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Host Cell Proteins Binding to Domain IV of the 5′ Noncoding Region of Poliovirus RNA

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Translation of poliovirus RNA occurs by the binding of ribosomes to an internal segment of RNA sequence within the 5′ untranslated region of the viral RNA. This region is predicted to consist of six domains (I to VI) that possess complex secondary and tertiary structures. Domain IV is a large region in which alterations in the sequence or structure markedly reduce translational efficiency. In this study, we employed RNA mobility shift assays to demonstrate that a protein(s) from uninfected HeLa cell extracts, as well as from neuroblastoma extracts, interacts with the domain IV structure. A mutation in domain IV caused reduced binding of HeLa cell proteins and reduced translation both in vitro and in vivo, suggesting that the binding of at least one of these proteins plays a role in the mechanism of viral translation. UV cross-linking indicated that a protein(s) with a size of ~40 kDa interacted directly with the RNA. Using streptavidin beads to capture biotinylated RNA bound to proteins, we were able to visualize a number of HeLa and neuroblastoma cell proteins that interact with domain IV. These proteins have molecular masses of ~39, ~40, and ~42 kDa.

Poliovirus is a member of the *Picornaviridae* family. It has a single-stranded, positive-sense, RNA genome of about 7,500 nucleotides (nt) which contains a highly structured 5′ noncoding region (5′ NCR) of 742 nt. The 5′ NCR of poliovirus is predicted to form six structured domains (domains I to VI; Fig. 1) (2, 32, 43, 44, 46, 51). Several studies have shown that the translation of poliovirus RNA occurs by internal ribosome binding in the 5′ NCR (reviewed in references 23, 52, and 59). The region of the 5′ NCR required for internal ribosome binding has been termed the internal ribosome entry site (IRES) (24) or the ribosome landing pad (40), and in this article, we will use the former convention.

The 5′ NCR contains signals for RNA replication and for internal ribosome binding; both processes involve interactions with proteins that depend on RNA structure. RNA replication signals are located in a cloverleaf structure formed by the first 100 nt at the 5′ terminus of the RNA (2) and appear to require a specific fit with viral replication proteins coded for by the homologous virus (47). The essential translation signals appear to constitute an independent structure located between nt 130 and 630 (15, 56). This internal segment can be exchanged among different viruses (1, 26) and can be transferred to reporter genes to confer a cap-independent, internal initiation mechanism of translation (57). The idea that the RNA replication and translation domains function independently has been challenged recently by Borman et al. (4), who suggested that replication signals are present in domains IV and V of the IRES. In addition, a previous report suggested that mutations in the most 5′ stem-loop structure (domain I) may affect translation (50).

The precise mechanism by which the poliovirus IRES directs the binding of ribosomes to the RNA, and the subsequent initiation of translation, remains largely unknown, although it has been postulated that the complex domains of the IRES interact to present short, conserved, unpaired regions of the RNA in a precise three-dimensional arrangement for contacts with specific proteins (23). A number of studies have demonstrated that small deletions, insertions, or substitutions within the IRES are detrimental to viral translation (6, 16, 29, 56, 57). Secondary mutations which restore disrupted domain structures tend to also restore IRES activity (16, 29), lending support to the idea that it is the conserved sequence of the unpaired regions of the loops that must be properly presented by the paired stem regions. Studies using mutants in which various domain structures were either partially or completely deleted demonstrated that the requirements for translation include only domains II, IV, and V and sequences in domain VI (6, 15, 41).

The majority of the effort toward an understanding of the role of the 5′ NCR domains involved in viral translation has, so far, focused on domains V and VI of the poliovirus IRES. Domain V is the site of a single base change which is a major determinant of the attenuation phenotype of the Sabin vaccine strains. The single nucleotide alteration apparently causes a defect in viral translation in neuronal cells (10, 31, 38, 39). Domain VI contains a noninitiating AUG triplet spaced critically downstream of a pyrimidine-rich sequence (Fig. 1) which appears to constitute the actual site of ribosome entry (23). A 52-kDa protein identified as the La autoantigen has been shown to interact with sequences in domain VI and to be necessary for the correct translation of poliovirus mRNA in vitro (35). This protein is abundant in HeLa cells but is found in only limiting quantities in rabbit reticulocyte lysates (RRL). A 57-kDa protein identified as polypyrimidine tract-binding protein, or PTB, was shown to bind to three regions of the poliovirus 5′ NCR which mapped within domains III, V, and VI (18, 19). The analysis of the functional role of PTB in poliovirus translation has been somewhat more difficult. Immunodepletion of PTB from HeLa cell extracts prevented the translation of poliovirus RNA, indicating a role for this protein in viral translation (18); although addition of purified PTB to these immunodepleted extracts failed to restore the translation of poliovirus mRNA, addition of recombinant PTB to extracts depleted with an RNA affinity column completely restored the capacity for IRES-driven translation (27). Since domain III is not essential to viral translation (6, 15, 41), the functional

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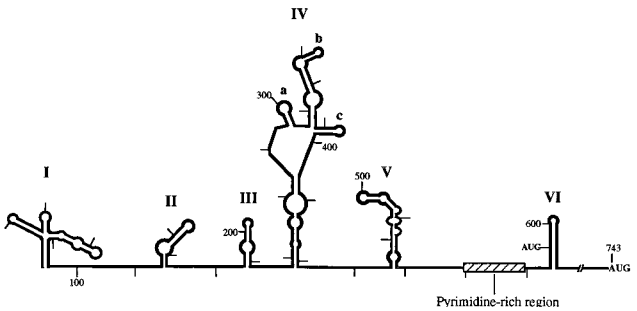

FIG. 1. Predicted structure of the poliovirus type 1 5′ NCR. Numbers refer to nucleotide positions relative to the 5′ end of the RNA. The AUG at position 743 represents the authentic poliovirus initiation codon. This structure is modified from one by Poyry et. al. (44), and the nucleotide numbering corresponds to that of the poliovirus type 3 sequence.

interactions may be with domains V and VI. Recently, Haller and Semler have shown data that indicate that domains V and VI may act synergistically to bind some proteins (17).

