26). Two cellular mRNAs, which encode immunoglobulin heavy-chain binding protein (Bip) and the Antennapedia protein of *Drosophila melanogaster*, have also been shown to use internal initiation of translation (30, 34). All picornavirus mRNAs that utilize IRES-dependent translation contain a polypyrimidine tract located at the 3′ border of the IRES sequences within the 5′-UTR. Recent evidence suggests that proper spacing between the polypyrimidine tract and the cryptic AUG at nucleotide 586 of the poliovirus 5′-UTR is important for viral translation (21, 23, 43).

Accurate translation of poliovirus mRNA in rabbit reticulocyte lysate requires HeLa cell proteins, suggesting the involvement of cellular proteins in internal initiation of translation (8, 14). A 50-kDa protein has been shown to interact with the RNA stem-loop structure located between nucleotides 186 and 221 in poliovirus type 1 RNA (33). The physiological significance of this binding is still unclear. Another protein, called p52, present in abundance in HeLa cells compared with rabbit reticulocytes has been found to specifically bind to the stem-loop structure between nucleotides 559 and 624 of type 2 poliovirus (31). The p52 protein appears to be identical to human La autoantigen (32). This nuclear protein, recognized by antibodies from patients with the autoimmune disorder lupus erythematosus, leaches out of the nucleus into the cytoplasm in poliovirus-infected HeLa cells. Cell extracts immunodepleted with La antibodies fail to promote cap-independent translation, and the addition of exogenous purified La protein corrects aberrant translation of poliovirus RNA in reticulocyte lysate which contains little or no p52 (32). UV cross-linking studies have demonstrated that another cellular protein, p57, interacts with the IRES elements of encephalomyocarditis virus (EMCV), foot-and-mouth disease virus, rhinovirus, poliovirus, and hepatitis A virus (5, 7, 9, 23, 24, 29, 40,

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42). It has recently been demonstrated that p57 binding to the IRES of EMCV is identical to that of polypyrimidine tract-binding protein (PTB), which presumably plays a role in nuclear splicing (20). Anti-PTB antibody inhibits the translation of EMCV and poliovirus RNAs, and thus PTB may be be directly involved in IRES-directed translation. In addition, two other cellular proteins with molecular masses of 38 and 48 kDa have previously been shown to specifically interact with RNA structures spanning nucleotides 286 and 456 of poliovirus. These two proteins are reported to be present in higher quantities in HeLa cells than in reticulocyte lysate and appear to be involved specifically in poliovirus translation (15). Another 54-kDa protein cross-links to a region between nucleotides 457 and 626 and is required for the translation of all mRNAs (15). A very recent report suggests a role for a 97-kDa protein in the IRES-dependent translation of human rhinovirus RNA (5). RNA-protein complex formation has also been demonstrated with the regions encompassing nucleotides 98 to 182 and 510 to 629 of poliovirus RNA (12). Taken together, these results suggest that picornavirus translation involves direct interaction between cellular factors and RNA sequences and secondary structures that leads to internal initiation. *trans*-acting proteins could direct ribosomes to enter the mRNA or could alter the RNA structure to facilitate ribosome binding.

In a previous study, we showed that yeast cells are incapable of translating poliovirus RNA both in vivo and in vitro and that this is a consequence of the 5′-UTR of virus RNA (10). It was found that the inhibitory effect was due to a *trans*-acting factor present in yeast lysate that can also inhibit the ability of HeLa cell extracts to translate poliovirus RNA. Initial characterization of this inhibitor showed that it binds to DEAE-Sephacel and elutes at 1 M potassium acetate. Its activity was heat stable and resistant to proteinase K digestion, phenol extraction, and DNase digestion. Interestingly, the inhibitor lost its activity when digested with RNase, suggesting RNA as the nature of the inhibitor (10).

Here we report the purification and nucleotide sequence of inhibitor RNA (I-RNA) from the yeast *Saccharomyces cerevisiae*. A synthetic oligonucleotide representing this sequence has been cloned. In vitro-expressed RNA from this clone appears to inhibit internal initiation but not cap-dependent translation. Using poliovirus 5′-UTR deletion mutants, we localized the sequences necessary for translation inhibition by this RNA to a fragment containing the IRES elements. UV cross-linking results demonstrate that the I-RNA derived from yeast binds to a number of cellular proteins believed to be involved in the internal initiation of translation. I-RNA competes with the 5′-UTR of poliovirus RNA for p52 protein and selectively inhibits the translation of virus mRNA without affecting host cell protein synthesis. The interaction between I-RNA and the protein factors needed for internal initiation appears to be due to the secondary structure of I-RNA, which closely resembles that of fragments of poliovirus 5′-UTR. Additionally, we report here that when transfected into HeLa cells, I-RNA efficiently inhibits the replication of poliovirus RNA presumably by blocking translation of the input virus RNA.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in spinner culture in minimal essential medium (GIBCO Laboratories) supplemented with 1 g of glucose per liter and 6% newborn calf serum. HeLa cell monolayers were grown in tissue culture flasks in minimal essential medium (GIBCO) supplemented with 5% fetal bovine serum. The *Saccharomyces cerevisiae* strain used, ABYSI (**a** *pra1 prb1 prc1 cps1 ade*), was kindly provided by D. Meyer (University of California, Los Angeles).

Extract preparation and in vitro translation. HeLa cell extracts were prepared as previously described (10, 46). In vitro translation under HeLa cell extract translation conditions was performed essentially as described elsewhere (46). Two micrograms of each mRNA was used with 80 μg of HeLa cell extract in a 25-μl reaction mixture in the presence of 25 μCi of [^{35}S]methionine (800 Ci/mmol; Amersham) and 40 U of RNasin (Promega).

Purification of yeast I-RNA. Yeast cell lysates were prepared as described previously (47) except that they were not treated with micrococcal nuclease. Lysates were loaded onto a DEAE-Sephacel (Pharmacia) column at 0.1 M potassium acetate and step eluted with buffers containing 0.3, 0.6, and 1 M potassium acetate. Fractions were dialyzed back to 0.1 M salt and assayed for translation-inhibitory activity. The 1 M fraction which showed inhibitory activity was subjected to DNase treatment and then to proteinase K digestion and phenol-chloroform extraction. RNA from this fraction was then isolated by alcohol precipitation. Yeast RNAs that copurified with the 1 M fraction were dephosphorylated and then 5′ end labeled by kinase reaction. Labeled RNA species were separated on a 20% acrylamide–8 M urea sequencing gel. Labeled and cold RNA bands run in parallel lanes were eluted from the gel as follows. Individual gel slices were soaked in 500 μl of elution buffer (2 M ammonium acetate and 1% sodium dodecyl sulfate [SDS]) at 37°C for 4 h. After brief centrifugation at room temperature, the supernatant was collected, extracted with phenol-chloroform (1:1), and alcohol precipitated in the presence of 20 μg of glycogen (Boehringer Mannheim Biochemicals). Precipitated RNA pellets were resuspended in nuclease-free water and tested for the ability to inhibit translation of P2CAT RNA (10) in a HeLa cell-free translation system.

I-RNA sequencing. Sequencing of yeast I-RNA was performed with an RNA sequencing kit from U.S. Biochemicals Corporation. End-labeled RNA was mixed with a base-specific ribonuclease-buffer combination (following the U.S. Biochemicals protocol), incubated at 50°C, and then loaded onto a 20% acrylamide–8 M urea sequencing gel.

Cloning of I-RNA. On the basis of the RNA sequence, a 60-nucleotide synthetic oligonucleotide was constructed. The sense- and antisense-strand-specific oligonucleotides were annealed and then cloned into the pGEM 3Z expression vector (Promega) between the *Hin*dIII and *Eco*RI sites in the polylinker region. The recombinant plasmid was named pSDIR (see Fig. 1).

In vitro transcription. mRNAs were transcribed in vitro by using T7 or SP6 promoters from different linear plasmids with T7 or SP6 RNA polymerases. The clone pSDIR was linearized with *Hin*dIII restriction enzyme and then transcribed with T7 RNA polymerase to generate I-RNA (sense transcript). Plasmids pG3CAT and P2CAT (10) and deletion mutants (see Fig. 2 and reference 37) were linearized with *Bam*HI, and runoff transcripts were generated with SP6 RNA polymerase. The plasmid pBIP-LUC construct was a gift from P. Sarnow (30) and was linearized with *Hpa*I enzyme and transcribed with T7 RNA polymerase. The poliovirus type 2 IRES-containing bicistronic construct pGEM CAT/p2-5′/Luc was a gift from N. Sonenberg and was linearized with *Xho*I before transcription with T7 RNA polymerase. Poliovirus 5′-UTR and the fragment spanning nucleotides 320 to 461 were subcloned between the *Hin*dIII and *Eco*RI sites of the pGEM 3Z vector (Promega). Both plasmids were linearized with *Hin*dIII and transcribed with T7 RNA polymerase.

Oligonucleotide templates. Oligodeoxyribonucleotide templates for transcription by T7 RNA polymerase were synthesized on an Applied Biosynthesis DNA synthesizer and then purified. Equimolar amounts of the 18-mer T7 primer oligonucleotide and template oligonucleotides were mixed in 0.1 M NaCl and annealed by heating at 100°C for 5 min and then slowly cooling to room temperature. The stem-loop B (SL-B), SL-C, and SL-G RNAs were synthesized in vitro following the method described above.

