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Specific Interactions of HeLa Cell Proteins with Proposed Translation Domains of the Poliovirus 5′ Noncoding Region

JOHN R. GEBHARD[1]* AND ELLIE EHRENFELD[1,2]

Departments of Cellular, Viral, and Molecular Biology[1] and Biochemistry,[2] University of Utah School of Medicine, Salt Lake City, Utah 84132

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To determine which sequences or structures in the poliovirus 5′ noncoding region (5′NCR) are involved in binding proteins used for internal ribosome binding and protein synthesis initiation, translation competition assays were performed in rabbit reticulocyte lysates in the presence and absence of HeLa cell extract. The results revealed two functional domains in the poliovirus 5′NCR. One, requiring nucleotides (nts) 457 to 626, binds proteins that are required for translation of all mRNAs and that are present in both reticulocyte lysates and HeLa cell extracts. Another, contained within nts 286 to 456, interacts with proteins that are specific for poliovirus translation and are present in HeLa cells but not in significant amounts in rabbit reticulocyte lysates. In order to detect HeLa cell proteins that interact stably with the 5′NCR of poliovirus, UV cross-linking was used. At least four major protein-RNA complexes were identified, three of which were shown by RNA competition analysis to bind specifically to defined domains within the 5′NCR. Protein A (54 kDa) cross-linked to RNA sequences and/or structures located between nts 457 and 626; proteins B (48 kDa) and C (38 kDa) bound to nts 286 to 456.

Poliovirus, an enterovirus of the *Picornaviridae* family, is a single-stranded RNA virus of positive polarity. The genome of poliovirus consists of approximately 7,500 nucleotides (nts) that include one large open reading frame preceded by an unusually long, highly structured, 5′ noncoding region (5′NCR) of 742 nts. The 5′NCR is highly conserved among the three poliovirus serotypes (21, 40, 47). The 5′ terminus of the genomic RNA is not capped but is covalently linked to a small viral protein, called VPg (13, 25). Poliovirus mRNA is identical to the genomic RNA except that it lacks VPg and terminates with 5′-pUp (15, 30).

Poliovirus infection results in the degradation of the p220 subunit of eukaryotic initiation factor 4F (12), which is responsible for identifying the 5′ end of capped mRNAs for binding to ribosomes. As a result, cap-dependent translation is inactivated, and cellular protein synthesis is inhibited. However, translation of picornaviral RNAs initiates by a cap-independent mechanism (reviewed in reference 44).

The 5′NCR of picornaviruses provides a unique challenge to the translational machinery of the cell. It is of considerable length and assumes marked secondary (24, 39, 43) and probably tertiary (1) structure, which would likely impede ribosome movement. In addition, the 5′NCR contains numerous AUGs (eight in the Mahoney strain of poliovirus type 1) upstream of the initiating codon. Mutational analysis has demonstrated that only the AUG located at nts 588 to 590, most proximal to the initiating AUG (nts 743 to 745), is essential for growth and translation of the virus (31). The 5′NCRs of poliovirus and the related encephalomyocarditis virus and foot-and-mouth disease virus are able to direct translation initiation when placed between two reporter cistrons, conclusively demonstrating that ribosomes initiate translation internally in these 5′NCRs (4, 18, 19, 34). Recently, a cellular mRNA encoding immunoglobulin heavy-chain binding protein has been shown to be translated by a cap-independent mechanism (42), involving internal ribosome binding as well (26).

To identify the *cis*-acting element(s) involved in internal translation initiation of poliovirus RNA, several genetic and molecular biological approaches have been applied. Site-directed mutations of 5′NCR sequences suggested that nts 140 to 630 included regions critical for translation of poliovirus RNA (10, 22, 48, 49). In vitro translation experiments have suggested that the region essential for internal initiation resides between nts 320 and 630 (5, 32–35). The conclusions from these studies were that one or more large structural elements in the 5′NCR regulated poliovirus RNA translation, rather than a small defined sequence element. This structure has been termed a ribosome landing pad (32) or an internal ribosome entry site (IRES) (18). The IRES functions to provide a site to which ribosomes bind, thus bypassing many of the apparent impediments to scanning in the 5′NCR.

The molecular events by which the IRES participates in internal translation initiation are not understood. It has been suggested that cellular proteins participate in binding of the 40S ribosomal subunit to the IRES. The protein(s) could act directly as a ribosome recognition site or act to alter the RNA structure to facilitate ribosome entry. In addition, ribosome entry could take place directly at the correct AUG (17, 20), or alternatively, the ribosome could scan from an internal entry site to the correct AUG (17, 44). It should be noted that the IRES for enteroviruses-rhinoviruses (e.g., poliovirus) and cardioviruses-aphthoviruses (e.g., encephalomyocarditis virus) differ considerably in their proposed 5′NCR secondary structures (17, 38, 39).

We are interested in identifying the cellular protein(s) that interacts with the 5′NCR of poliovirus RNA, which may facilitate its translation. In other laboratories, proteins have been detected that bind to short segments of poliovirus 5′NCR RNA. A 50-kDa protein, isolated from HeLa cell extracts, was shown to interact with nts 186 to 221 via a transient covalent adduct (29). In addition, gel retardation

* Corresponding author.

assays reveal a number of cytoplasmic protein interactions with truncated 5′NCR RNAs (9). One of these is eukaryotic initiation factor 2, a protein that was independently implicated in correct translation start site selection in vitro (46). A 52-kDa cellular protein specifically binds to nts 559 to 624 (28). Recently, a 57-kDa protein which bound to the 5′NCR of encephalomyocarditis virus was shown to bind specifically to the 5′NCR of poliovirus as well (20). Neither the functions of these proteins nor the physiological significance of their interaction with poliovirus 5′NCR RNA fragments has been elucidated.