Domain IV is the largest and most complex of the regions in the poliovirus 5′ NCR. It has remained largely uncharacterized in terms of its interactions with cellular proteins and the potential involvement of these proteins in the translation mechanism. Small insertions and deletions in conserved domain IV sequences have been shown to abrogate translation and give rise to lethal and small-plaque phenotypes. Three domain IV substructures are predicted to be located within the larger RNA structure (IVa, b, and c; Fig. 1). The domain IVc substructure is not necessary for translation, and the IVa substructure is important but not essential (53). Small mutations in the domain IVb substructure are lethal to the virus and have a negative effect on translation (53, 56). Using RNA mobility shift analysis, Dildine and Semler (7) demonstrated that domain IV RNA (formerly referred to as stem-loop E) interacted with HeLa cell proteins. These data indicated that much of the domain IV sequence or structure was important for protein binding. Gebhard and Ehrenfeld used UV cross-linking to demonstrate the specific interaction of a number of cellular proteins with the 5′ NCR, including two with molecular masses of 38 and 48 kDa whose binding mapped to domain IV (14). Importantly, the domain IV RNA sequences appeared to interact with proteins that were involved in poliovirus translation but that did not affect the translation of cellular mRNAs. Furthermore, the sequences (nt 286 to 456) appeared to play a role in the translation of viral proteins in HeLa cell extracts but not rabbit reticulocyte extracts. Unfortunately, the cross-linking approach precluded isolation of these proteins for biochemical analyses.

In this report, we demonstrate that the intact poliovirus IRES can compete for protein binding with a small 240-nt RNA representing the entire domain IV of the poliovirus 5′ NCR, indicating that the small RNA can be used as an affinity probe to selectively isolate proteins and protein complexes that interact with this region of the intact IRES. RNA mobility shift assays were used to demonstrate that a lethal mutation in domain IV had a greatly reduced affinity for these proteins. This mutation abolished over 90% of the translation directed by the poliovirus 5′ NCR fused to a luciferase reporter sequence, suggesting that the binding of at least one of these proteins plays a role in viral translation. UV cross-linking of the RNA-protein complex from the mobility shift analysis demonstrated the direct interaction of the RNA with a protein(s) with a molecular mass of approximately 40 kDa. By using a novel biotin-streptavidin assay that detects protein-RNA as well as protein-protein interactions, we were able to visualize a number of proteins which interacted with domain IV sequences. These proteins have molecular masses of ~39, ~40, and ~42 kDa.

MATERIALS AND METHODS

Plasmids. pT220-460, described previously (7), contains nt 220 to 460 of the Mahoney strain of poliovirus type 1 cDNA under the control of the T7 promoter. pT325Mut is identical to pT220-460 except for the insertion of an AGT triplet at nt 325 by PCR mutagenesis as described previously (20, 22). pT7PV1, described previously (16), contains the entire cDNA of the Mahoney strain of poliovirus type 1 cDNA under the control of the T7 promoter. pT7PV1-325 is identical to pT7PV1 except for the insertion of an AGT triplet at nt 325 by PCR mutagenesis. pTMLuc was constructed by J. Gebhard (13) and contains the firefly luciferase gene fused, in frame, to the AUG codon at nt 743 of the complete 5′ NCR of poliovirus type 1. pTMLuc-325 is identical to the pTMLuc construct except for the insertion of an AGT triplet at nt 325 by PCR mutagenesis. pGEM-4Z(PV 1-72) was constructed by O. Richards (45) and contains cDNA representing the first 72 nt of poliovirus RNA. Transcription with T7 RNA polymerase gives rise to an 87-nt RNA containing 15 nt of the pGEM sequence and the 3′-terminal 72 nt of poliovirus negative-strand RNA.

Poliovirus plaque assays. HeLa cell monolayers grown (in minimal essential medium supplemented with 6% fetal calf serum) to 80 to 90% confluency in 60-mm-diameter dishes were transfected either by a modified DEAE-dextran-mediated precipitation technique (30, 58) or by using DOTAP reagent (Boehringer Mannheim) according to the manufacturer's instructions. Cells were incubated under semisolid agar at 33 or 37°C for 2 to 4 days and stained with crystal violet (21). If no plaques were observed in the initial transfection, the transfection was repeated with a liquid rather than a semisolid overlay. On day 4 or 5 posttransfection, the liquid supernatants were harvested and used to infect HeLa cell monolayers which were then overlaid with semisolid agar.

Preparation of HeLa, NGP, and rabbit reticulocyte extracts. The HeLa and NGP cell extracts used in all of the experiments were prepared as described previously (5) from either HeLa S3 or NGP cells adapted to suspension culture. RRL were obtained from Green Hectares (Oregon, Wis.) and fractionated as described previously (5).

Preparation of RNA substrates. The pT220-460 and pT325Mut circular DNA plasmids were linearized with *Hin*dIII, and pT7PV1 was linearized with *Sfu*I. pTMLuc and pTMLuc-325 were linearized with *Eco*RI. The pGEM circular plasmid was linearized with *Rsa*I. pGEM-4Z (PV 72-1) was linearized with *Eco*RI. RNA transcripts were synthesized by transcribing linearized DNA in 20-μl reaction mixtures with the MEGAscript T7 kit from Ambion. The synthesis was done in the presence of 3 μCi of [^{3}H]UTP to facilitate quantitation. After transcription, the RNAs were extracted with phenol and precipitated with ethyl alcohol before quantitation. ^{32}P-labeled RNAs were synthesized with the MAXIscript T7 kit (Ambion). Fifty microcuries of [^{32}P]CTP (3,000 Ci/mmol) was added to 20-μl transcription reaction mixtures. Unincorporated nucleotides were removed by passage over a G-50 Sephadex Quick Spin column (Boehringer Mannheim). Biotinylated RNA was synthesized by adding 1.25 μl of 20 mM biotinylated UTP (Bio-11-UTP from ENZO Diagnostics) to a 20-μl MEGA script transcription reaction mixture. Following transcription, the RNA was precipitated with ethyl alcohol. RNAs were analyzed on 1% agarose gels or on denaturing 6% polyacrylamide gels containing 8 M urea and visualized by ethidium bromide staining or autoradiography.

RNA mobility shift assays. RNA mobility shift assays were performed as described previously (7, 28). Briefly, 10-μl reaction mixtures consisting of different amounts of cell extract in a buffer of 40 mM KCl, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.1), 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, heparin (0.25 μg/μl), and *Escherichia coli* tRNA (1 μg/μl) were incubated at 30°C for 10 min prior to the addition of labeled RNA. After the addition of 0.5 to 1.0 pmol of labeled probe, the reaction mixtures were incubated for an additional 15 min at 30°C, and this was followed by the addition of 3 μl of 50% glycerol per 10-μl reaction mixture. The reaction mixtures were then applied to 4% polyacrylamide gels (nondenaturing) which had been prerun for 30 min at 280 V. The gels were run at 50 to 200 V at 4°C until bromophenol blue markers had migrated 75% of the gel length. The gels were then dried and subjected to autoradiography.