RNA transfection. Poliovirus RNA (type 1 Mahoney) was isolated from infected HeLa cells as described elsewhere (11). Synthetic I-RNA or poliovirus RNA was mixed with carrier yeast tRNA to yield a total of 20 μg of RNA per transfection reaction. RNA samples were then mixed with 30 μg of Lipofectin (GIBCO-BRL) and 20 U of RNasin (Promega) and incubated for 30 min at room temperature. Finally, the samples were mixed with 4 ml of minimal essential medium (GIBCO) containing 2.5% fetal bovine serum and were added to petri dishes containing 70 to 80% confluent HeLa monolayer cells. Cells were then incubated at 37°C in a CO_2 incubator for 24 h.

In vivo labeling and immunoprecipitation. For in vivo labeling of proteins after transfection, cells were preincubated in methionine-free medium (GIBCO) for 40 min at 37°C. Then 100 μCi of *trans*-labeled methionine (specific activity, >1,000 Ci/mmol) was added to each plate, and incubation was continued for another hour. [^{35}S]methionine-labeled HeLa cell extract was prepared as described previously (45). In vivo-labeled viral proteins in transfected cells were detected by immunoprecipitation with poliovirus anti-capsid antibody (purchased from American Type Culture Collection). Immunoprecipitations were performed overnight at 4°C with 5 μl of anti-capsid antibody in a 500-μl reaction volume containing 1× RIPA buffer (5 mM Tris [pH 7.9], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). Immune complexes were precipitated with protein A-Sepharose (75 μl of a 20% solution in RIPA buffer plus 0.2% bovine serum albumin) and then analyzed on an SDS–14% polyacrylamide gel as described previously (10).

Mobility shift electrophoresis assay. HeLa S10 cytoplasmic extract was prepared by collecting the supernatant after centrifugation of the HeLa cell translation extract at 10,000 × *g* for 30 min at 4°C. Fifty micrograms of S10 extract was preincubated at 30°C for 10 min with 4 μg of poly(dI-dC) (Pharmacia) in a 15-μl reaction mixture containing 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 25 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 3.8% glycerol, and 2 mM dithiothreitol. For competition experiments, 25- to 100-fold excesses of unlabeled competitor RNAs were added to the reaction and incubated for 10 min at 30°C. Finally, 5 to 10 fmol of labeled RNA probe was added to respective reaction mixtures and incubation was continued for another 30 min at 30°C. The nonspecific RNA used in competition assays was the sequence of the polylinker region (*Eco*RI to *Hin*dIII) of the pGem 3Z vector (Promega). Three microliters of gel loading dye was added to the reaction mixture to a final concentration of 10% glycerol and 0.2% each of bromophenol blue and xylene cyanol. RNA-protein complexes were then analyzed on a 4% polyacrylamide gel (39:1 ratio of acrylamide:bis) in 0.5× TBE.

UV-induced cross-linking. Forty to fifty fmol of ^{32}P-labeled RNA probes (8×10^4 cpm) was incubated with 50 to 100 μg of S10 extract of HeLa cells as described above. After the binding reaction was complete, samples were irradiated with UV light from a UV lamp (multiband UV, 254/366NM model U GL; 25UVP, Inc.) at a distance of 3 to 4 cm for 10 min. Unbound RNAs were then digested with a mixture of 20 μg of RNase A and 10 U of RNase T_1 at 37°C for 30 min. Products were then analyzed on SDS–14% polyacrylamide gels.

Nucleotide sequence accession number. The nucleotide sequence for yeast I-RNA has been submitted to the EMBL database library under accession number X76506.

RESULTS

Purification of yeast I-RNA. The inhibitor from yeast cells capable of specifically inhibiting IRES-dependent translation from poliovirus RNA was initially purified by passage through a DEAE-Sephacel column (10). The inhibitor bound strongly to the column and was eluted by washing the column with 1 M potassium acetate. Previous results from our laboratory showed that the activity of the inhibitor was not sensitive to proteinase or DNase treatments or to phenol extraction (10). Thus, further purification of the DEAE-Sephacel-purified inhibitor involved DNase and proteinase K digestion followed by phenol-chloroform extraction. Finally, RNA obtained by alcohol precipitation of the aqueous phase was labeled with [γ-^{32}P]ATP at the 5′ end and RNA bands were resolved by 20% polyacrylamide–8 M urea gel electrophoresis. Each RNA band was eluted from gel slices and was assayed for its ability to inhibit IRES-mediated translation from a poliovirus 5′-UTR-chloramphenicol acetyltransferase (CAT) construct but not from a CAT construct known to initiate translation in a cap-dependent manner (38). A single band which migrated as a 60-nucleotide RNA was associated with inhibitory activity. This RNA was sequenced with a commercially available sequencing kit. Figure 1A shows the sequence of the 60-nucleotide RNA. On the basis of the RNA sequence, sense- and antisense-strand-specific deoxyoligonucleotides were synthesized, annealed, and then cloned into the pGEM 3Z expression vector between the *Hin*dIII and *Eco*RI sites in the polylinker region. The recombinant plasmid was named pSDIR (Fig. 1B). Transcription by T7 RNA polymerase from the linearized plasmid resulted in the synthesis of I-RNA. When analyzed by gel electrophoresis, a single RNA band of approximately 71 nucleotides was detected (Fig. 1C). This consisted of the 60-nucleotide yeast I-RNA as well as 10 extra nucleotides with the *Eco*RI site from the 5′ polylinker region and 1 nucleotide at the 3′ end from the *Hin*dIII site.

To determine whether RNA synthesized from the synthetic clone was active, its effect on translation from a CAT construct containing poliovirus 5′-UTR at the 5′ end of the CAT gene (P2CAT) was determined. Both partially purified inhibitor from yeast and purified inhibitor transcribed from pSDIR inhibited translation from the P2CAT RNA in vitro in a HeLa cell extract (Fig. 1D, lanes 4, 5, and 6). However, translation from CAT RNA (cap-dependent translation) was not significantly inhibited by the presence of these inhibitors (Fig. 1D, lanes 1, 2, and 3). Quantitation of CAT translation by densitometry showed no significant inhibition from the pG3CAT construct (Fig. 1D, lanes 2 and 3), while P2CAT translation was inhibited 40 and 60% (lanes 5 and 6), respectively. Thus, I-RNA synthesized from the synthetic clone was active in specifically inhibiting poliovirus IRES-dependent translation as previously found with partially purified inhibitor from yeast cells (10).

Poliovirus 5′-UTR sequences required for inhibition by yeast I-RNA. To determine whether specific sequences within the 5′-UTR of poliovirus RNA are required for yeast I-RNA to inhibit IRES-dependent translation, a number of deleted 5′-UTR-CAT constructs were obtained from the laboratory of