In order to detect cellular proteins which interact with the poliovirus 5′NCR and may be involved in poliovirus protein synthesis, we first sought to identify which RNA sequences and/or structures were important, using in vitro translation competition. The results of these studies distinguish two domains in the proposed poliovirus IRES: one domain interacts with proteins specifically required for translation of poliovirus RNA, and a second domain binds general translation factors. UV cross-linking was used to detect proteins which stably interacted with the 5′NCR. The binding probe was an RNA fragment that contained the entire 5′NCR, in order to include proteins whose interactions might depend on higher-order RNA secondary or tertiary structures. Specific binding domains were mapped by binding competition experiments with shorter 5′NCR RNAs based on the results from translation competition studies. Two proteins interacted specifically with the proposed poliovirus RNA translation domain, while a third interacted with the domain important in translation of other mRNAs.

MATERIALS AND METHODS

Plasmids. pTPOV-2954 contains the first 2,954 nts of the Mahoney strain of poliovirus type I cDNA under the control of the T7 promoter (5). pRA NS, which contains a truncated vesicular stomatitis virus (VSV) NS gene under the control of the SP6 promoter, was the kind gift of A. Banerjee (7). pCAT/Z is a bicistronic vector which contains the chloramphenicol acetyltransferase (CAT) gene and *lacZ* gene fragment under the control of the T7 promoter and was kindly provided by Xi-Yu Jia.

Preparation of HeLa cell extract. The HeLa cell extract used in both the UV cross-linking and in vitro translation studies was prepared from HeLa S3 cells as described previously (6).

Preparation of RNA substrates. pTPOV-2954, pRA NS, and pCAT/Z cDNAs were linearized with the appropriate restriction endonucleases, and DNA fragments containing the phage promoter and adjacent sequences were purified from agarose gels. These DNA templates were transcribed (27) with either 120 μCi of [α-^{32}P]UTP (1.6×10^{11} μCi/mol) per 50 μl or 3.6 μCi of [^{3}H]UTP (2.5×10^{7} μCi/mol) per 100 μl as the radiolabeled nucleotide. Reaction mixes were treated with RNase-free DNase I (10 U) (Stratagene), and transcripts were extracted with phenol-chloroform. Unincorporated nucleotides were removed by spin column chromatography with Sephadex G-50 by the method of Maniatis et al. (27). RNAs were analyzed for length and integrity on 1% agarose gels containing methylmercury hydroxide and visualized by autoradiography or ethidium bromide staining. RNA concentrations were calculated from the specific activity of the radiolabeled nucleotide in the transcription reaction mix.

UV cross-linking of RNA and protein. The protocol for analysis of RNA-protein interactions is based on the UV cross-linking studies of Garcia-Blanco et al. (14). The binding buffer contained 8.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4), 3 mM $MgCl_2$, 1.3 mM ATP, 5 mM creatine phosphate, 27 mM KCl, 1 mM dithiothreitol, and 4% glycerol. A mixture of 10 μg of *Escherichia coli* 16S and 23S rRNA (Boehringer Mannheim) and 6 to 15 μg of HeLa S10 cell extract in binding buffer was incubated for 6 min at room temperature. The mixture was then added to the RNA probe (0.5×10^{6} to 1.0×10^{6} cpm/1.5 ng) to give a final volume of 15 μl and incubated for 25 min at 30°C. After binding, the reaction mixtures were irradiated in 96-well microtiter plates placed 4.5 cm from the UV light source (5,200 mW/cm^2) of a Stratalinker 1800 UV cross-linker (Stratagene). Unprotected RNA was digested with 1 μg of pancreatic RNase A (Sigma) for 15 min at 30°C. The cross-linked RNA-protein complexes were separated by electrophoresis on 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels (23). The gels were stained, dried, and autoradiographed.

When required, competitor RNAs were included with the RNA probe, and the protein mixture was added to the combined RNAs.

In vitro translation. Translation studies were performed with either poliovirion RNA or in vitro-transcribed RNA transcripts to program micrococcal nuclease-treated rabbit reticulocyte lysates (RRL; Promega). Each 20-μl translation reaction mix contained 0.2 μg of mRNA and 10 μCi of [^{35}S]methionine (1,000 Ci/mmol). Where indicated, reaction mixes were supplemented with a micrococcal nuclease-treated HeLa S10 extract constituting 10 to 15% of the final volume. This was the amount of extract required to minimize the appearance of aberrant initiation products. After a 2-h incubation at 30°C, the products were analyzed on 10% polyacrylamide–SDS gels. Autoradiograms on preflashed film were subjected to laser densitometry to quantitate the changes in band intensity.

RESULTS

Effect of truncated RNAs on translation of poliovirus 5′NCR-containing RNAs. The poliovirus 5′NCR functions not only in translation of poliovirus proteins, but in RNA replication and perhaps RNA packaging and assembly as well. An in vitro translation competition assay was developed in order to identify regions of the poliovirus 5′NCR which may bind cellular proteins used in poliovirus RNA translation. We reasoned that if poliovirus 5′NCR RNA fragments bound cellular translation components, they would compete with RNAs containing the complete 5′NCR and thereby cause a reduction in the translation of the latter RNAs. Furthermore, since factors in HeLa cell extracts promote efficient utilization of the correct initiation site for translation of poliovirus RNA (6, 11, 36, 45), performing the translation competition in RRL in both the presence and absence of HeLa cell extract might implicate those 5′NCR domains that interact with specific HeLa cell proteins in the translation reaction.

