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Functional Interaction of Heterogeneous Nuclear Ribonucleoprotein C with Poliovirus RNA Synthesis Initiation Complexes

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We had previously demonstrated that a cellular protein specifically interacts with the 3' end of poliovirus negative-strand RNA. We now report the identity of this protein as heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2. Formation of an RNP complex with poliovirus RNA was severely impaired by substitution of a lysine, highly conserved among vertebrates, with glutamine in the RNA recognition motif (RRM) of recombinant hnRNP C1, suggesting that the binding is mediated by the RRM in the protein. We have also shown that in a glutathione *S*-transferase (GST) pull-down assay, GST/hnRNP C1 binds to poliovirus polypeptide 3CD, a precursor to the viral RNA-dependent RNA polymerase, 3D^{pol}, as well as to P2 and P3, precursors to the nonstructural proteins. Truncation of the auxiliary domain in hnRNP C1 (C1ΔC) diminished these protein-protein interactions. When GST/hnRNP C1ΔC was added to *in vitro* replication reactions, a significant reduction in RNA synthesis was observed in contrast to reactions supplemented with wild-type fusion protein. Indirect functional depletion of hnRNP C from *in vitro* replication reactions, using poliovirus negative-strand cloverleaf RNA, led to a decrease in RNA synthesis. The addition of GST/hnRNP C1 to the reactions rescued RNA synthesis to near mock-depleted levels. Furthermore, we demonstrated that poliovirus positive-strand and negative-strand RNA present in cytoplasmic extracts prepared from infected HeLa cells coimmunoprecipitated with hnRNP C1/C2. Our findings suggest that hnRNP C1 has a role in positive-strand RNA synthesis in poliovirus-infected cells, possibly at the level of initiation.

Picornaviruses effectively subvert host cell functions and efficiently complete their intracellular life cycle in spite of the limited coding capacity of their relatively small single-stranded, positive-sense RNA genomes. Poliovirus (PV), the prototypic member of *Picornaviridae*, contains a single open reading frame within its 7.4-kb genomic RNA which encodes a 247-kDa polyprotein that is processed by virus-encoded proteinases into numerous intermediate and mature polypeptides. In addition to polyprotein processing, further strategies that successfully expand the biochemical activities encoded in this monocistronic genomic RNA include having distinct activities for precursors and mature polypeptides, embedding multiple activities in a single polypeptide, and recruiting cellular proteins for viral replication functions. After translation and polyprotein processing to generate functional nonstructural viral proteins, replication of positive-sense RNAs occurs via a negative-sense intermediate that ultimately produces new genomes and translation templates for the production of progeny virions. The virus-encoded RNA-dependent RNA polymerase (RdRp), 3D^{pol}, plays a central role in both negative-strand RNA synthesis and in replication of the genomic RNA.

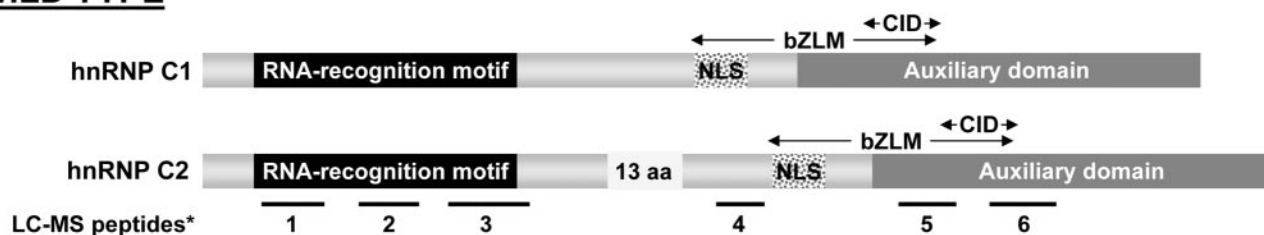
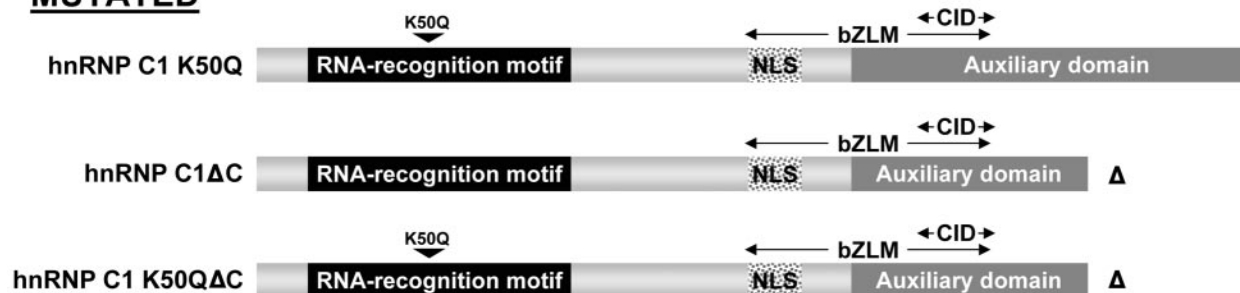
Picornavirus researchers have postulated that a *cis*-acting replication determinant, required for efficient template selection to initiate positive-strand RNA synthesis, may be present