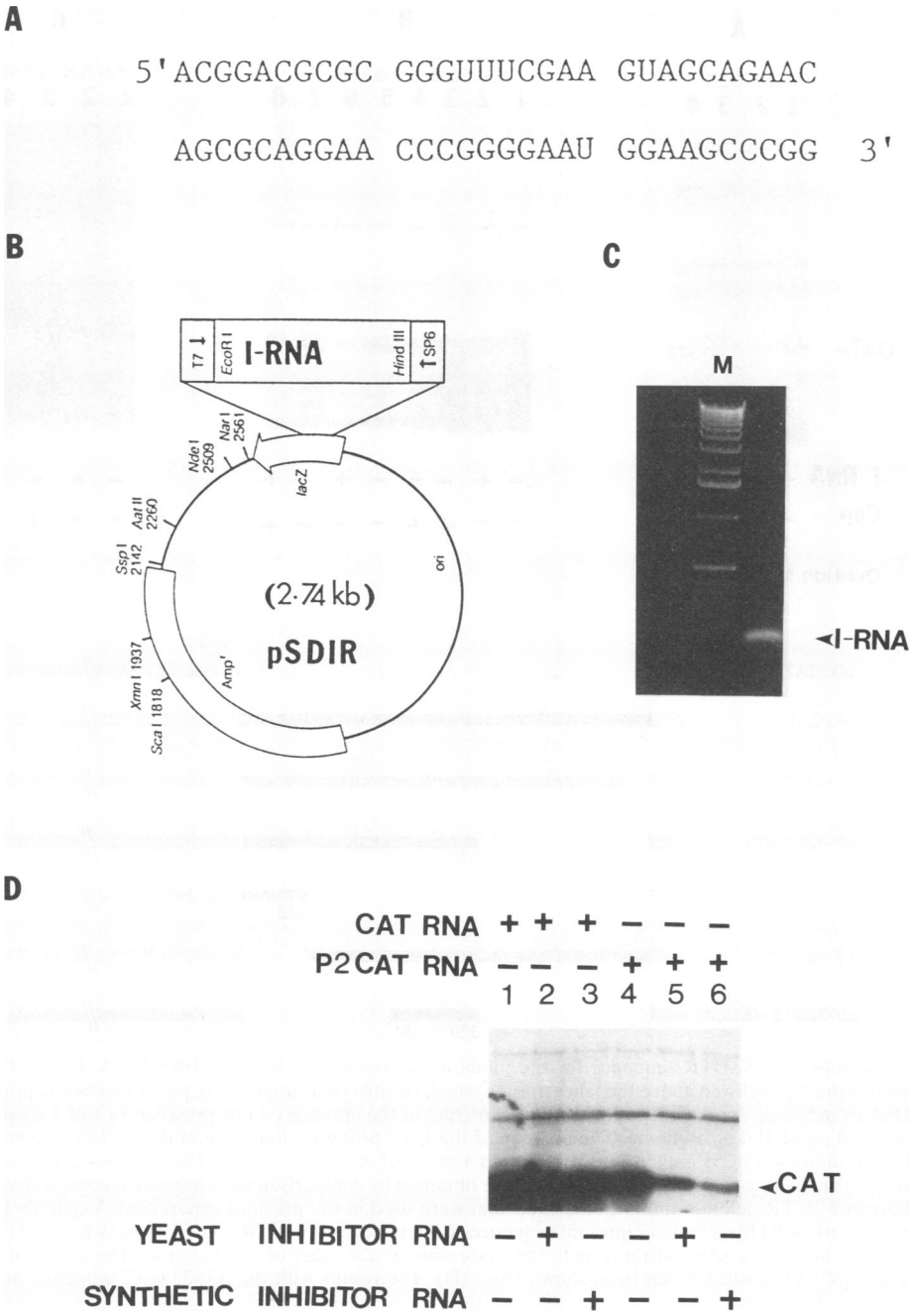

FIG. 1. Nucleotide sequence, cloning, expression, and activity of yeast I-RNA. (A) Sequence of the 60-nucleotide purified yeast I-RNA was determined as described in Materials and Methods. 5′ and 3′ termini are indicated. (B) Illustration of the pSDIR plasmid expressing I-RNA. The positions of the *Hin*dIII and *Eco*RI restriction endonuclease sites are shown. T7 and SP6, the locations of the respective promoters; arrows show the direction of transcription. (C) I-RNA (sense transcript) was transcribed in vitro with T7 RNA polymerase from plasmid pSDIR linearized with *Hin*dIII restriction enzyme. Four micrograms of synthesized RNA was mixed with denaturing gel loading dye (U.S. Biochemicals), heated at 55°C for 10 min, and then analyzed on a 1.2% agarose gel along with the 1-kb ladder DNA marker (BRL) (lane M) under conditions for the analysis of DNA samples. The position of the I-RNA band is indicated. (D) In vitro translation of pG3CAT and P2CAT RNA in HeLa cell translation lysates was performed in the absence (−) or presence (+) of I-RNA. Two micrograms of pG3CAT RNA or P2CAT RNA was added per reaction containing 80 μg of HeLa cell lysates. Approximately 4 μg of partially purified yeast I-RNA or 1 μg of synthetic I-RNA was used in reactions as indicated. The location of the CAT gene product is shown by an arrowhead.

N. Sonenberg (McGill University). Figure 2D shows the deleted 5′-UTR-CAT constructs used for this purpose. The inhibitor efficiently inhibited translation from the P2CAT construct but not from the pG3CAT (or pCAT) construct (Fig. 1D). Almost complete inhibition was observed when the Δ5′-33/CAT construct was translated in the presence of I-RNA (data not shown). Deletion of the first 320 nucleotides from the 5′ end of the UTR did not have a significant effect on the

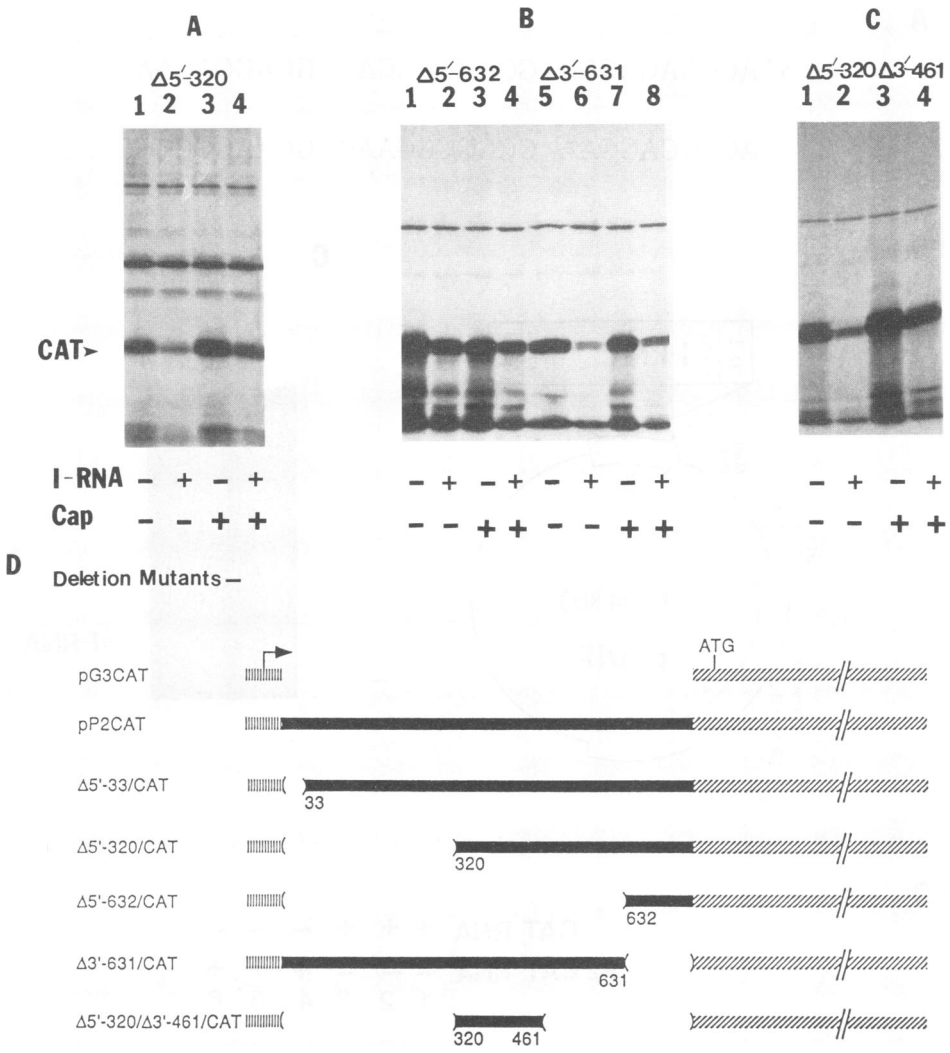

FIG. 2. Requirement of poliovirus 5′-UTR sequence for the inhibition of translation by yeast I-RNA. (A, B, and C) HeLa cell translation lysates were used to translate the RNAs listed above the lanes in each panel. In vitro translations were performed with approximately 2 μg of either capped or uncapped RNA as indicated for each deletion mutant construct in the absence (−) or presence (+) of 1 μg of purified I-RNA. Each reaction mixture contained 80 μg of HeLa cell lysate. The position of the CAT protein is indicated at the far left. Quantitation of band intensity for the CAT protein showed inhibition of 55 and 30% in lanes 2 and 4 of panel A; 10, 15, 74, and 25% in lanes 2, 4, 6, and 8 of panel B; and 78 and 39% in lanes 2 and 4 of panel C, respectively. These values were obtained by comparison with respective controls (without I-RNA). (D) The diagram shows the poliovirus 5′-UTR deletion mutant constructs that were used in the previous experiment. Vertically hatched boxes represent SP6 RNA polymerase promoters, solid black boxes represent sequences from poliovirus 5′-UTR, and diagonally hatched boxes indicate CAT gene coding sequences. A number beneath a plasmid represents the nucleotide at the edge of the deletion. The effect of I-RNA on the in vitro translation of pG3CAT and p2CAT constructs has been shown (Fig. 1D). The results with the Δ5′-33/CAT construct are not shown.

ability of the inhibitor to inhibit translation from the Δ5′-320/CAT construct (Fig. 2A, lanes 1 and 2). Significant inhibition of translation was observed in the presence of the inhibitor (Fig. 2A, lanes 1 and 2). Some of the inhibition observed could be reversed when template RNA was capped prior to translation (Fig. 2A, lanes 3 and 4). A similar result was obtained with the Δ3′-631/CAT construct; almost 75% inhibition of translation from this construct was observed in the presence of the inhibitor and the addition of capped RNA reversed the inhibition of translation to only 25% compared with that of the control (Fig. 2B, lanes 5 to 8). In contrast, translation from the Δ5′-632/CAT construct was almost unaffected by the presence of the inhibitor (Fig. 2B, lanes 1 and 2). Thus, almost the entire IRES region of virus RNA (nucleotides 320 to 631) was required for the inhibitor to efficiently inhibit poliovirus IRES-dependent translation. A very interesting observation was that translation from the construct Δ5′-320/Δ3′-461/CAT, containing only nucleotides 320 to 461 of the UTR, was significantly inhibited by I-RNA (Fig. 2C, lanes 1 and 2). This inhibition can be overcome to a significant extent by capping RNA prior to translation (Fig. 2C, lanes 3 and 4). The stimulation of translation by capping deleted RNAs appears to be due to cap-dependent translation as cap analogs inhibit stimulated translation (data not shown). Although almost complete inhibition of IRES-dependent translation by the inhibitor required almost all of the UTR, significant inhibition was observed with a construct containing only nucleotides 320 to 461 of the virus UTR. Because capping RNAs prior to translation prevented

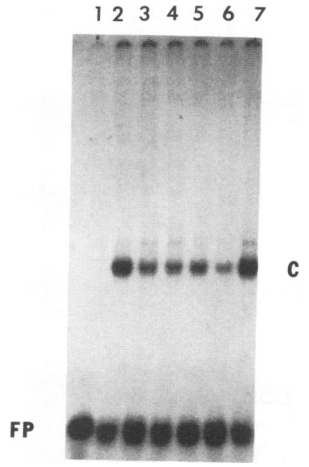

FIG. 3. I-RNA forms a gel-retarded complex which is inhibited by 5′-UTR of poliovirus RNA. ^{32}P-labeled I-RNA was incubated with HeLa S10 extract in the absence (lane 2) or presence of an unlabeled 25- or 50-fold molar excess of I-RNA (lanes 3 and 4, respectively) or 5′-UTR (lanes 5 and 6, respectively) or of a 50-fold molar excess of nonspecific RNA (lane 7). Protein-RNA complexes were analyzed on a nondenaturing gel. In lane 1, S10 extract was not added, i.e., free probe (FP). C, complex C.