In vitro translation assays. Translation assays with 12.5-μl reaction mixtures were performed in the presence of either wild-type or mutant poliovirus RNA sequences as described previously (36, 37). The reaction mixtures were incubated for 16 h at 33°C.

Transfection of HeLa cell monolayers. HeLa cell monolayers were grown (in minimal essential medium supplemented with 6% fetal calf serum) to 80 to 90% confluency in 60-mm-diameter dishes. The cells were washed twice with phosphate-buffered saline (PBS) and transfected with 5 μg of either DNA or RNA by means of the DOTAP reagent described above.

Luciferase assays. Monolayers of HeLa cells that were 80 to 90% confluent and that were transfected with luciferase-producing constructs were washed

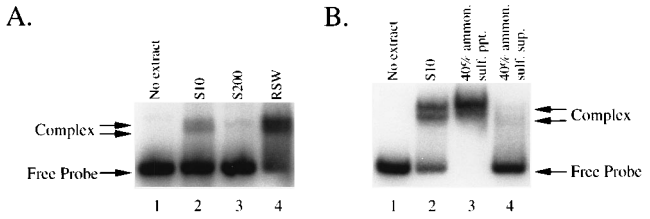

FIG. 2. Domain IV RNA mobility shifts by different HeLa cell extract fractions. The indicated fractions of HeLa cell extracts were incubated with ^{32}P-labeled domain IV RNA, and the resulting RNA-protein complexes were resolved from free RNA on nondenaturing polyacrylamide gels. (A) S10 refers to the supernatant of cytoplasmic extract centrifuged at 10,000 × *g* for 10 min, S200 refers to the supernatant of the S10 fraction centrifuged at 200,000 × *g* for 60 min, and RSW refers to the ribosomal salt wash (5). (B) RNA mobility shift analysis of ammonium sulfate fractions of the HeLa cell RSW.

twice with PBS. Cell lysis buffer (250 μl; Promega) was added and incubated at room temperature for 15 min. Following incubation, the monolayers were scraped and collected in 1.5-ml tubes and centrifuged. The supernatant was assayed by mixing 20 μl of it with 100 μl of luciferase assay reagent (Promega) and immediately reading the sample in a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

In situ UV cross-linking of RNA and protein. UV cross-linking of ^{32}P-labeled RNAs to protein was carried out as described previously (34). An RNA mobility shift assay with ^{32}P-labeled domain IV RNA and protein from the 40% ammonium sulfate precipitate fraction of HeLa cell ribosomal salt wash (RSW) was performed as described above. After electrophoresis, the gel was irradiated in a UV Stratalinker for 30 min and then exposed to X-ray film, and the gel region that contained the complex was excised. The gel slice was treated with a solution of 1 mg of RNase A per ml in 0.5× Tris-borate-EDTA (49) at 37°C for 1 h. The gel slice was then removed from the solution and placed in a 2× sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (49) and incubated for 30 min at 37°C, and this was followed by a 10-min incubation at 65°C. The gel slice was then embedded in the stacking gel of a denaturing 10% polyacrylamide gel and subjected to electrophoresis followed by autoradiography.

Isolation of proteins interacting with domain IV RNA sequences by affinity binding to biotinylated RNA. Reaction mixtures (100 μl) in RNA mobility shift buffer (described above) contained approximately 3 μg of biotinylated probe consisting of nt 220 to 460 of poliovirus RNA (~40 pmol), 200 μg of protein, 25 μg of heparin, and 74 μg of yeast RNA. After 15 min at 30°C, the reaction mixture was added to 400 μl of Streptavidin MagneSphere Paramagnetic Particles (Promega) which had been washed five times with RNA mobility shift buffer without heparin or yeast RNA. Binding was allowed to occur for 10 min at room temperature. The complex was then washed five times with the same buffer. Following the last wash, 30 μl of 2× SDS-PAGE sample buffer was added to the beads and incubated for 10 min at room temperature to dissociate proteins from the RNA. The sample containing the eluted proteins was then boiled and subjected to SDS-PAGE at 200 V in a 10% gel until the bromophenol blue marker had run off the gel. The gels were stained with either silver stain or Coomassie blue and dried.

RESULTS

Proteins binding to domain IV RNA sequences. To characterize potentially important cell protein interactions with domain IV of the poliovirus 5′ NCR, we used the previously described plasmid pT220-460, in which nt 220 to 460 of poliovirus type 1 had been cloned downstream of a T7 promoter (7). Digestion of this plasmid with the restriction enzyme *Hin*dIII followed by transcription with T7 RNA polymerase leads to the production of an ~240-nt RNA which includes the sequences predicted by computer modeling to constitute the domain IV structure.

^{32}P-labeled RNA was incubated in the presence of equal amounts of protein from different HeLa cell extract fractions. RNA-protein complexes formed in the presence of these cellular proteins were detected by the use of nondenaturing polyacrylamide gels (Fig. 2). When 20 μg of protein from the S10 fraction of uninfected HeLa cells was incubated with labeled domain IV RNA, the RNA was retarded in its mobility and appeared as a doublet (Fig. 2A, lane 2). Incubation of the

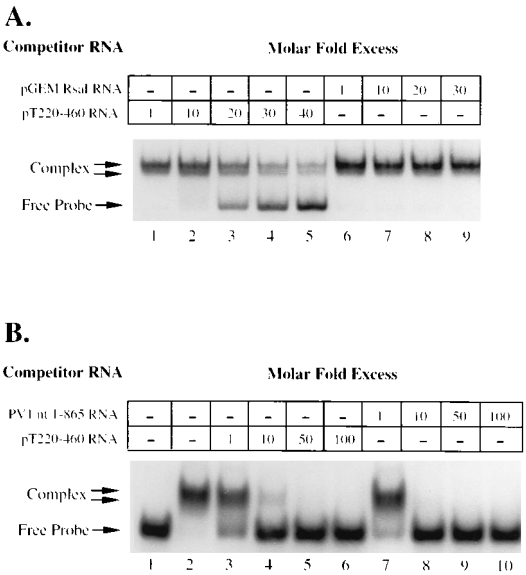

FIG. 3. Competition RNA mobility shift assays. (A) ^{32}P-labeled domain IV RNA was incubated with the 40% ammonium sulfate precipitate fraction of HeLa cell RSW in the presence of either excess unlabeled domain IV RNA or excess unlabeled RNA consisting of ~150 nt of pGEM-derived RNA, as indicated. RNA-protein complexes were resolved from free RNA on nondenaturing polyacrylamide gels. (B) ^{32}P-labeled domain IV RNA was incubated with the 40% ammonium sulfate precipitate fraction of HeLa cell RSW in the presence of either excess unlabeled domain IV RNA or excess unlabeled RNA consisting of nt 1 to 865 of poliovirus type 1 RNA, as indicated. The sample loaded in lane 1 has no extract. RNA-protein complexes were resolved from free RNA on nondenaturing polyacrylamide gels.