within the 3'-terminal 100 nucleotides of PV negative-strand RNA (5, 6, 44, 47, 49). The complementary sequences, in the context of the positive-strand RNA, form a secondary structure termed the "cloverleaf" (stem-loop I) that has been shown to be important for viral RNA replication (3, 4, 41). The nucleotides at the 3' end of the negative-strand RNA have also been hypothesized to be important for viral replication since these sequences are similarly predicted to form a cloverleaf structure capable of binding viral and cellular proteins comprising the RNA synthesis initiation complex (5, 49).

To identify host factors important in the picornavirus intracellular life cycle, investigators have focused on RNA-interacting proteins. Roehl and Semler (49) used *in vitro* biochemical methods and a genetic approach to identify cellular proteins potentially involved in the formation of replication complexes at the 3' end of PV negative-strand RNA, where initiation of positive-strand RNA synthesis likely occurs. Two proteins, with molecular masses of 36- and 38-kDa, present in cytoplasmic extracts from PV-infected HeLa cells, were shown to UV cross-link to RNA encompassing various portions of the 3' end of PV negative-strand RNA. The 36- and 38-kDa proteins were detected by UV cross-linking ca. 3 h postinfection and were increasingly accessible to UV cross-linking at later times postinfection (49). The specificity of the interaction between the 36-kDa protein and the 3' end of PV negative-strand RNA was confirmed by demonstration of a dose-dependent decrease in complex formation in the presence of increasing molar excess of unlabeled homologous competitor RNA (49). Although we have not carried out a further analysis on the 38-kDa polypeptide, we report here the identity of the 36-kDa cellular

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WILD TYPE**MUTATED*****Peptides Identified by LC-MS**

peptide 1 (residues 18 – 29)	VFIGNLTLVVK
peptide 2 (residues 51 – 61)	GFQFVQYVNER
peptide 3 (residues 74 – 89)	MIAGQVLDINLAAEPK
peptide 4 (residues 143 – 151)	VPPPPPIAR
peptide 5 (residues 188 – 198)	LKGDDLQAIKK
peptide 6 (residues 205 – 216)	QKVDSLLENLEK

FIG. 1. Schematic representation of wild-type and mutated hnRNP C proteins. Only the hnRNP C1 portions of the GST (amino terminus) fusion proteins are depicted. Each protein contains a single RRM, a nuclear localization signal (NLS), a bZLM, a CID, and an acidic auxiliary domain.

protein as heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2.

The hnRNP C proteins are among the most abundant nuclear proteins (~100 million copies per nucleus [16]) involved in mRNA biogenesis (17) and belong to the large family of RNP motif RNA-binding proteins containing amino acid sequences highly conserved among vertebrates. The two C isoforms, hnRNP C1 and C2, are produced by alternative mRNA splicing. They form the C protein heterotetramer [(C1)₃C2] that comprises approximately one-third of the protein mass of mammalian 40S hnRNP particles. hnRNP C2 contains an additional 13 amino acids and is expressed at one-third the level of hnRNP C1 (31). Each isoform (see Fig. 1) has an RNA recognition motif (RRM), a basic leucine zipper-like motif (bZLM), a nuclear localization signal, a nuclear retention signal, and an acidic auxiliary domain thought to be involved in protein-protein interactions that serve to increase the specificity of RNA binding (21, 34). The presence of the additional 13 amino acids in hnRNP C2 enhances the specificity between the RRM, the bZLM, or the full-length protein and its ligand, allowing each to bind specifically to sequences with high affinity (31). An oligomerization domain (the C1-C1 interaction domain [CID]) has also been identified in hnRNP C1 (54).

Although hnRNP C is primarily a nuclear protein in cells, Gustin and Sarnow reported that three different hnRNP proteins—A1, K, and C—relocalize to the cytoplasm during PV and human rhinovirus infection (22, 23) via a mechanism unrelated to the induction of apoptosis. Belov et al. have determined that PV infection increases nuclear envelope permeability, resulting in relocalization of nuclear proteins to the cytoplasm, as well as cytoplasmic proteins to the nucleus (9). The findings of Belov et al. also indicated that PV-induced permeability does not require caspase-9, a protease that is involved in nuclear permeabilization during apoptosis.