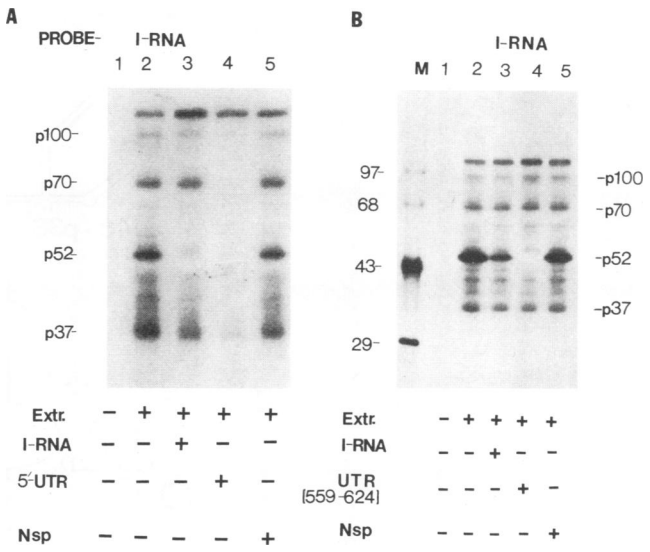

FIG. 4. I-RNA binds proteins that interact with poliovirus 5′-UTR. UV cross-linking of ^{32}P-labeled I-RNA with HeLa cell proteins was performed as described in Materials and Methods. (A) The numbers to the left refer to the approximate molecular masses of the proteins that interact with I-RNA. For competition studies, 100-fold molar excess of unlabeled competitor RNA was added to the binding reaction. The competitor RNAs used were I-RNA (lane 3), 5′-UTR (lane 4), and nonspecific (Nsp) RNA (lane 5). (B) The numbers to the left refer to the molecular masses of protein markers (BRL) (lane M). The numbers to the right correspond to the molecular masses of proteins which cross-link to the labeled I-RNA probe. A 100-fold molar excess of each unlabeled competitor RNA, I-RNA (lane 3), UTR(559-624) RNA (lane 4), and nonspecific (Nsp) RNA (lane 5), was used in binding reactions. Extr., HeLa extract.

the inhibition of translation to a significant extent, cap-dependent translation was probably not affected by the inhibitor, a result consistent with our previous observations (10).

I-RNA forms a gel-retarded complex that is inhibited by UTR. Theoretically, I-RNA could inhibit IRES-dependent translation by two possible mechanisms. It could bind to some sequences in the UTR as antisense RNA or it could bind protein factors needed for the internal entry of ribosomes, thus inhibiting IRES-dependent translation. The possibility that yeast I-RNA acts by binding protein factors required for IRES-dependent translation was plausible for the following reasons. First, purified RNA was not complementary to 5′-UTR sequences nor did it hybridize with virus 5′-UTR (data not shown). Secondly, inhibition of the translation of poliovirus RNA could be overcome by the addition of increasing concentrations of HeLa extract but not of poliovirus RNA or 5′-UTR sequence (10). Therefore, ^{32}P-body-labeled I-RNA probe was prepared and mixed with HeLa S10 extracts, and the resulting RNA-protein complexes were analyzed by nondenaturing polyacrylamide gel electrophoresis. As shown in Fig. 3, lane 2, a single complex (denoted C) was clearly evident. The addition of increasing concentrations of unlabeled I-RNA inhibited the formation of the labeled complex (Fig. 3, lanes 3 and 4). A similar result was obtained when unlabeled poliovirus 5′-UTR was used for competition (Fig. 3, lanes 5 and 6). A nonspecific RNA, however, was not able to compete with labeled I-RNA (Fig. 3, lane 7). Thus, I-RNA was able to form a gel-retarded complex with HeLa S10 proteins(s) that can be specifically competed with by virus 5′-UTR sequences. It should be noted that a significant amount of complex C remained in the presence of cold homologous I-RNA competitor (Fig. 3, lanes 3 and 4). This result is consistent with the UV cross-linking studies discussed below, showing that many of the cross-linked bands could not be specifically inhibited by cold I-RNA competitor. The precise reason for this observation is unclear at present.

I-RNA binds proteins that interact with poliovirus 5′-UTR. To determine if specific polypeptides that interact with virus 5′-UTR also interact with yeast I-RNA, a series of UV cross-linking experiments were performed. In these experiments, body-labeled I-RNA was first incubated with HeLa S10 extract and then cross-linked by using UV light. After ribonuclease treatment, protein-nucleotide complexes were analyzed by SDS-polyacrylamide gel electrophoresis. When ^{32}P-labeled I-RNA was used to cross-link proteins in HeLa extract, a number of polypeptides with approximate molecular masses of 100, 70, 52, and 37 kDa were detected (Fig. 4A and B, lanes 2). The addition of unlabeled I-RNA successfully competed with the 52-kDa band (Fig. 4A, lane 3). It is unclear at present why significant amounts of the 100-, 70-, and 37-kDa bands remain in the presence of unlabeled I-RNA (Fig. 4A, lane 3). When unlabeled poliovirus 5′-UTR was used as a competitor, the 52-kDa band as well as the 100-, 70-, and 37-kDa bands was completely inhibited (Fig. 4A, lane 4). In contrast, the addition of unlabeled nonspecific RNA was unable to inhibit any of the polypeptides cross-linked to I-RNA (Fig. 4A, lane 5). Because a 52-kDa protein has previously been shown to interact with a specific region of virus 5′-UTR (nucleotides 559 to 624), we determined whether an RNA containing this sequence [called UTR(559-624)] was able to inhibit the 52-kDa band cross-linked to labeled I-RNA. As shown in Fig. 4B, lane 4, unlabeled UTR(559-624) completely inhibited the formation of the I-RNA–52-kDa complex. Unlike 5′-UTR, UTR(559-624) only inhibited the 52-kDa band (compare lanes 4 in Fig. 4A and B). These results suggest that yeast I-RNA binds a 52-kDa protein similar to that bound by poliovirus 5′-UTR nucleotides 559 to 624.

Yeast I-RNA competes with SL-G for protein binding.

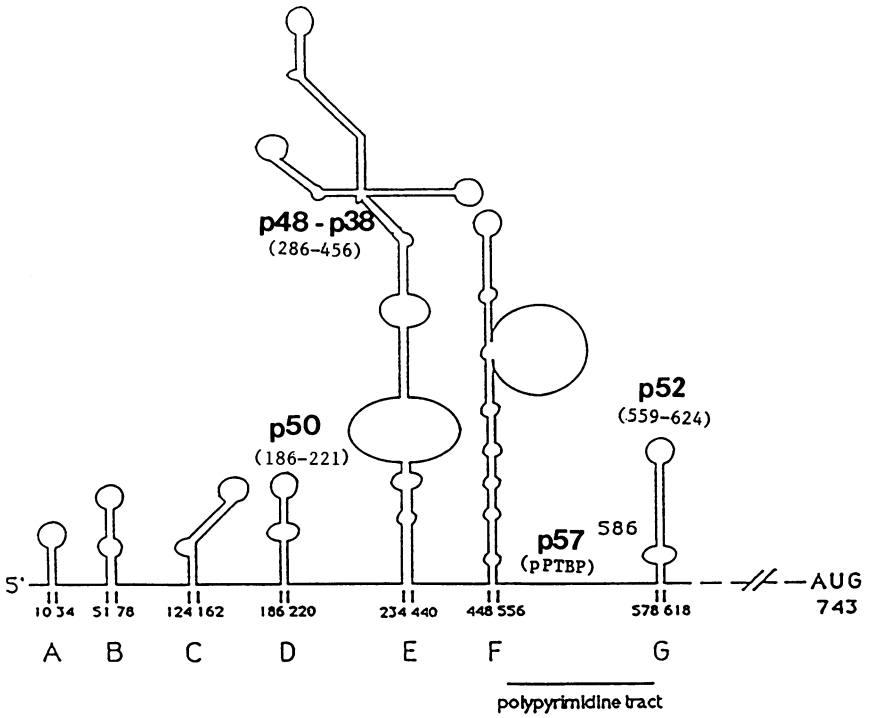

FIG. 5. Predicted secondary structure of 5′-UTR of poliovirus RNA. This figure illustrates the probable interaction of various structured RNA domains with cellular proteins. Molecular masses of the cellular proteins with their possible sites of interaction (indicated by nucleotide numbers within parentheses) are shown. This figure is a modified version of secondary structure predictions by Pilipenko et al. (43), Jackson et al. (21), and Dildine et al. (13).