An RNA transcript containing nts 1 to 2954 (Fig. 1) of poliovirus RNA sequence was translated in an unsupplemented RRL. This transcript (PV-2954) included the entire poliovirus 5′NCR plus the first 2,211 nts, coding for the viral capsid protein precursor. The major product of translation, shown in Fig. 2A, lane a, was the predicted 84-kDa portion of capsid protein precursor sequences; in addition, numerous smaller products resulting primarily from aberrant internal initiations were formed, as has been described previously

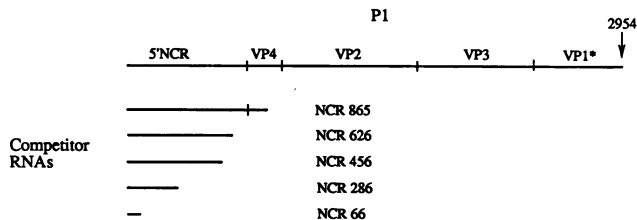

FIG. 1. Schematic representation of the poliovirus RNA reporter transcript (PV-2954) and the 5'NCR truncated poliovirus RNAs used for in vitro translation competition.

(5, 16). The proportion of translation products from "correct" initiation was increased by the addition of a HeLa cell extract to the translation reaction mix (Fig. 2A, lane b). It should be noted that different batches of RRL manifest marked variability in their abilities to select the correct initiation site for translation of poliovirus RNA in unsupplemented reaction mixes.

Translations were performed in the presence of increasing molar concentrations of the truncated RNA fragments, of the same polarity as the mRNA (Fig. 1), in order to determine whether these fragments bound proteins necessary for protein synthesis. Each truncated RNA cntains contiguous sequences from the poliovirus RNA starting at nt 1 and ending at the nucleotide whose number is given in Fig. 1. Addition of NCR626 RNA produced a marked, dose-dependent reduction in translation (Fig. 2A, lanes c to f). Similarly, NCR456 RNA interfered with translation of PV-2954 RNA (Fig. 2A, lanes g to j), either by binding essential proteins required for poliovirus translation or by some other mechanism. In contrast, neither NCR286 nor NCR66 had any effect on translation of PV-2954 RNA (Fig. 2B). The lack of competition by these RNAs may be due either to a loss of a binding site(s) or an alternate structure assumed by the RNA, so that the translation proteins are not bound. The reduction in translation observed upon addition of some transcripts to the translation reaction mix was not due to the

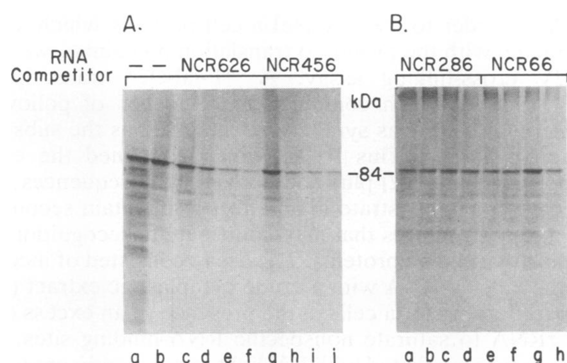

FIG. 2. In vitro translation of PV-2954 RNA in the presence and absence of competitor RNA. PV-2954 RNA was translated in RRL supplemented with HeLa cell extract in the presence of the indicated competitor RNA. Panel A, lane a, contained no HeLa cell extract. (A) Translation of PV-2954 RNA alone (lanes a and b) or with a 1-, 5-, 10-, or 25-fold molar excess of NCR626 RNA (lanes c through f, respectively) or NCR456 RNA (lanes g through j, respectively). (B) Translation of PV-2954 RNA with competitor RNA NCR286 (lanes a through d) or NCR66 (lanes e through h) at a 1-, 5-, 10-, and 25-fold molar excess, respectively. The 84-kDa protein product expected from translation of PV-2954 is indicated.

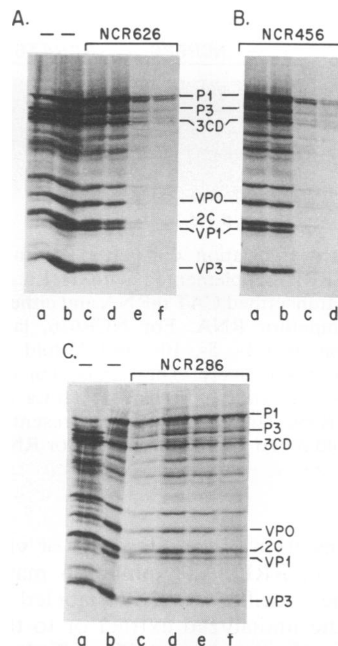

FIG. 3. In vitro translation competition with poliovirion RNA. RRL supplemented with HeLa cell extract (except for lane a in panels A and C) was added to virion RNA and either (A) NCR626, (B) NCR456, or (C) NCR286 competitor RNA at a 0-, 0.5-, 1-, 10-, and 25-fold molar excess (panels A and C, lanes b through f, respectively) or a 0.5-, 1-, 10-, and 25-fold molar excess (panel B, lanes a through d, respectively).

presence of small amounts of double-stranded RNA in the transcript preparation, since further addition of poly(I): poly(C) (25 or 50 μg/ml) did not relieve the inhibition.

A heterologous RNA competitor was used to confirm the specificity of the effect observed with the longer poliovirus RNA fragments. An antisense luciferase RNA of 600 nts, when added at 25-fold molar excess to PV-2954, had no effect on PV-2954 translation (not shown). Thus, the 5'NCR region between nts 287 and 626 appears to bind proteins needed for translation of poliovirus RNAs and thus constitutes a translation domain.

Similar experiments were performed with the truncated NCR RNAs as competitors against full-length poliovirion RNA in RRL supplemented with HeLa cell extract (Fig. 3). Again, NCR626 and NCR456 (Fig. 3A and B) appeared to compete for essential translation proteins, while NCR 286 (Fig. 3C) and NCR66 (not shown) did not compete.