labeled domain IV RNA with 20 μg of protein from RSW from the same extract also showed a retardation in the electrophoretic mobility of the probe (Fig. 2A, lane 4). Results of mobility shift assays performed with the postribosomal supernatant (S200) fraction of HeLa cells showed variable, but very limited, RNA binding activity (Fig. 2A, lane 3). When labeled domain IV RNA was incubated with the 40% ammonium sulfate precipitate of an HeLa cell RSW fraction, the shifted complex appeared as a single band with a slower mobility than that of the doublet seen with the S10 (Fig. 2B, lane 3). When domain IV RNA was incubated with lesser amounts of protein from the 40% ammonium sulfate precipitate fraction, the shifted RNA-protein complex appeared as a doublet (Fig. 3). The binding occurred even in the presence of KCl concentrations as high as 1 M, although to a lesser extent (data not shown). Incubation of the probe with the 40% ammonium sulfate supernatant fraction of the RSW resulted in only a very slight mobility shift which was probably due to incomplete separation from the fractionation of the RSW (Fig. 2B, lane 4). The binding activity of proteins from nuclear extracts of HeLa cells was not examined.

The specificity of the interaction of the domain IV RNA with the proteins from HeLa cell extracts was determined by competition RNA mobility shift assays. Labeled domain IV RNA was incubated with the 40% ammonium sulfate precipitate of the RSW of uninfected HeLa cells in the presence of increasing amounts of unlabeled competitor RNA. When nonspecific RNA from pGEM was used as the competitor, there was no competition for binding (Fig. 3A, lanes 6 to 9). Domain IV RNA, used as the unlabeled competitor, competed efficiently for binding (Fig. 3A, lanes 1 to 5; Fig. 3B, lanes 1 to 6). One potential problem with these assays is that the domain IV RNA may not fold into the same structure as that formed in

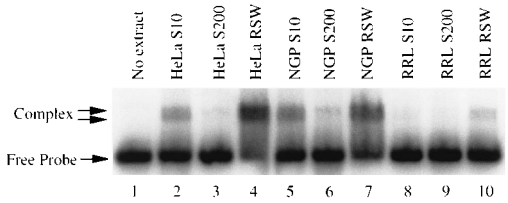

FIG. 4. RNA mobility shift analysis of neuroblastoma cell extract and RRL fractions. Indicated fractions from either neuroblastoma (NGP) cells or RRL were incubated with ^{32}P-labeled domain IV RNA. S10, S200, and RSW refer to protein fractions as described in the legend to Fig. 2. RNA-protein complexes were resolved from free RNA on nondenaturing polyacrylamide gels.

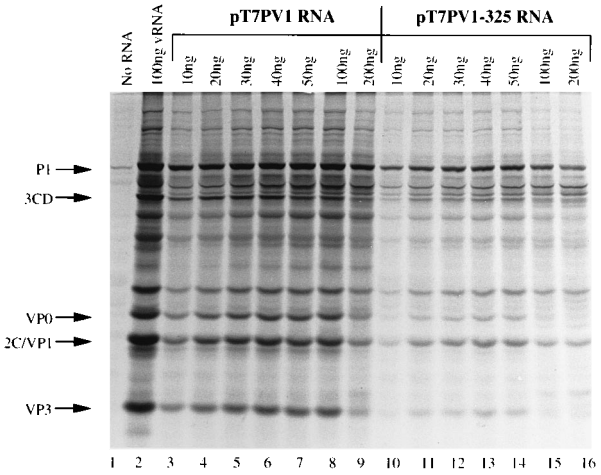

FIG. 5. In vitro translation of poliovirus RNA derived from cDNA containing either wild-type or mutant sequences. The indicated amounts of RNA of either the wild-type (pT7PV1) or mutant (pT7PV1-325) RNA sequences were used to direct translation in a HeLa cell extract. Translation of poliovirion RNA (vRNA) was used to identify viral proteins, some of which are indicated on the left.

the context of the complete 5′ NCR. To address this, we used unlabeled RNA which consists of poliovirus nt 1 to 865 and which includes the complete 5′ NCR as the competitor. As can be seen from Fig. 3B (lanes 7 to 10), this RNA competed efficiently for the binding of the HeLa cell proteins, indicating that the small domain IV RNA interacted with proteins similarly to the RNA containing the complete 5′ NCR.

It has been observed previously that the efficiency of translation of poliovirus RNA in vitro varies markedly on the basis of the source of the cellular extracts. For example, poliovirus RNA is translated quite efficiently in HeLa cell extracts, but the same RNA is translated poorly in RRL (5, 8). Importantly, while the correct initiation codon of poliovirus RNA is selected accurately in HeLa cell extracts, a number of products resulting from aberrant translation initiation are found when in vitro translations are performed with RRL. One possible explanation for this difference in translation efficiency is that proteins present in one extract are not present, or are present in limiting quantities, in other extracts. To examine this issue, we performed RNA mobility shift assays by incubating ^{32}P-labeled domain IV RNA with equal amounts of protein from the S10, S200, and RSW fractions of extracts from HeLa and NGP (a neuroblastoma cell line) cells as well as from RRL (Fig. 4). Extracts from the S10 and RSW fractions of NGP cell preparations were able to retard the mobility of the labeled domain IV RNA in a manner similar to that of the corresponding fractions from HeLa cell extracts (Fig. 4; compare lanes 5 and 7 with 2 and 4). As with the HeLa cell S200 fraction, the NGP S200 fraction was unable to retard the mobility of the labeled RNA (Fig. 4; compare lanes 3 and 6). Equal amounts of protein from rabbit reticulocyte extracts were able to shift only a very small amount of material (Fig. 4, lanes 8 to 10). This result suggests either an absence or a lower abundance of the domain IV specific binding proteins in RRL by comparison with the protein levels in HeLa and neuroblastoma extracts.