Poliovirus 5′-UTR contains a number of thermodynamically stable stem-loop structures that are believed to play important roles in virus RNA replication and translation (Fig. 5). Nucleotides 186 to 221, which constitute SL-D, have been shown to bind a 50-kDa protein (p50) (33). SL-G, representing poliovirus 5′-UTR sequences from nucleotides 559 to 624, binds to a 52-kDa protein (p52) which has recently been identified as the human La protein (32). The results presented immediately above suggested that I-RNA interacts with p52, which normally binds to SL-G within the virus 5′-UTR. To confirm that similar proteins may be interacting with I-RNA and SL-G, the following competition experiments were performed. ^{32}P-labeled 5′-UTR was incubated with HeLa S10 extract either alone or in the presence of various unlabeled competing RNAs (e.g., 5′-UTR, SL-G, I-RNA, SL-B, SL-C, and nonspecific RNA). The resulting complexes were then analyzed by UV cross-linking studies. RNA sequences representing SL-B and SL-C were used as negative controls in these experiments as these sequences are not likely to interact with p52. When poliovirus 5′-UTR was used as the labeled probe, almost complete inhibition of the formation of the 52-kDa protein-nucleotidyl complex was observed with unlabeled UTR, I-RNA, and SL-G (Fig. 6A, lanes 3 to 6). Unlabeled SL-B, SL-C, and nonspecific RNA were relatively ineffective in inhibiting the binding of 5′-UTR and p52 (Fig. 6A, lanes 7 to 9). Only unlabeled UTR RNA inhibited the formation of all labeled bands, whereas I-RNA and SL-G specifically inhibited formation of the p52 band.

When SL-G was used as the labeled probe, a doublet migrating to 52 and 54 kDa was detected (Fig. 6B, lane 2). Homologous competition with unlabeled SL-G completely inhibited the formation of these complexes (Fig. 6B, lane 3). Almost 80% inhibition of the formation of these UV cross-linked complexes was observed in the presence of unlabeled I-RNA (Fig. 6B, lane 4). However, no inhibition was observed when nonspecific, SL-B, or SL-C RNA was used as a competitor (Fig. 6B, lanes 5 to 7). Taken together, these results suggest that similar proteins having approximate molecular masses of 52 kDa interact with I-RNA and SL-G.

The results presented above suggested that the inhibition of poliovirus IRES-dependent translation by I-RNA may be mediated at least in part by its interaction with the 52-kDa protein. To investigate how I-RNA inhibits translation from

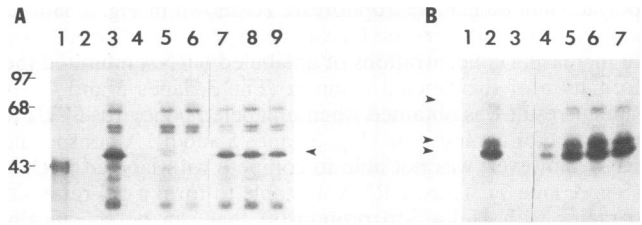

FIG. 6. Competition UV cross-linking studies with ^{32}P-labeled 5′-UTR (A) and UTR(559-624) (B) probes. (A) UV cross-linking experiments were performed in the absence (lane 3) or presence of unlabeled 5′-UTR (lane 4), SL-G [UTR(559-624)] (lane 5), I-RNA (lane 6), SL-B [UTR(51-78)] (lane 7), SL-C [UTR(124-162)] (lane 8), and nonspecific RNA (lane 9). Lane 2 contained the probe but no S10 extract. Lane 1 shows the migration of marker proteins. (B) UV cross-linking was performed in the absence (lane 2) or presence of unlabeled SL-G (lane 3), I-RNA (lane 4), SL-B (lane 5), SL-C (lane 6), and nonspecific RNA (lane 7). Lane 1 contained the probe but no S10 extract.

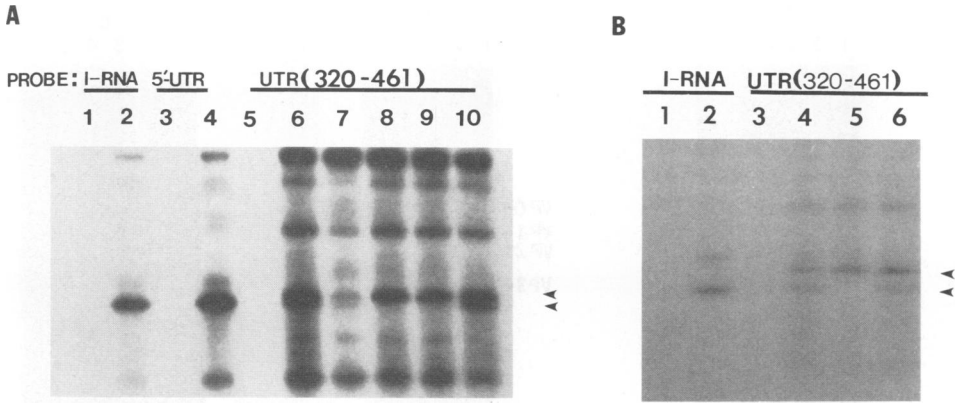

FIG. 7. Competition UV cross-linking studies with ^{32}P-labeled UTR(320-461) probe. (A) ^{32}P-labeled UTR(320-461) probe was incubated with HeLa extracts in the absence (lane 6) or presence of unlabeled 5′-UTR (lane 7), SL-G (lane 8), I-RNA (lane 9), and nonspecific RNA (lane 10). Lane 5 contained the ^{32}P-labeled UTR(320-461) probe but no S10 extract. ^{32}P-labeled I-RNA probe was incubated with (lane 2) or without (lane 1) HeLa S10 extract. ^{32}P-labeled 5′-UTR probe was incubated with (lane 4) or without (lane 3) HeLa S10 extract. (B) ^{32}P-labeled I-RNA (lanes 1 and 2) or UTR(320-461) (lanes 3 to 6) was used for UV cross-linking experiments. Lanes: 1, no extract; 2, HeLa S10 extract; 3, no extract; 4, S10 extract; 5 and 6, same as lane 4 except that unlabeled SL-G RNA or unlabeled nonspecific RNA was used as the competitor, respectively. Arrowheads indicate the positions of p54 and p52.

the Δ5′-320/Δ3′-461/CAT construct (Fig. 2), which does not contain the p52-binding site, an RNA representing poliovirus sequence nucleotides 320 to 461 was used as the probe in UV cross-linking experiments. As shown in Fig. 7A, lane 6, incubation of the labeled probe with HeLa S10 extract resulted in the formation of a number of protein-nucleotidyl complexes. Among these bands was a doublet with approximate molecular masses of 54 and 52 kDa (indicated in the figure by arrowheads). The lower band of the doublet comigrated with protein-nucleotidyl complexes formed with labeled IRNA (Fig. 7A, lane 2) and 5′-UTR (lane 4). To further determine the nature of the lower band of this doublet, competition experiments were performed with unlabeled UTR, I-RNA, and SL-G (the p52-binding site). The addition of unlabeled UTR inhibited the formation of many of the bands except the uppermost and lowermost bands (Fig. 7A, lane 7). In contrast, when unlabeled SL-G (the p52-binding site) was used as the competitor, only the formation of the lower band of the doublet was inhibited (Fig. 7A, lane 8). A similar result was obtained when unlabled I-RNA was used as the competitor (Fig. 7A, lane 9). However, the addition of unlabeled, nonspecific RNA did not result in the disappearance of any complex (Fig. 7A, lane 10). Homologous competition with unlabeled UTR(320-461) produced results similar to those with the whole UTR (Fig. 7A, lane 7, and data not shown). Because the protein-nucleotidyl complexes (54 and 52 kDa) in the doublet were poorly resolved in Fig. 7A, a similar experiment in which the bands were better resolved by running the gel for a longer time was performed (Fig. 7B). Some of the protein-nucleotidyl complexes in Fig. 7B were not present in high quantities in this experiment because a lower concentration of S10 was used here than was used in Fig. 7A. Additionally, the specific activity of the labeled probe in this experiment was much lower than that used in the previous experiment. These results suggest that a polypeptide that interacts with SL-G is also capable of interacting with nucleotides 320 to 461. Additionally, I-RNA is able to compete with UTR(320-461) for binding to this protein.