Specificity of translation competition. Proteins that interact with poliovirus RNAs to promote translation might be general translation factors, required for translation of all mRNAs, or they might be specific for the interactions that result in cap-independent internal initiation mediated by the poliovirus IRES. In order to distinguish these two classes of RNA-protein interactions, the truncated poliovirus NCR RNAs were tested for their abilities to interfere with translation of a nonviral, uncapped mRNA coding for CAT. CAT mRNA is translated extremely efficiently in RRL (Fig. 4A, lane a). Addition of HeLa cell extract in the same concentrations used in the experiments shown in Fig. 2 and 3 significantly inhibited CAT mRNA translation (Fig. 4A, lane b). The reason for this effect is unknown, but we have repeatedly seen apparent inhibition by crude HeLa cell

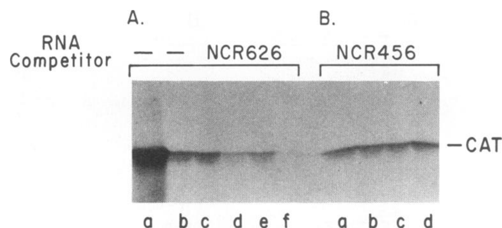

FIG. 4. In vitro translation competition with a heterologous RNA template. RRL supplemented with HeLa cell extract was added to in vitro-transcribed CAT mRNA and either (A) NCR626 or (B) NCR456 competitor RNA. For NCR626, lanes c through f represent addition of a 1-, 5-, 10-, and 25-fold molar excess of competitor RNA, respectively. The minus sign indicates that no competitor RNA was added. In lane a, HeLa cell extract was omitted. For NCR456, lanes a through d represent addition of a 1-, 5-, 10-, and 25-fold molar excess of competitor RNA, respectively.

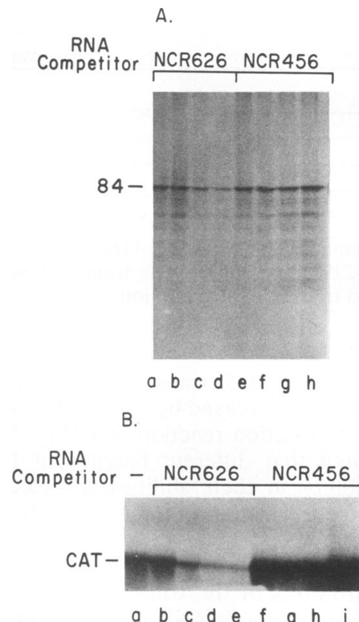

FIG. 5. In vitro translation competition in the absence of correcting concentrations of HeLa cell extract. (A) RRL was supplemented with HeLa cell extract for translation of PV-2954 RNA plus NCR 626 (lanes a through d) or NCR456 (lanes e through h) competitor RNAs at a 1-, 5-, 10-, and 25-fold molar excess, respectively. This amount of HeLa cell extract was insufficient to correct aberrant initiation on poliovirion RNA. (B) RRL with no HeLa cell extract was used to translate CAT mRNA plus NCR626 (lanes b through e) or NCR456 (lanes f through i) competitor RNAs at a 1-, 5-, 10-, and 25-fold molar excess, respectively. Translation in the absence of competitor RNA is shown in lane a.

extracts on translation of other cellular or viral (non poliovirus) mRNAs in RRL. The inhibition may be due to a reduction in the specific activity of labeled methionine by dilution from the undialyzed extract or to the presence of inhibitory factors in the extract. The effects of addition of NCR626 and NCR456 on translation of CAT mRNA under these conditions were determined (Fig. 4). As observed with poliovirus RNAs in the presence of NCR626 competitor RNA, CAT mRNA translation was substantially reduced by this RNA (Fig. 4A, lanes c to f); a 25-fold molar excess of NCR626 RNA caused an 18-fold reduction in translation, and 5- and 10-fold molar excesses of RNA produced a 4- to 5-fold reduction. No reduction in CAT synthesis was effected by NCR456 RNA (Fig. 4B, lanes a to d). This is in sharp contrast to the strong competitive effect that NCR456 RNA had on translation of poliovirus RNAs (compare Fig. 2 and 3). Inclusion of NCR286 or NCR66 in CAT mRNA translation reaction mixes also had no effect (not shown). The same pattern of translation inhibition was obtained for VSV mRNA as for CAT mRNA. These results suggest that nts 456 to 626 participate in the formation of some RNA structure or sequence element that binds proteins required for translation of other mRNAs, but that elements including nts 286 to 456 bind proteins specific for translation of poliovirus RNAs.

Translation in the absence of HeLa cell extract. The above experiments were performed in RRL supplemented with HeLa cell extracts and did not distinguish between RNA competition for proteins in the RRL or in the HeLa extract. To address this question, translation competition experiments were performed in the absence of HeLa cell extract or in the presence of extract in amounts which were insufficient to correct the translation profile of poliovirus RNA. Figure 5A shows that under the latter conditions, NCR626 again effectively reduced translation of PV-2954 RNA (about 60% reduction), but NCR456 failed to compete with the template. The same results were obtained when the effects of the competitor RNAs on translation of CAT mRNA in the absence of HeLa cell extract supplement were analyzed (Fig. 5B). In the absence of the HeLa cell extract, NCR626 RNA is an effective competitor while NCR456 RNA is not, with both poliovirus and nonviral templates.

Thus, it appears that a domain including nts 456 to 626 in the poliovirus 5′NCR binds general translation factors that are present in reticulocyte lysates as well as in HeLa cell extracts; nts 286 to 456, on the other hand, appear to be required to bind proteins that are specific for the poliovirus sequence and which are present in HeLa cells but not in RRL in significant amounts.