Protein binding to domain IV correlates with translational activity. A number of studies have demonstrated the binding of host proteins to regions of the poliovirus 5′ NCR important for viral translation (for a review, see reference 9). Both the La and PTB proteins have been shown to be involved in the translation of viral RNAs, although the biochemical roles of these proteins in the translation mechanism have not been elucidated (3, 19, 25, 35, 55). Although the binding of a protein(s) to domain IV RNA displays sequence and/or structure specificity (Fig. 3), we sought evidence that these proteins play a role in viral translation or in other viral functions. In the absence of either antibody against the specific protein(s) of interest or purified protein(s), mutational analysis of the binding region was utilized to provide a functional test for the formation of a ribonucleoprotein complex with domain IV RNA. We therefore examined a previously reported lethal mutation in domain IV (56). This lesion (a 3-nt insertion at nt 325 of the PV1 5′ NCR) was chosen with the expectation that such a mutation might affect protein binding, thus providing a connection among protein interaction with domain IV RNA, viability, and translation initiation.

To confirm the effect of this mutation at nt 325 on virus viability, we reconstructed the insertion into our poliovirus type 1 full-length cDNA to produce the pT7PV1-325 clone. As reported previously (56), RNA with this mutation was unable to produce viable virus (data not shown). Although the mutation at nt 325 was lethal for virus growth, it was unclear if the lethal defect affected viral translation or replication. Translation and replication defects can be difficult to separate from each other since the replication of poliovirus is linked to the translation of the RNA and the subsequent production of functional RNA polymerase. To test for a defect in translation, full-length RNA transcripts from either wild-type poliovirus cDNA or cDNA containing the mutation at nt 325 were used in translation experiments in HeLa cell extracts (Fig. 5). pT7PV1 RNA was translated to yield a pattern of viral proteins similar to that seen with virion RNA (Fig. 5; compare lane 2 with lanes 3 to 9). Increasing the amount of RNA in the translation reaction demonstrated an optimum of about 100 ng for input RNA. In contrast, RNA from pT7PV1-325 was translated at lower efficiencies at all concentrations used and showed an optimum for translation of about 40 to 50 ng of input RNA (Fig. 5, lanes 10 to 16). These data indicated that the translation of pT7PV1-325 RNA was impaired compared with that of pT7PV1 RNA but that under the assay conditions used, translation still occurred.

To determine whether protein binding to the domain IV structure was affected by the mutation at nt 325, we incorporated the 3-nt insertion into the pT220-460 construct to create the pT325Mut construct. ^{32}P-labeled RNA made from this construct was used in the RNA mobility shift assay and was compared with wild-type domain IV RNA (Fig. 6A). At the highest concentration of protein from the 40% ammonium sulfate precipitate of RSW, wild-type domain IV RNA was shifted entirely to a doublet as shown above. However, when RNA from pT7PV1-325 was used in the reaction, only a small

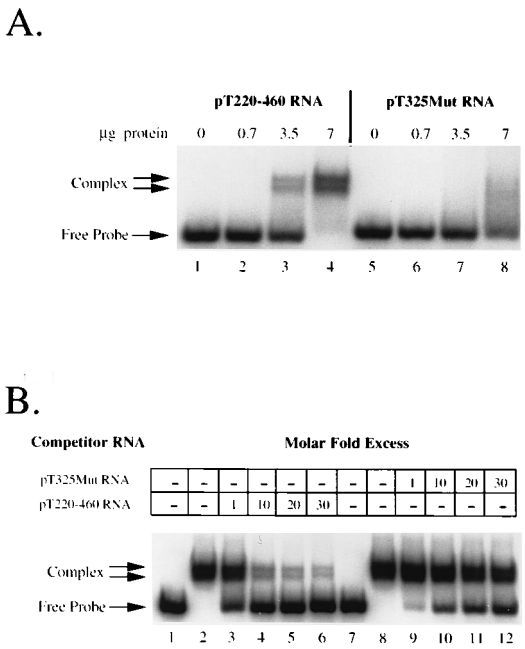

FIG. 6. RNA mobility shift analysis of pT220-460 and pT325Mut RNA sequences. (A) Increasing amounts of protein from the RSW fraction of HeLa cell extracts were incubated with ^{32}P-labeled domain IV RNA. Lanes 1 and 5 contain no protein. (B) Competition RNA mobility shift assay with pT220-460 and pT325Mut RNA sequences. ^{32}P-labeled pT220-460 RNA was incubated with 14 μg of the 40% ammonium sulfate precipitate fraction of HeLa cell RSW in the presence of increasing molar excesses of either unlabeled pT220-460 or pT325Mut RNA as indicated. Lanes 1 and 7 contain no protein.

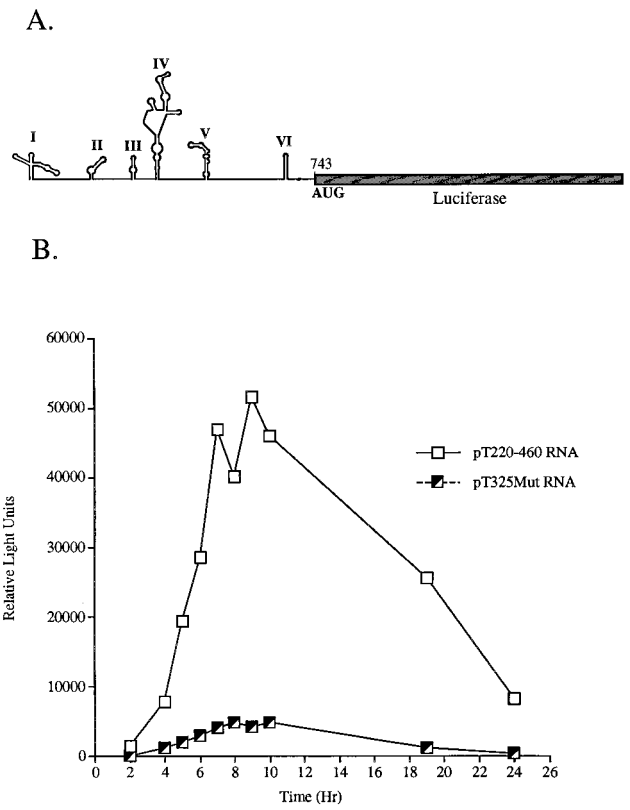

FIG. 7. (A) Diagrammatic representation of pTMLuc RNA. The shaded bar represents the firefly luciferase gene. (B) Luciferase activity assays of cells transfected with either pTMLuc or pTMLuc-325 RNA. Cells were incubated for the indicated times, harvested, and assayed as described in Materials and Methods. Activity is represented as relative light units. Results for each time point are the averages of two independent assays.

amount of material was shifted at the highest concentration of protein. These data suggested that the affinity of the pT325Mut RNA for the HeLa cell binding factors was greatly reduced.