Yeast I-RNA inhibits IRES-mediated translation in vitro. We previously showed that partially purified yeast I-RNA specifically inhibited the translation of monocistronic RNAs containing poliovirus 5′-UTR. However, cap-dependent translation of either CAT RNA or yeast mRNA was not affected by this inhibitor (10). To examine if this cloned and purified RNA preferentially inhibits internal initiation of translation, its effect on translation from a bicistronic messenger was determined. For this purpose, a bicistronic construct containing CAT and luciferase genes flanked by the poliovirus 5′-UTR was obtained from N. Sonenberg. The initiation of translation occurring internally from the poliovirus 5′-UTR would result in the synthesis of luciferase, whereas cap-dependent translation would normally produce CAT protein. In uninfected

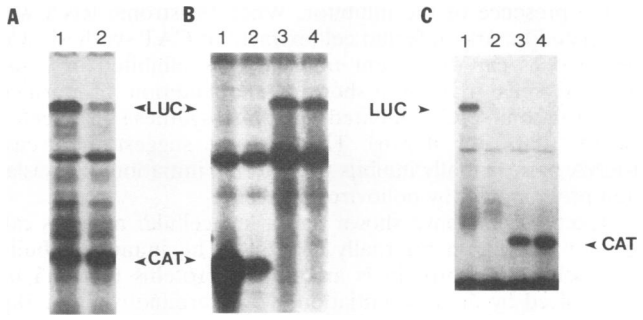

FIG. 8. I-RNA inhibits internal initiation of translation in vitro. (A) A bicistronic construct containing the CAT and luciferase (LUC) genes flanked by type 2 poliovirus 5′-UTR was translated in vitro in HeLa cell lysates in the absence (lane 1) or presence (lane 2) of 1 μg of I-RNA. Products were analyzed on an SDS-14% polyacrylamide gel. (B) p2CAT RNA (lanes 1 and 2) and capped pGEMLuc mRNA (lanes 3 and 4) (2 μg each) were translated in vitro in HeLa cell lysate in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 μg of I-RNA. (C) The pBIP-LUC construct containing the 5′-UTR of Bip mRNA linked to a reporter (LVC) gene was translated in vitro in HeLa cell lysates in the absence (lane 1) or presence (lane 2) of 1 μg of yeast inhibitor. As a control, the pG3CAT construct was also translated in the absence (lane 3) or presence (lane 4) of the yeast inhibitor. Products were analyzed on an SDS-14% polyacrylamide gel. Arrowheads indicate the positions of the LUC gene product and the CAT gene product.

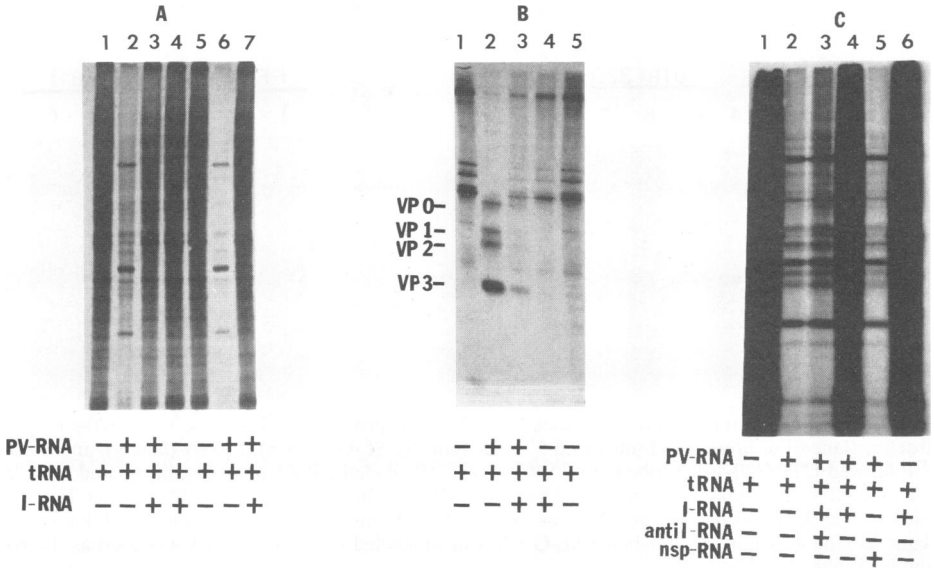

FIG. 9. I-RNA inhibits the translation of poliovirus RNA in vivo. HeLa monolayer cells were transfected with virus RNA alone, I-RNA alone, or virus RNA and I-RNA together. After transfection, cells were labeled with [^{35}S]methionine and in vivo-labeled proteins were analyzed on an SDS-14% polyacrylamide gel either directly (A) or after immunoprecipitation with anti-capsid antibody (B) as described in Materials and Methods. (A) RNAs added to each transfection reaction are indicated. Transfection reaction mixtures contained 1 or 2 μg of poliovirus RNA (PV-RNA) and 3 μg of I-RNA. (B) Immunoprecipitation of the in vivo-labeled proteins from the transfection reactions in lanes 1 to 5 of panel A. The positions of the poliovirus capsid proteins are indicated on the left. (C) PV-RNA (1.5 μg) was transfected either alone or together with I-RNA and/or antisense I-RNA as indicated (3 μg each). The total RNA concentration in each transfection was adjusted to 20 μg by adding carrier tRNA.

HeLa cell extracts, translation from the capped bicistronic message produced both CAT and luciferase proteins (Fig. 8A, lane 1). In the presence of purified yeast inhibitor, significant inhibition of luciferase synthesis was observed, whereas the synthesis of CAT was almost completely unaffected. Quantitation of the labeled CAT and luciferase bands showed that while luciferase synthesis was inhibited by over 70% compared with the control, no inhibition of CAT synthesis was observed in the presence of the inhibitor. When bicistronic RNA was translated in virus-infected cell extract, no CAT synthesis was detected as cap-dependent translation is inhibited in virus-infected cells (data not shown). The addition of purified inhibitor completely inhibited luciferase synthesis in infected extract (data not shown). These results suggest that yeast I-RNA preferentially inhibits the internal initiation of translation programmed by poliovirus 5′-UTR.

Recent results have shown that a few cellular mRNAs can initiate translation internally (30, 34). The immunoglobulin heavy-chain Bip protein is among the proteins that can be synthesized by internal initiation. To determine whether Bip synthesis can be specifically inhibited by I-RNA, a construct containing the 5′-UTR of Bip mRNA linked to a reporter gene (luciferase) was obtained from P. Sarnow (University of Colorado). The translation of this mRNA in HeLa extracts generated the luciferase protein (Fig. 8C, lane 1). The addition of yeast I-RNA completely inhibited luciferase synthesis from this RNA construct (Fig. 8C, lane 2). As expected, cap-dependent translation from a CAT construct was not at all inhibited under the same conditions (Fig. 8C, lanes 3 and 4). In fact, CAT translation was significantly stimulated over that of the control as previously observed (10). These results suggest that yeast I-RNA is capable of inhibiting internal initiation from a cellular mRNA.

To examine the possibility that luciferase translation from the Bip UTR construct was nonspecifically inhibited by I-RNA, a monocistronic luciferase construct which did not contain the Bip IRES sequence was obtained. Translation from this construct is known to occur by a cap-dependent mechanism. Translation from this RNA was inhibited by only 7% in the presence of the inhibitor compared with the control (Fig. 8B, lanes 4 and 3, respectively). Under similar conditions, translation from the poliovirus IRES containing the P2CAT construct was inhibited by 72% compared with the control (Fig. 8B, lanes 1 and 2).

Yeast I-RNA inhibits the translation of poliovirus RNA in vivo. Previous studies from our laboratory (10) and the results presented here suggested that yeast I-RNA inhibits virus IRES-dependent translation in vitro. To determine whether I-RNA inhibits the translation of poliovirus RNA in vivo, poliovirus RNA was transfected into HeLa cells singly or together with purified yeast RNA. Proteins were labeled by the addition of [^{35}S]methionine, and the synthesis of viral proteins was monitored by direct analysis of cell extracts (Fig. 9A) or by immunoprecipitation of viral capsid proteins by anti-capsid antisera (Fig. 9B). In the absence of added virus RNA and I-RNA, the synthesis of cellular proteins was evident (Fig. 9A, lane 1). When virus RNA (1 μg) was transfected into cells, the synthesis of distinct viral proteins was observed (Fig. 9A, lane 2). In addition, the background of host cell proteins diminished considerably because of the shutoff of host cell protein synthesis by poliovirus (Fig. 9A, lane 2). When I-RNA was cotransfected with virus RNA (1 μg) into HeLa cells, no detectable synthesis of viral proteins was observed and host cell protein synthesis was restored (Fig. 9A, lane 3). The expression of I-RNA alone did not interfere with the synthesis of cellular proteins (Fig. 9A, lane 4). Figure 9A, lane 5, shows that the carrier tRNA used in transfection experiments had no effect on cellular protein synthesis. The transfection of cells with an increased amount of poliovirus RNA (2 μg) resulted in the synthesis of viral proteins and a more pronounced shutoff of

cellular protein synthesis (Fig. 9A, lane 6). However, in the presence of I-RNA, viral protein synthesis was inhibited and host cell protein synthesis was restored (Fig. 9A, lane 7) to the level seen in the control reaction. The results shown in Fig. 9B confirm that viral protein synthesis was inhibited in cells containing I-RNA. The synthesis of viral capsid proteins was inhibited in cells cotransfected with virus RNA and I-RNA (Fig. 9B, lane 3). Thus, yeast I-RNA efficiently inhibited translation from poliovirus RNA in vivo. Although not shown here, the protection of monolayer cells from the cytolytic effects of poliovirus infection in the presence of yeast I-RNA paralleled the restoration of host cell protein synthesis seen in lanes 3 and 7 of Fig. 9A.