Interaction of cellular proteins with the 5′NCR of poliovirus RNA detected by UV cross-linking. The above experiments suggested protein binding by specific domains of poliovirus RNA. In order to identify HeLa cell proteins which stably interacted with the proposed translation domain(s), we used a UV cross-linking assay. A ^{32}P-labeled transcript of pTPOV-2954, which contained nts 1 to 865 of poliovirus RNA (NCR865), was synthesized and used as the substrate for cross-linking. This RNA, which contained the entire 5′NCR (nts 1 to 742) plus additional coding sequences, was selected as the substrate in an effort to maintain secondary or tertiary structures that may contribute to recognition and binding by specific proteins. The assay consisted of incubating the labeled RNA with a crude cytoplasmic extract (S10) prepared from HeLa cells in the presence of an excess of *E. coli* rRNA to saturate nonspecific RNA-binding sites. The mixture was irradiated with UV light to covalently cross-link proteins bound in close contact with the labeled RNA. After cross-linking, the mixture was digested with pancreatic RNase A. Proteins cross-linked to the RNA substrate became radioactive by label transfer from the RNA and were detected by autoradiography after SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 6 shows a typical pattern of proteins covalently cross-linked to NCR865 by UV irradiation. The figure demonstrates the effect of increasing concentrations of the S10 extract with a constant amount of NCR865 RNA. Four

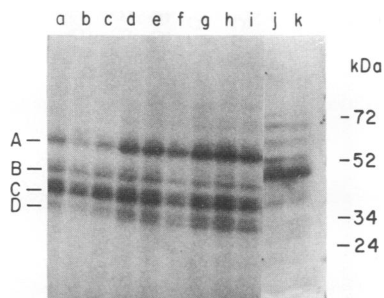

FIG. 6. UV cross-linking of proteins to poliovirus NCR865 RNA. Cross-linking was performed as described in Materials and Methods with 5, 10, 15, 25, 36, 45, 55, 60, or 72 μg of HeLa cell extract (lanes a through i, respectively) or 5 and 10 μl of commercial RRL (lanes j and k, respectively). The numbers refer to molecular masses of poliovirus protein standards. Lanes j and k were spliced from the same gel as lanes a through i. The positions of proteins A through D are indicated.

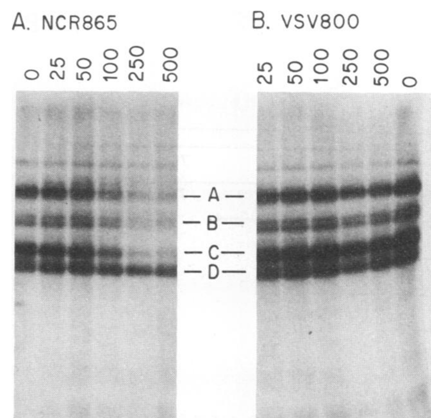

FIG. 7. UV cross-linking of HeLa cell proteins to NCR865 RNA in the presence of competitor RNA. HeLa cell extract was added to ^{32}P-NCR865 RNA in the presence of either (A) ^{3}H-NCR865 RNA or (B) ^{3}H-VSV800 RNA. The numbers above the lanes indicate the molar excess (fold) of each competitor RNA.

major proteins, which usually appeared as doublets, were detected. Their mobilities on SDS-polyacrylamide gels indicate molecular weights of approximately 54,000, 48,000, 38,000, and 35,000. The cross-linked proteins did not represent the most abundant proteins in the extract, and the pattern did not reflect the total protein profile in the extract. Increasing the concentration of protein extract increased the amount of cross-linking to some proteins (e.g., bands labeled A and D in Fig. 6), whereas some proteins appeared to saturate the RNA-binding sites even at the lowest concentration tested (Fig. 6, lane a, bands B and C). These differences may reflect different amounts of particular proteins in the extract or may indicate differing affinities of the proteins for the NCR865 RNA. Some additional proteins appeared as faint bands, indicating low levels of cross-linking, at much higher concentrations of protein (>120 μg of protein; data not shown). These complexes were not analyzed further. For a given extract, the cross-linked protein profile was reproducible in different experiments; different HeLa cell extract preparations showed some variability in the relative intensities of particular proteins, although the same general pattern was always evident.

The labeling of proteins was dependent upon UV irradiation; no RNA-protein complexes were detected without irradiation. A time course experiment demonstrated that the pattern of cross-linked proteins seen in Fig. 6 was evident after only 3 min of irradiation, reached maximal intensities at 14 min, and showed no further increase in signals when cross-linking was extended to 30 min. RNase digestion was required to detect individual protein species, since its omission resulted in a smear of unbound labeled RNA plus numerous unresolved protein-RNA complexes. Pronase treatment following RNase digestion abolished the pattern, as did omission of cell extract. Inclusion of rRNA successfully eliminated a large background of presumably nonspecific RNA-binding proteins. Increasing the concentration of rRNA up to a 1,500-fold molar excess over the NCR865 RNA failed to eliminate the binding of proteins A through D to NCR865 RNA (data not shown). Adjusting the salt concentration of the reaction mix during binding and irradiation from 25 to 100 mM KCl had no effect on the pattern of cross-linked proteins.

Figure 6 also shows the profile of proteins in an RRL that bind and cross-link to NCR865 RNA (lanes j and k). These lysates are frequently used for in vitro translation of poliovirus RNA but have been shown to be deficient in some factor(s) required for accurate initiation at the correct AUG (6, 11, 36, 46). The proteins that bind NCR865 RNA in the RRL display a different profile from those that bind it in HeLa cells. Some, however, have similar mobilities (e.g., the protein comigrating with A); it is not known whether these are homologous proteins.