To examine the relative differences in the binding of host cell proteins between pT220-460 RNA and RNA from the pT325Mut clone, competition RNA mobility shift assays were performed. When unlabeled pT220-460 RNA was used to compete for protein binding to labeled homologous RNA, as little as a 10-fold molar excess was sufficient to compete for all of the binding (Fig. 6B, lanes 2 to 6). A small amount of nonreversible residual binding that cannot be competed for, even at a 100-fold molar excess (data not shown), remains. Further experiments showed that this residual binding occurred only when a competitor was added after the labeled RNA was already allowed to interact with proteins in the extract. When a competitor and labeled probe were added simultaneously, no residual binding was seen (data not shown). When unlabeled pT325Mut RNA was used as the competitor in the same reaction, it was unable to fully compete for binding even at a 30-fold molar excess (Fig. 6B, lanes 8 to 12), although at a 100-fold molar excess, most of the binding was competed for (data not shown). Examination of these results shows that about 10- to 30-fold more of the pT325Mut RNA than the wild-type RNA was necessary to compete for binding (Fig. 6B; compare lanes 3 and 12).

Although both the in vitro translation data and the RNA mobility shift assays suggested that there was a defect in the binding of host cell factors to domain IV RNA, these experiments were done under conditions which may not accurately mimic those found in vivo. Importantly, the concentrations of binding proteins may differ and affect the relative levels of binding seen in our assays. To address this problem, we made two constructs, one in which the luciferase gene was fused to the AUG start codon of the poliovirus 5′ NCR (pTMLuc) and another which is identical except for the presence of the mutation at nt 325 (pTMLuc-325) (Fig. 7A). By introducing these RNAs into HeLa cells and assaying the level of luciferase produced, we could obtain quantitative information about the effects of this mutation on viral translation in vivo.

HeLa cells were transfected with 5 μg of RNA derived from either the pTMLuc or pTMLuc-325 construct and were incubated at 37°C. At either 1- or 2-h intervals, the cells were washed with PBS and lysed with luciferase assay buffer and a fraction of the lysate was assayed for luciferase activity (Fig. 7B). Over a 9-h time period, the activity of both constructs rose linearly, but the level of activity from the pTMLuc-325 construct was only about 10% of that from the wild-type pTMLuc RNA. This suggests that the lethal phenotype of this mutation in the full-length viral RNA can be attributed to a translation defect. This defect is likely due to a reduced affinity of the mutated domain IV structure for HeLa cell proteins.

Characterization of proteins which interact with domain IV RNA sequences. To characterize the cellular protein(s) which interacts with the domain IV RNA sequences, we used an in situ UV cross-linking assay. Following the resolution of RNA-protein complexes on nondenaturing gels, these complexes were subjected to UV cross-linking, excised, and then digested with RNase to effect transfer of the label to the bound protein(s), which was then resolved by SDS-PAGE. When the RSW fraction of the HeLa cell extracts was used in this assay,

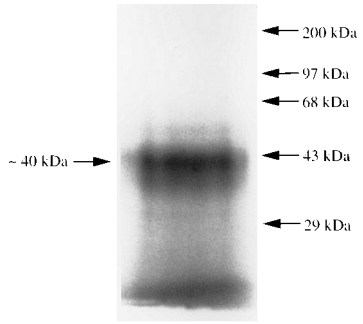

FIG. 8. Protein(s) cross-linked to domain IV RNA. ^{32}P-labeled domain IV RNA was incubated with the RSW of the HeLa cell S10 fraction. Following resolution on nondenaturing acrylamide gels, the complex was UV cross-linked, excised, and treated with RNase and the labeled protein(s) was resolved by application to a denaturing acrylamide gel. Molecular mass markers are indicated to the right of the figure.

a diffuse band with a size of approximately 40 kDa was visualized by autoradiogram (Fig. 8). The poor resolution and diffuse nature of the band migrating in a gel are commonly seen after in situ cross-linking. We were unable to visualize any bands with higher molecular weights, even after longer exposures of the film (data not shown), but we did see a smear of lower-molecular-weight material which was presumably due to either UV-induced degradation of the labeled proteins or RNA-bound lower-molecular-weight proteins.

While the above-described UV cross-linking assay provides information about proteins which contact the RNA directly, it does not allow the detection of indirect, or protein-protein, interactions. To circumvent this problem, we utilized biotinylated RNA to isolate proteins which interact with domain IV of the poliovirus 5′ NCR. Biotinylated RNA was prepared by incorporating Bio-11-UTP, in which a biotin molecule is covalently linked to the UTP via an 11-carbon linker, in the transcription reaction with T7 RNA polymerase. Biotinylated UTP was included in the reaction to give a biotinylated UTP: UTP ratio of 1:7. In preliminary experiments, this was shown by RNA mobility shift analysis to be the highest ratio at which the mobility shift and, by inference, protein binding were unaffected by the biotin moiety (data not shown). The biotinylated RNA was then used in a binding reaction similar to that used for the RNA mobility shift assay. The protein-RNA complex was isolated with magnetic streptavidin beads. After the magnetic bead–RNA-protein complex had been washed, the protein was separated from the RNA by incubation in 2× SDS-PAGE sample buffer and then was analyzed by SDS-PAGE.