Figure 9C represents an experiment similar that shown in Fig. 9A except that two important controls are included. First, the transfection of an unrelated nonspecific RNA similar in size to I-RNA along with poliovirus RNA did not result in the inhibition of viral protein synthesis (compare lanes 2 and 5 in Fig. 9C). Secondly, the translation-inhibitory effect of I-RNA in cotransfected cells (Fig. 9C, lane 4) was reversed by including equimolar amounts of antisense I-RNA during transfection (lane 3). These experiments suggested that the inhibition of virus translation in cotransfected cells is mediated by I-RNA. It should be noted that similar amounts of intracellular poliovirus RNA were detected in cells transfected with poliovirus RNA alone and in cells transfected with a mixture of poliovirus RNA and I-RNA, suggesting that the stability of poliovirus RNA is not altered significantly in cells containing I-RNA (data not shown).

DISCUSSION

Poliovirus RNA is poorly translated in the yeast *S. cerevisiae* both in vivo and in vitro (10). A previous study from our laboratory identified an RNase-sensitive activity from yeast cells which was able to inhibit (in *trans*) the translation of poliovirus RNA in HeLa cell extracts. The inhibitor had no effect on cap-dependent translation of a number of mRNAs. Because naturally uncapped poliovirus RNA uses a cap-independent mode of translation which utilizes the internal entry of ribosomes, it was thought that the yeast inhibitor specifically inhibited IRES-dependent translation. Here we report the purification of a small RNA molecule (60 nucleotides) from yeast cells which appears to specifically inhibit IRES-mediated translation programmed by poliovirus RNA. This small RNA inhibitor (I-RNA) has been sequenced, and a synthetic clone has been prepared according to the RNA sequence. RNA derived from the synthetic clone by transcription with T7 RNA polymerase blocks the translation of poliovirus RNA both in vivo and in vitro. Several lines of evidence suggest that purified yeast RNA specifically inhibits the internal initiation of translation mediated by poliovirus IRES. First, deletion analysis of poliovirus 5′-UTR has shown that I-RNA requires internal ribosome entry site sequences to efficiently inhibit the translation of poliovirus 5′-UTR-CAT RNAs (Fig. 2). Secondly, using a bicistronic construct which contains the poliovirus IRES between two cistrons, we have shown that translation from the second cistron is specifically inhibited by I-RNA, whereas translation from the first cistron is not affected at all by the inhibitor (Fig. 8). The translation of poliovirus RNA in vivo is inhibited when cells are cotransfected with virus RNA and I-RNA (Fig. 9). This inhibition can be reversed by the inclusion of an equimolar amount of antisense I-RNA during cotransfection. Additionally, translation mediated by the 5′-UTR of Bip mRNA, which is known to initiate translation internally, is also inhibited by I-RNA (Fig. 8). Finally, I-RNA appears to specifically interact with at least one polypeptide (p52) believed to be involved in the internal initiation of translation (Fig. 4 and 6).

How I-RNA inhibits the translation of poliovirus mRNA is not clear at present. Our UV cross-linking studies suggest that I-RNA interacts with a protein with an apparent molecular mass of 52 kDa present in HeLa S10 extract. We assume, although we have not shown, that this protein is similar to the p52 protein which was originally described as binding to the poliovirus sequence of nucleotides 559 to 624 (SL-G). The results presented in Fig. 4 and 6 suggest that this 52-kDa protein (p52) may also bind to a region of poliovirus UTR(320-461) in addition to the region spanning nucleotides 559 to 624. This is similar to what has recently been reported for p57, a polypeptide which has been identified as PTB. This protein has been shown to bind to three noncontiguous sites on poliovirus RNA, nucleotides 7 to 286 (site 1), 443 to 539 (site 2), and 630 to 730 (site 3) (19). p52 binding to multiple regions of poliovirus RNA may be important for virus RNA translation. In such a case, it is conceivable that the binding of p52 by I-RNA may lead to the inhibition of viral protein synthesis. The binding of p52 to poliovirus RNA sequence 320 to 461 may be important in view of the studies indicating that SL-D and -G may not be absolutely necessary for virus translation in vivo (17, 18, 41). It should be noted that cell extracts immunodepleted of p52 fail to promote cap-independent translation (32). Future studies with purified p52 and anti-p52 antiserum should enable us to determine if indeed p52 binds to nucleotides 320 to 461 of poliovirus RNA. At present, the possibility that the 52-kDa polypeptide that interacts with nucleotides 320 to 461 of virus RNA is not related to p52 (i.e., the La autoantigen) cannot be excluded.

It is clear from deletion analysis that the minimum 5′-UTR sequence required for the inhibition of translation appears to be nucleotides 320 to 461 (Fig. 2C). This sequence potentially forms two stem-loop structures, SL-E and SL-F (Fig. 5). Dildine and Semler have shown that part of SL-E (nucleotides 260 to 415) is capable of forming RNA-protein complexes with extracts prepared from many cell types (13). UV cross-linking studies by Gebhard and Ehrenfeld have shown that two proteins with apparent molecular masses of 48 and 38 kDa bind to the region covering SL-E and -F and that these proteins are believed to be important for virus translation (15). The relationship between the 52-kDa protein that binds to nucleotides 320 to 461 (Fig. 7) and the 48-kDa protein seen by Gebhard and Ehrenfeld remains unclear at present.

Purified yeast I-RNA is able to inhibit virus replication in cells transfected with poliovirus RNA presumably by inhibiting virus translation (Fig. 9). First, poliovirus-specific proteins are not detected in cells cotransfected with poliovirus mRNA and I-RNA. Secondly, host protein synthesis is restored in cotransfected cells, demonstrating the absence of viral proteins in these cells. Finally, immunoprecipitation with anti-capsid antibody confirms the absence of viral proteins in cells transfected with I-RNA. In addition, the translation (viral)-inhibitory effect of I-RNA in cotransfected cells can be reversed by including equimolar amounts of antisense I-RNA during transfection (Fig. 9C). Although we have not determined whether the synthesis of specific cellular proteins is inhibited in vivo, gross cellular protein synthesis does not appear to be affected by the presence of this inhibitor. Why do we see almost complete restoration of host cell protein synthesis (i.e., the lack of productive infection) although only 15 to 20% of cells are transfected with I-RNA? The answer lies in the fact that liposome-mediated cotransfection results in a high percentage of transfected cells which carry both virus RNA and I-RNA. In

other words, there are very few cells that are transfected with virus RNA alone during cotransfection. This results in almost total inhibition of viral protein synthesis. In contrast, cells transfected with virus RNA alone are able to produce progeny virions which cause spreading infection, eventually resulting in the lysis of almost all cells. Thus, in the absence of I-RNA, we see an amplified effect of virus infection.

Since both virus RNA (5′-UTR) and I-RNA bind similar proteins involved in the internal initiation of translation, is there any sequence homology between these two RNAs? We found no significant homology between I-RNA and poliovirus mRNA. We have searched for sequences similar to the I-RNA sequence by using FASTA (35) on a Biovax copy of the Genetics Computer Group-formatted GenBank files for three databases, namely, virus RNA, structural RNA, and plants, including yeasts (Sept. 1993 version). Interestingly, 89.5% homology (with a 19-nucleotide overlap) with a region of the Japanese encephalitis virus genome was found. Similarly, 70 to 80% homology was observed with different regions of the genomes of herpes simplex virus, Sindbis virus, Epstein-Barr virus, dengue virus, and influenza virus. Compared with the structural RNA database, the most striking homology (79 to 100%, with an 11- to 19-nucleotide overlap) was with 16S rRNAs from a variety of organisms. I-RNA also has extensive homology (93.8%, with a 16-nucleotide overlap) with 5S rRNA of trypanosomes. Comparison with the plant database, which includes yeasts, showed 93.3% homology (15-nucleotide overlap) with the yeast genes of histones H4.1 and H3. The only mRNA found to have homology with I-RNA was maize superoxide dismutase 3 isoenzyme (100% homology, with a 13-nucleotide overlap). Considering the shortness of the overlapping sequences, the significance of these findings is unclear at present.

It is worth noting that an antisense RNA with an exact complementary sequence of I-RNA is as efficient in binding p52 as the sense I-RNA molecule (data not shown). This result, taken together with the fact that there is no apparent sequence homology with poliovirus RNA, suggests that the secondary structure of I-RNA may play a crucial role in the inhibition of internal initiation of translation. Indeed, two computer-predicted secondary structures of I-RNA that are thermodynamically relatively stable could be generated (data not shown). These structures partly resemble the p52-binding site on poliovirus mRNA. Thus, it may be the secondary structure rather than the primary sequence of I-RNA that is responsible for its ability to bind p52 or other proteins necessary for the internal initiation of translation.