Specificity of protein complex formation with the 5′NCR of poliovirus RNA. The specificity of the protein interactions with NCR865 RNA was examined through the use of homologous (NCR865) and heterologous (VSV800) RNAs in competition experiments. A subsaturating amount (15 μg) of HeLa cell protein extract was added to labeled NCR865 RNA premixed with increasing amounts of unlabeled RNA, and the reaction mixes were cross-linked and processed as in Fig. 6. Figure 7A shows a reduction in labeled proteins A through C by competition from a 50-fold or greater molar excess of the same NCR865 unlabeled RNA. Under these conditions, reduction in complex D was not observed. When another RNA, representing the first 800 nts of the VSV NS mRNA (VSV800), was used to compete for protein binding to the poliovirus NCR865 RNA, no reduction in the formation of any protein complexes was observed (Fig. 7B). A second heterologous RNA, CAT mRNA, also did not reduce the intensity of the protein labeling pattern produced with NCR865 (not shown). In contrast, intact poliovirion RNA competed for the binding of proteins A through C at the same excess molar concentrations as did NCR865 RNA itself (not shown). These results demonstrate that the poliovirus 5′NCR binds specifically to proteins A through C.

Mapping of protein binding sites to specific domains of poliovirus 5′NCR. To localize specific regions of the poliovirus 5′NCR that interacted with proteins A through C, equilibrium competition experiments similar to those illustrated in Fig. 7 were performed, utilizing truncated 5′NCR poliovirus RNAs as competitor RNAs against labeled NCR865 RNA. The rationale for this experiment is that if the truncated RNAs retained binding sites for specific proteins, they would compete with the complete NCR865 RNA and reduce the cross-linking of that subset of proteins to the labeled 865-nt probe. The nested set of truncated RNAs used as competitors are shown in Fig. 8A and are similar to those used in the translation competition. Competition cross-

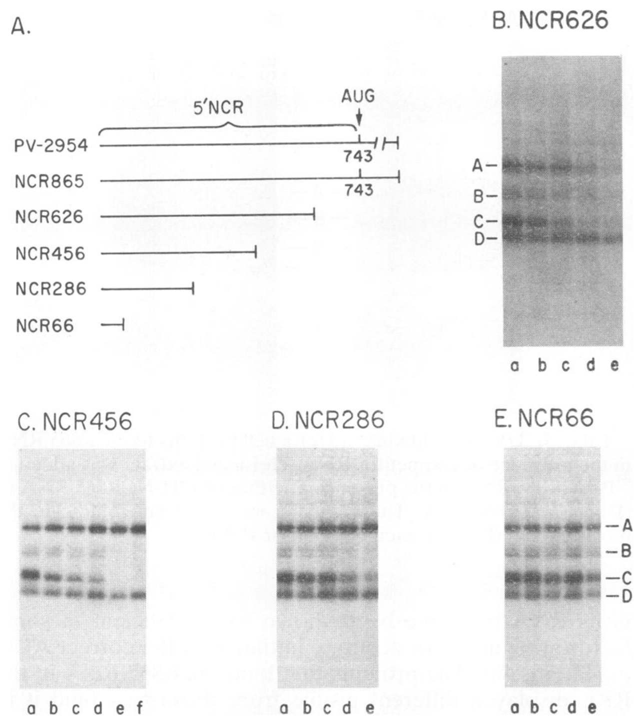

FIG. 8. Mapping of protein cross-linking to specific domains of NCR865 RNA. (A) Schematic representation of the truncated poliovirus 5'NCR RNAs used in the mapping studies. The initiation codon at nt 743 is indicated. UV cross-linking competition studies were performed as described in Materials and Methods. (B through E) Separate competition studies of each indicated competitor RNA with labeled NCR865 RNA. Lanes a through e in panels B, D, and E represent a 25-, 50-, 100-, 250-, and 500-fold molar excess of each competitor RNA over NCR865 RNA, respectively. In panel C, these reactions are shown in lanes b through f, respectively; lane a had no competitor RNA.

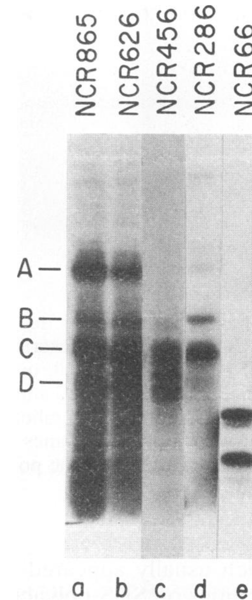

FIG. 9. UV cross-linking of HeLa cell proteins to competitor RNAs. The RNAs indicated at the top of each lane were uniformly labeled and UV cross-linked as described in Materials and Methods, using 40 μg of HeLa cell extract per 15-μl reaction mix. All lanes were spliced from the same gel. Lane c was a short exposure (2 h, versus 14 h for other lanes) in order to demonstrate the binding pattern.

linking in the presence of each competitor RNA is shown in Fig. 8B through E. Figure 8B demonstrates that an RNA containing 5'NCR nts 1 to 626 (NCR626) competes successfully with radiolabeled NCR865 for all of the proteins (A through C) that are affected by self-competition with nts 1 to 865 (Fig. 7A). On a molar basis, competition by NCR626 was only slightly less effective than that by NCR865. These data tentatively assign the binding sites for these proteins between nts 1 and 626 and suggest that the shorter RNA is able to form the same structures and/or to expose the same sequences recognized by the proteins as the larger RNA. It is still possible that nts 626 to 865 contain additional binding sites. Nts 1 to 626 have been shown previously to contain all of the information required for cap-independent translation and internal initiation of poliovirus RNA (1, 17). This RNA also showed the ability to interfere with translation (Fig. 2A).

NCR456 RNA yielded different results when used in competition experiments (Fig. 8C). This RNA effectively competed with NCR865 RNA for proteins B and C; however, NCR456 did not compete for the binding of protein A, even at a 500-fold molar excess. These data suggest that the binding site for protein A lies between nts 456 and 626 and localizes more precisely the binding domain for proteins B and C between nts 1 and 456. A 52-kDa HeLa cell protein was previously reported by Meerovitch et al. (28) to interact specifically with poliovirus RNA nts 559 to 624. The position of the protein A binding site and its apparent molecular weight are consistent with its being the same protein as that described by Meerovitch et al.