In initial experiments, various fractions from HeLa cell extracts were incubated with biotinylated UTP-labeled domain IV RNA. The protein-RNA complexes were isolated and washed, and the proteins were resolved by PAGE. The proteins on these gels were then visualized with silver stain. The use of biotinylated domain IV RNA in the reaction showed the interaction of several polypeptides enriched in the RSW fraction of HeLa cells (Fig. 9A, lane 4). The major species of proteins detected have molecular masses of ~39, ~40, and ~42 kDa. These proteins were not visualized in the absence of RNA (Fig. 9A, lane 1). The S200 fraction contained limited amounts of the 42- and 40-kDa proteins. This result is consistent with the results seen following RNA mobility shift analysis (Fig. 2). The molecular weights of these proteins are in general agreement with those of the proteins labeled in the UV cross-linking experiment (Fig. 8), although it was not clear from the

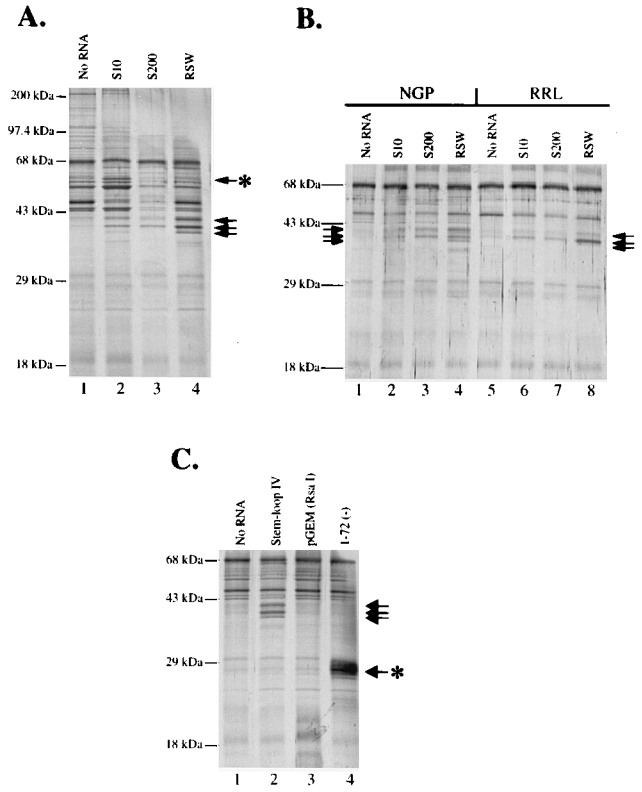

FIG. 9. (A) Capture of HeLa cell proteins bound to biotinylated domain IV RNA. Biotinylated domain IV RNA was prepared as described in Materials and Methods. This RNA was incubated with the indicated protein fractions (S10, S200, and RSW) from HeLa cells. Protein-RNA complexes were isolated by binding to magnetic streptavidin beads. The complexes were disrupted by incubation in SDS-PAGE sample buffer, and the proteins were resolved on denaturing 10% polyacrylamide gels. Molecular mass markers are shown on the left. Arrows indicate the positions of specific binding proteins found in the RSW fraction. A specific binding protein found in the S10 fraction is indicated with an asterisk. (B) Binding of neuroblastoma and RRL proteins to biotinylated domain IV RNA. Biotinylated domain IV RNA was incubated with the indicated fractions from either neuroblastoma or RRL extract. The resulting RNA-protein complexes were isolated with magnetic streptavidin beads, and the proteins were resolved on denaturing polyacrylamide gels. Molecular mass markers are shown on the left. Arrows indicate the positions of specific binding proteins. (C) Binding of HeLa cell proteins to nonspecific biotinylated RNAs. An ~150-nt biotinylated RNA derived from pGEM or an 87-nt RNA consisting of nt 72-1 of the poliovirus negative strand (numbered by positive-strand convention) was incubated with the RSW fraction from HeLa cells. Protein-RNA complexes were isolated by binding to magnetic streptavidin beads. The complexes were disrupted by incubation in SDS-PAGE sample buffer, and the proteins were resolved on denaturing 10% polyacrylamide gels. Molecular mass markers are shown on the left. Arrows indicate the positions of specific binding proteins. A binding protein which interacts with the 1-72(−) RNA is indicated (*).

UV cross-linking how many proteins were in direct contact with the labeled RNA. An additional specific interaction of an ~60-kDa protein from the S10 fraction of the HeLa cell lysates was also seen by the biotinylated RNA-protein binding method (Fig. 9A, lane 2 [marked with an asterisk]). We have not pursued the characterization of this protein.

When extracts from NGP cells were used in the biotinylated RNA assay, the distribution and molecular weights of the major proteins bound to the RNA were similar to those seen with HeLa cell extracts (compare Fig. 9B, lanes 2 to 4, with Fig. 9A, lanes 2 to 4). The intensity of the bands appears to be lower than that obtained with HeLa cell extracts, but this assay is not quantitative for the amount of protein in the extract. When RRL fractions were used, the 40-kDa protein band was easily

visualized but the 39- and 42-kDa bands were present at only very low levels. Again, it is difficult to compare the total amounts of each protein present in the HeLa and RRL fractions.

The specificity of protein binding to the biotinylated domain IV RNA was demonstrated by a control reaction which contained no RNA (Fig. 9A, B, and C, lane 1 of each figure) and by the use of either a biotinylated RNA that was transcribed from pGEM vector sequences and that was similar in size to that from domain IV or RNA from the poliovirus negative-strand RNA sequence complementary to nt 1 to 72 of the positive strand (Fig. 9C). The nonspecific RNAs did not interact with the 39-, 40-, or 42-kDa proteins. The fragment (nt 1 to 72) from the poliovirus negative strand may interact specifically with a lower-molecular-weight protein, but this has not been investigated.

DISCUSSION

Poliovirus translation occurs via internal ribosome binding mediated by the interaction of host cell proteins with the 5′ NCR of the viral RNA. Although domain IV of the poliovirus 5′ NCR plays a major role in viral translation, we have only just begun to thoroughly characterize the host cell proteins that interact with it or the role of those proteins in viral translation. Previously, RNA mobility shift analysis and UV cross-linking demonstrated that at least two proteins from HeLa cells appeared to specifically interact with the domain IV RNA (7, 14). In this report, we confirmed that a cellular protein(s) from the 0 to 40% ammonium sulfate precipitate of the RSW fraction of uninfected HeLa cells interacts with domain IV RNA. This cellular protein(s) was able to shift the labeled RNA to a more slowly migrating species that resolved as a doublet at lower protein concentrations and as a more slowly migrating single band at higher concentrations following PAGE (Fig. 2 and 3).