The normal function of I-RNA in yeast cells remains unknown at present. Altman et al. previously reported that a strain of *S. cerevisiae* can translate (albeit inefficiently) UTR-CAT gene fusions present as the second gene in bicistronic RNAs (2). An intriguing possibility is that yeast cells contain genes which initiate translation internally and that I-RNA can be used to regulate the expression of these genes posttranscriptionally. So far, two cellular mRNAs, one encoding the immunoglobulin heavy-chain Bip protein and the other encoding the *Drosophila* Antennapedia protein, have been shown to be translated by a mechanism involving internal ribosome entry. We have shown here that purified I-RNA inhibits the in vitro translation programmed by Bip 5′-UTR (Fig. 8). In studies to be published elsewhere, we have shown that the inhibitor specifically inhibits the translation of other picornaviruses, including rhino-, hepatitis A, and Theiler's murine encephalomyelitis viruses. Thus, both a cellular RNA and a number of picornavirus RNAs known to initiate translation internally are inhibited by the same inhibitor. Future studies should utilize yeast I-RNA as a probe to understand in detail the mechanism(s) of the internal initiation of translation in eukaryotes.

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REFERENCES

1. **Agol, V. I.** 1991. The 5′ untranslated region of picornaviral genomes. Adv. Virus Res. **40:**103–180.
2. **Altman, M., S. Blum, J. Pelletier, N. Sonenberg, T. M. A. Wilson, and H. Trachsel.** 1990. Translation initiation factor dependent extracts from *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1050:**155–159.
3. **Bandyopadhyay, P. K., C. Wang, and H. L. Lipton.** 1992. Cap-independent translation by the 5′ untranslated region of Theiler's murine encephalomyelitis virus. J. Virol. **66:**6249–6256.
4. **Belsham, G. J., and J. K. Brangwyn.** 1990. A region of the 5′ noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. J. Virol. **64:**5389–5395.
5. **Borman, A., M. T. Howell, J. G. Patton, and R. J. Jackson.** 1993. The involvement of a splicesome component in internal initiation of human rhinovirus RNA translation. J. Gen Virol. **74:**1775–1788.
6. **Borman, A., and R. J. Jackson.** 1992. Initiation of translation of human rhinovirus RNA: mapping the internal ribosome entry site. Virology **188:**685–696.
7. **Borovjagin, A. V., M. V. Ezrokhi, V. M. Rostapshov, T. Y. U. Ugarova, T. F. Bystrova, and I. N. Shatsky.** 1991. RNA-protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA. Nucleic Acids Res. **19:**4999–5005.
8. **Brown, B. A., and E. Ehrenfeld.** 1979. Translation of poliovirus RNA in vitro: changes in cleavage pattern and initiation sites by ribosomal salt wash. Virology **97:**376–405.
9. **Chang, K. H., E. A. Brown, and S. M. Lemon.** 1993. Cell type-specific proteins which interact with the 5′ nontranslated region of hepatitis A virus RNA. J. Virol. **67:**6716–6725.
10. **Coward, P., and A. Dasgupta.** 1992. Yeast cells are incapable of translating RNAs containing the poliovirus 5′-untranslated region: evidence for a translational inhibitor. J. Virol. **66:**286–295.
11. **Dasgupta, A.** 1983. Purification of host factor required for *in vitro* transcription of poliovirus RNA. Virology **128:**245–251.
12. **del Angel, A., G. Papvassiliou, C. Fernandez-Tomas, S. J. Silverstein, and V. R. Racaniello.** 1989. Cell proteins bind to multiple sites within the 5′-untranslated region of poliovirus RNA. Proc. Natl. Acad. Sci. USA **86:**8299–8303.
13. **Dildine, S. L., and B. L. Semler.** 1992. Conservation of RNA protein interactions among picornaviruses. J. Virol. **66:**4364–4376.
14. **Dorner, H. A., B. L. Semler, R. J. Jackson, R. Hanecek, E. Duprey, and E. Wimmer.** 1984. In vitro translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. J. Virol. **50:**507–514.
15. **Gebhard, J. R., and E. Ehrenfeld.** 1992. Specific interactions of HeLa cell proteins with proposed translation domains of the poliovirus 5′ noncoding region. J. Virol. **66:**3101–3109.
16. **Glass, M. J., X. Jia, and D. Summers.** 1993. Identification of the hepatitis A virus internal ribosome entry site: *in vivo* and *in vitro* analysis of bicistronic RNAs containing HAV 5′ noncoding region. Virology **193:**842–852.
17. **Haller, A. A., J. H. C. Nguyen, and B. L. Semler.** 1993. Minimum internal ribosome entry site required for poliovirus infectivity. J. Virol. **67:**7461–7471.

18. **Haller, A. A., and B. L. Semler.** 1992. Linker scanning mutagenesis of the internal ribosome entry site of poliovirus RNA. J. Virol. **66**:5075–5086.
19. **Hellen, C. U. T., T. V. Pestova, M. Litterst, and E. Wimmer.** 1994. The cellular polypeptide p57 (pyrimidine tract-binding protein) binds to multiple sites in the poliovirus 5′ nontranslated region. J. Virol. **68**:941–950.
20. **Hellen, C. U. T., G. W. Witherell, M. Schmid, S. H. Shin, T. V. Pestova, A. Gil, and E. Wimmer.** 1993. A cytoplasmic 57 kd protein (p57) that is required for translation of picornavirus RNA by internal ribosome entry is identical to the nuclear polypyrimidine tract binding protein. Proc. Natl. Acad. Sci. USA **90**:7642–7646.
21. **Jackson, R., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15**:477–483.
22. **Jang, S. K., H.-G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
23. **Jang, S. K., T. V. Pestova, C. U. T. Hellen, G. W. Witherell, and E. Wimmer.** 1990. Cap-independent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. Enzyme (Basel) **44**:292–309.
24. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57 kD RNA-binding protein. Genes Dev. **4**:1560–1572.
25. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. Vander Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization, and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
26. **Kohara, K. T., N. Iizuka, M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66**:1476–1483.
27. **Kozak, M.** 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol. Rev. **47**:1–45.
28. **Luz, N., and E. J. Beck.** 1990. A cellular 57 kDa protein binds to two regions of the internal initiation site of foot-and-mouth disease virus. FEBS Lett. **269**:311–314.
29. **Luz, N., and E. Beck.** 1991. Interaction of a cellular 57-kilodalton protein with the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **65**:6486–6494.
30. **Macejak, D. G., and P. Sarnow.** 1991. Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. Nature (London) **353**:90–94.
31. **Meerovitch, K., J. Pelletier, and N. Sonneberg.** 1989. A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3**:1026–1034.
32. **Meerovitch, K., Y. V. Svitkin, H. S. Lee, F. Lejbkowicz, D. J. Kenan, E. K. L. Chan, V. I. Agol, J. D. Keene, and N. Sonenberg.** 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. J. Virol. **67**:3798–3807.
33. **Najita, L., and P. Sarnow.** 1990. Oxidation-reduction sensitive interaction of a cellular 50-kD protein with an RNA hairpin in the 5′ noncoding region of the poliovirus genome. Proc. Natl. Acad. Sci. USA **87**:5846–5850.
34. **Oh, S. K., M. P. Scott, and P. Sarnow.** 1992. Homeotic gene *Antennapedia* mRNA contains 5′-noncoding sequences that confer translational initiation by internal ribosome binding. Genes Dev. **6**:1643–1653.
35. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
36. **Pelletier, J., M. E. Flynn, G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Mutational analysis of upstream AUG codons of poliovirus RNA. J. Virol. **62**:4486–4492.
37. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Translational efficiency of poliovirus mRNA: mapping inhibitory *cis*-acting elements within the 5′-noncoding region. J. Virol. **62**:2219–2227.
38. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5′-noncoding region. Mol. Cell. Biol. **8**:1103–1112.
39. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334**:320–325.
40. **Pelletier, J., and N. Sonenberg.** 1989. Internal binding of eucaryotic ribosomes on poliovirus RNA: translation in HeLa cell extracts. J. Virol. **63**:441–444.
41. **Percy, N., G. J. Belsham, J. K. Brangwyn, M. Sullivan, D. M. Stone, and J. W. Almond.** 1992. Intracellular modifications induced by poliovirus reduce the requirement for structural motifs in the 5′-noncoding region of the genome involved in internal initiation of protein synthesis. J. Virol. **66**:1695–1701.
42. **Pestova, T. V., C. U. T. Hellen, and E. Wimmer.** 1991. Translation of poliovirus RNA: role of an essential *cis*-acting oligopyrimidine element within the 5′ nontranslated region and involvement of a cellular 57-kilodalton protein. J. Virol. **65**:6194–6204.
43. **Pilipenko, E. V., A. P. Gmyl, S. V. Maslova, Y. V. Svitkin, A. N. Sinyakov, and V. I. Agol.** 1992. Prokaryotic-like cis-elements in the cap-independent internal initiation of translation on picornavirus RNA. Cell **68**:119–131.
44. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78**:4887–4891.
45. **Ransone, L. J., and A. Dasgupta.** 1987. Activation of double-stranded RNA-activated protein kinase in HeLa cells after poliovirus infection does not result in increased phosphorylation of eucaryotic initiation factor-2. J. Virol. **61**:1781–1787.
46. **Rose, J. K., H. Trachsel, K. Leong, and D. Baltimore.** 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. Proc. Natl. Acad. Sci. USA **75**:2732–2736.
47. **Rothblatt, J. A., and D. I. Meyer.** 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertase in a homologous cell-free system. Cell **44**:619–628.
48. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond.** 1989. New model for the secondary structure of the 5′ noncoding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207**:379–392.