To further define the binding region for proteins B and C, two additional truncated RNAs were prepared (Fig. 8A). NCR286 was not an effective competitor for protein cross-linking to NCR865 RNA, except at very high molar excess concentrations, when weak competition for proteins B and C was observed (Fig. 8D). This might indicate that NCR286 contains some sequence or structure element to which these proteins can bind, but additional sequences are likely required to form an optimal structure that allows high-affinity protein-RNA interactions. Clearly, proteins B and C interact more strongly with nts 1 to 456 than nts 1 to 286. Finally, an RNA comprising only the first 66 nts of the 5'NCR was used as a competitor RNA (Fig. 8E). NCR66 failed to reduce protein complex formation of any proteins cross-linked to NCR865.

The truncated RNAs used as competitors in the above cross-linking experiments were tested directly for their abilities to bind and cross-link proteins in the HeLa cell cytoplasmic extract (Fig. 9). NCR865 and NCR626 demonstrated similar patterns, cross-linking primarily to proteins A through D (Fig. 9, lanes a and b). This confirms the competition results, which suggested that both of these RNAs bound to this set of proteins. When NCR456 was used as a labeled RNA substrate, protein A no longer bound, whereas proteins B through D still showed cross-linking (Fig. 9, lane c); in addition, several new cross-linked proteins were detected, demonstrating that the truncated 456-nt RNA presents some structural element(s) not present in the longer RNAs. Failure to form protein complex A is consistent with the results of the competition experiments (Fig. 8C).

NCR286 RNA cross-linked efficiently to proteins B and C (Fig. 9, lane d), despite being able to compete with NCR865 only very poorly for these proteins (Fig. 8C). Competition experiments with a 200- to 500-fold molar excess of NCR865 to labeled NCR286 showed competition of the longer RNA for both proteins B and C (not shown). This may illustrate an important aspect of the poliovirus 5'NCR structure. Both NCR456 and NCR286 bound protein complexes B and C, yet only NCR456 could efficiently compete for these proteins against the entire 5'NCR (NCR865). This suggests that the structure adopted by nts 1 to 456 presents a better binding site(s) for these proteins than that assumed by nts 1 to 286 and that the structure bound in NCR456 is likely quite similar to that formed by that region in the context of the longer NCR865 RNA. In addition, the unmapped protein complex D was not formed with NCR286. Finally, NCR66, which failed to compete for any protein binding with NCR865, also failed to demonstrate any cross-linking to proteins A through D (Fig. 9, lane e). Instead, NCR66 cross-linked to approximately two to four low-molecular-weight proteins which did not show any binding to any of the larger RNAs. Similar proteins from an RRL also bound to NCR66 (not shown). It is unlikely that this binding is functionally significant.

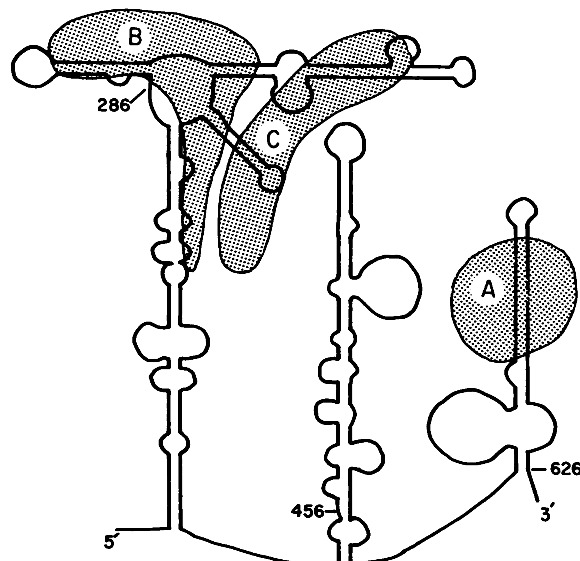

FIG. 10. Schematic diagram of HeLa cell proteins bound to nts 230 to 626 of poliovirus 5'NCR. The structure of the RNA was taken from the report of Agol (1).

DISCUSSION

The 5'NCR of poliovirus RNA serves multiple functions. This region is known to function in ribosome binding and translation (5, 10, 31, 32, 34, 35, 46, 48, 49), and it may also be involved in RNA replication (2, 3) and assembly and packaging of RNA into virions. It is not known whether host cell factors play a role in the latter processes, but several lines of evidence suggest that *trans*-acting cellular proteins are important for translation initiation from the IRES (6, 8, 11, 45). We sought to determine which RNA sequences or structures serve as elements for interacting with translation proteins and to identify some of these proteins. Competition translation analysis demonstrated that RNA sequences or structures contained in the region between nts 286 and 626 were required to bind factors needed for translation (Fig. 2 and 3). Identification of this domain correlates well with the findings of other investigations designed to identify *cis*-acting elements in poliovirus RNA that regulate translation (reviewed in reference 1). Further analyses, however, demonstrated that poliovirus RNA nts 457 to 626 competed for proteins required for translation of all mRNAs tested, while nts 286 to 456 competed specifically for poliovirus RNA translation and had no effect on translation of CAT or other mRNAs (Fig. 2 through 4). This suggests that contained within nts 286 to 456 is a sequence or structure which interacts with proteins specifically required for poliovirus translation. In addition, those factors that interacted specifically with poliovirus RNA were present in HeLa cell extracts but were either absent or greatly reduced in reticulocyte lysates, whereas the general translation factors bound by nts 456 to 626 were present in both cell types (Fig. 5). Perturbations in nts 456 to 626 might impair translation by reducing or destroying binding sites needed by ribosomes or general translation proteins.