A concern in experiments utilizing small RNA probes for mobility shifts or other protein binding studies is that the small RNA may not present the same structure as that formed by the identical sequence within the full-length viral 5′ NCR. We used an RNA containing nt 1 to 743 of the 5′ NCR and extending into the poliovirus coding region to nt 865 to compete for the protein(s) binding to the domain IV RNA (Fig. 3). The results from this competition indicated that although the isolated domain IV RNA likely represents a population of RNAs containing different conformations, at least a significant fraction of molecules adopts a conformation which interacts with proteins found to bind to the full-length poliovirus 5′ NCR. Although such folding was not demonstrated directly here, this result suggests that the RNA fragment (nt 220 to 460) folds into a secondary and/or tertiary structure similar to that present in the full-length RNA. The competition experiments with the full-length 5′ NCR RNA also suggest that at least for the proteins involved in the mobility shift assay, domain IV was the only structure required for binding. It is possible, however, that a construct containing other domains along with domain IV would interact with new, or additional, host cell proteins. Indeed, this has recently been shown to be the case for domains V and VI, which appear to interact with a 36-kDa protein only when both domains are present (17).

It is assumed that a *trans*-acting factor(s) required for the efficient utilization of the poliovirus IRES is variably present in cells of different tissue origins, which results in cell specificity for poliovirus translation. For example, a major determinant in the differential growth rates of Sabin vaccine strains of poliovirus in neuronal cells has been suggested to be reduced translation efficiency caused by a single point mutation in domain V (10, 38, 39). One explanation for this effect is that limiting amounts of an essential translation factor in these cells prevents productive binding to the structure formed by the weakened base pair interactions. A second explanation is that a neuronal cell-specific translation factor present in normal quantities manifests differentially reduced binding to the Sabin RNA. Similarly, the temperature-sensitive phenotype conferred by the 5′ NCR mutation in Sabin type 2 virus is expressed more strongly in some cell types than in others (33). The RNAs derived from Sabin strains of poliovirus are translated at lower efficiencies than those of their parental strains in Krebs ascites cell extracts as well (54). A second example of cell specificity for poliovirus RNA translation is the poor utilization of poliovirus RNA template by RRL; supplementation of these lysates with extracts from HeLa cells increased the accuracy and stimulated the efficiency of translation (5, 8, 35, 42). Our attempts to identify cell-specific proteins that bind domain IV RNA by RNA mobility shift experiments in neuroblastoma cell extracts gave results similar to those seen with HeLa extracts (Fig. 4), suggesting that there are no neuronal cell-specific factors which interact directly with domain IV sequences. This is not unexpected since the mutation at nt 480 which leads to reduced translation in the Sabin vaccine strain is not in the domain IV sequence. Similar experiments done with RRL fractions demonstrated a greatly reduced binding activity compared with that for HeLa cell extracts, indicating that the level of proteins which interact with domain IV RNA sequences may be limiting in reticulocytes.

The 5′ NCR of poliovirus has been shown to interact with a large number of host cell proteins (9). Some of these appear to be involved in replication, and some appear to be involved in translation. Others, as of yet, have no demonstrated function. These latter proteins may be involved in the viral life cycle or may simply interact strongly and specifically with poliovirus RNA sequences in vitro but play no role in vivo. In this study, a functional role in viral translation of proteins which interact with domain IV RNA sequences was suggested by the use of a lethal mutation at nt 325. This 3-nt insertion mutation leads to a defect in translation (Fig. 5 and 7) and affects the ability of one or more proteins to interact with the RNA as demonstrated by RNA mobility shift analysis (Fig. 6). The change in the affinity of the protein(s) for the domain IV sequences may be due to a change in a specific binding sequence or a change in the local secondary structure of domain IV. Given the nature of most RNA binding proteins, which may recognize structure more than sequence, the latter explanation seems likely (11). The mutation does not lead to a total loss of binding activity but rather induces a significant reduction in affinity.

The complete loss of viability conferred by the mutation at nt 325 was surprising since translation was reduced by only about 90% in the luciferase reporter gene assay. One possibility is that this level of translation is insufficient to initiate a successful round of replication. Recently, it has been demonstrated that sequences within the poliovirus IRES, including nt 500 to 502 (domain V) and a region (nt 313 to 374) in domain IV, are involved in poliovirus RNA replication (4). The region in domain IV encompasses the mutation at nt 325, and our data coupled with the results of Borman et al. (4) suggest that the mutation may affect both translation and replication. If this is the case, then a major defect in translation may combine with a replication defect to lead to a lethal phenotype. As the work by Borman et al. (4) demonstrates, the dissociation of translation and replication defects is difficult. We have not yet investigated the contribution of a potential replication defect

to the lethal phenotype of virus containing the mutation at nt 325.

In this study, we utilized a method by which biotinylated RNA-protein complexes can be isolated and the proteins can be characterized. The advantage of this method is that protein-protein interactions, as well as direct protein-RNA interactions, can be visualized. One of the potential problems with UV cross-linking is that it can be used only to look at proteins that interact directly with the RNA in a manner which is sensitive to the assay. Biotinylated DNA has been used previously to isolate DNA-binding proteins from yeast cells and other organisms (12, 48). The very strong interaction between biotin and streptavidin allows for the isolation and purification of DNA-protein (or RNA-protein) complexes from crude cell extracts. When biotinylated domain IV RNA was incubated with proteins from a HeLa cell RSW fraction, three proteins were found to interact specifically with the domain structure (Fig. 9). These proteins had molecular masses of ~39, ~40, and ~42 kDa. One or more of the proteins visualized by this technique may be the same as the ~40-kDa protein seen by UV cross-linking (Fig. 8), although we have not yet demonstrated this directly. It is possible that only a subset of the proteins seen by the biotinylated RNA affinity assay would UV cross-link to the domain IV RNA, since only some of these proteins may interact directly with the RNA in a manner detected by UV cross-linking. Proteins which are not detected by UV cross-linking may interact via protein-protein interactions with a protein bound directly to the domain IV RNA. The biotinylated RNA method employed here would still allow us to visualize these proteins and their interaction with the domain IV sequences.

Overall, this study has provided strong evidence that proteins which interact with domain IV RNA are involved in the translation of poliovirus RNA. We have identified three polypeptides that bind specifically to domain IV RNA sequences and which may not have been seen previously by UV cross-linking methods. We are currently pursuing biochemical techniques which will allow us to identify these proteins and to demonstrate directly their involvement in the translation mechanism.

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