The suggestion that 5'NCR RNA fragments inhibited poliovirus RNA translation by sequestering essential proteins led us to investigate whether we could demonstrate specific protein binding to these regions of RNA. We used UV cross-linking and competition assays to demonstrate and localize the binding of several cellular proteins to the poliovirus 5'NCR. Four major protein-RNA complexes were identified, each of which appeared to migrate as doublets in SDS-PAGE, after RNase digestion (Fig. 6). It is not clear whether the migration of each RNA-bound protein as two closely spaced bands results from the cross-linking and/or RNase digestion, or whether the bands represent two different RNA-bound proteins of similar molecular weight. In most experiments, the more slowly migrating band in the complex called protein C appeared to be more sensitive to competition by RNA fragments than the faster-migrating band (e.g., Fig. 7A and 8B and C). By this criterion, the two bands called protein C may be two different proteins. The doublets comprising the RNA-bound proteins A, B, and D always behaved similarly in competition experiments. We have designated each pair of bands with a single-letter name until further studies determine whether there are two different proteins in each complex.

A protein of about 54 kDa (protein A) cross-linked to RNA sequences and/or structures located between nts 457 and 626. This region encompasses a conserved hairpin loop (Fig. 10) that has been shown to be important for efficient translation in an RRL supplemented with HeLa cell extract (5). Meerovitch et al. (28) reported that p52, a HeLa cell protein that interacts specifically with nts 559 to 624 of the 5'NCR of poliovirus RNA, was deficient in RRL, and suggested that this deficiency may account for the aberrant in vitro translation pattern exhibited by poliovirus RNA in RRL. The RNA-binding protein A described in this report may be the same protein identified by Meerovitch et al. A protein with similar mobility that binds the poliovirus 5'NCR RNA was detected in RRL (Fig. 6). We have observed that different RRLs contain variable amounts of the 54-kDa protein, as indicated by variable intensities of cross-linked material migrating at the position of protein A. If this protein is partly or wholly responsible for the RRL deficiency for translation of poliovirus RNA, the finding of variable amounts of protein A (p52) in different lysates is consistent with the observed variation in the abilities of RRL to support poliovirus RNA

translation in the absence of HeLa cell supplements (6, 11, 36, 46).

Two other proteins, B (48 kDa) and C (38 kDa), cross-linked to poliovirus RNAs containing nts 286 to 456. This region contains a highly organized structure with multiple stem-loop formations (24, 39, 41, 43) and is considered an essential part of the IRES used to direct ribosomes to the internal translation initiation site (34). Figure 10 shows a model for the proposed secondary structure of the poliovirus 5'NCR, adapted from that derived by Agol (1). Proteins A through C are indicated schematically, interacting with nucleotide sequences in the domains suggested by this study. Although it is possible that proteins other than those detected by UV cross-linking bind poliovirus RNA and may be responsible for translation competition, the results are consistent with the presence of protein A in both HeLa cell extract and RRL, whereas proteins B and C were not detected or were deficient in RRL. Since proteins B and C affect translation specifically of sequences adjacent to the poliovirus 5'NCR, it is possible that these proteins function in the novel, cap-independent, internal ribosome binding for initiation of translation by poliovirus RNA.

In the cross-linking studies described here, we chose to use a relatively long RNA substrate (865 nts) that included the entire 5'NCR plus some coding sequences, in the hope that this RNA would fold into a three-dimensional structure similar to what is formed by the complete viral RNA. This might be an essential feature in the binding of proteins that normally interact with viral RNA. The success of this approach was confirmed by the ability of full-length virion RNA to compete with the substrate NCR865 for the binding of proteins to the same extent as NCR865 competed with itself. Smaller fragments, however, were used in the competition experiments designed to map the protein-binding sites. The caveat in interpreting the competition experiments is that a truncated RNA might contain the sequences utilized for specific protein binding but might adopt an altered conformation, so that the structure recognized by the proteins is not found. In this case, a protein might fail to bind to a truncated RNA even though the nucleotides constituting its binding site were present in that RNA. For this reason, the demonstrated presence of a binding site on a truncated RNA is more reliable than the loss of a binding site.

When the smaller RNA fragments were tested directly for their abilities to bind proteins in the HeLa cell extract, fragment NCR456 appeared to cross-link to several new proteins not bound by the longer RNA, and NCR66 bound exclusively several low-molecular-weight proteins not observed previously (Fig. 9). The cross-linking of smaller fragments to unique proteins not cross-linked by the longer RNAs illustrates the danger in using small RNA fragments to mimic protein-binding sites without performing competition experiments with the intact RNA.

Molecular genetic analyses of the poliovirus 5'NCR have shown that mutations in the region between nts 286 and 456 are not easily tolerated. Nucleotide alterations in this region were lethal (10, 48) or produced viruses with a small-plaque or temperature-sensitive phenotype (22, 48). Further analysis of the small-plaque viruses indicated that one aspect of the growth defect was in translation (48). It would be of interest to determine whether the proteins B and C identified in this study interact less well with the 5'NCR of these mutant viruses. Mutations in the downstream domain (nts 457 to 626) were more readily tolerated, although some mutations were lethal (10, 22, 48); in fact, deletion of nts 564 to 726 resulted in recovery of viable virus (22). This result appears to contradict the view that the binding of protein A (or any other proteins) to nts 457 to 626, or the binding of p52 to nts 559 to 624 (28), could be important for translation of viral RNA. A possible explanation has been advanced by Pilipenko et al. (37), who identified a tandemly repeated sequence element in this region that was predicted to generate a similar stem-loop p52 binding site when the deleted RNA folded.

In conclusion, we have identified proteins that interact with specific regions of the poliovirus 5'NCR that are implicated in internal ribosome binding for translation. The identification of proteins that function in poliovirus RNA translation represents an initial step toward understanding the mechanism of cap-independent, internal ribosome binding, as well as in elucidating the function of these proteins in uninfected cells.

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