Since PV positive-strand RNA synthesis almost certainly commences at or near the 3' end of negative-strand RNA, distinct *cis*-acting elements within this region may be required to confer specificity to the initiation step of genomic RNA replication. Interaction of viral or cellular proteins, localized in intracellular membranous complexes, with these specific RNA elements may selectively mediate initiation of PV genomic replication. We provide here evidence that at least one cellular protein (hnRNP C1) interacts with the 3' end of PV negative-strand RNA and serves a functional role in viral RNA replication. Mutagenesis of a highly conserved lysine in the RRM of hnRNP C1 to glutamine significantly reduced RNP complex

formation, indicating that the RNA binding is dependent on the RRM of the protein. Glutathione *S*-transferase (GST) pull-down assays demonstrated that GST/hnRNP C1 binds to 3CD, the immediate precursor to the viral RdRp, 3D^{pol}, as well as to P2 and P3, primary viral precursors to the nonstructural proteins. Truncation of the auxiliary domain in hnRNP C1 (C1ΔC), removing the C-terminal 40% of this region, significantly diminished these protein-protein interactions. To determine whether these RNA-protein and protein-protein interactions are biologically significant, we performed *in vitro* translation and RNA replication assays in HeLa S10 cytoplasmic extracts in the presence of recombinant wild-type and mutated GST/hnRNP C1. When GST/hnRNP C1ΔC was added, we observed an ~50% reduction in RNA synthesis compared to reactions supplemented with wild-type protein. In lieu of depletion, we attempted to sequester endogenous hnRNP C in the HeLa cytoplasmic extracts used for our *in vitro* replication assay by adding short RNAs representing the PV negative-strand cloverleaf in increasing molar excess over viral template RNAs. In the presence of the maximum concentration of short RNAs, we detected a significant decrease in the synthesis of single-stranded RNA. The addition of recombinant GST/hnRNP C1 restored RNA synthesis to levels nearly equivalent to those observed in the absence of hnRNP C sequestration. Finally, we demonstrated that antibody specific for hnRNP C1/C2 coprecipitates PV RNA of both polarities from cytoplasmic extracts from PV-infected HeLa cells. Collectively, these findings suggest that hnRNP C1 has a role in PV positive-strand RNA synthesis.

MATERIALS AND METHODS

Preparation of PV RNA probes. *In vitro* transcriptions were carried out with bacteriophage T7 RNA polymerase as previously described (48) A 100- μ l *in vitro* transcription reaction contained the following reagents (final concentrations): 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 10 mM NaCl, 10 mM dithiothreitol, 100 U of RNasin (Promega), GTP, ATP, and CTP (0.5 mM each), UTP (25 μ M unlabeled; 50 μ M of [α -³²P]UTP), and 40 U of RNA polymerase. Reactions were incubated for 2.5 h at 37°C, treated with 3 U of RNase-free DNase for 20 min at 37°C, phenol-chloroform extracted, chloroform extracted, ethanol precipitated (0.3 M sodium acetate), washed with 70% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. After further purification by Chroma spin column (Clontech), each RNA was quantitated by the number of counts incorporated.

pT7N66 (49) and pT7N66-D5-10 (47), the transcription templates for RNAs corresponding to the 3' end of PV negative-strand RNA, were prepared by digestion with MseI. The RNA probe transcribed from pT7N66- is referred to here as 66-RNA and the RNA probe transcribed from pT7N66-D5-10 is referred to as 66-Δ5-10 RNA.

Preparation of HeLa cell cytoplasmic extracts. Cytoplasmic extracts from uninfected HeLa cells were prepared as described elsewhere (8, 36, 52).

Preparation of cytoplasmic extracts from PV-infected HeLa cells. HeLa (S3) cells grown in suspension culture medium (S-MEM Joklik, antibiotic/antimycotic, 5% newborn calf serum) at a concentration of 5 × 10⁵ cells/ml were pelleted and washed with phosphate-buffered saline (PBS). The cells were resuspended in suspension culture medium without newborn calf serum to a density of 10⁷ cells/ml and infected with a passage 3 stock of PV1 at a multiplicity of infection of 25. After a 30-min adsorption at room temperature, newborn calf serum (5%) and 30 mM HEPES-KOH (pH 7.4) were added to the medium, along with additional S-MEM to bring the cell concentration to 5 × 10⁶ cells/ml.

Equal volumes of cells were harvested at various time points postinfection. After the harvest, the cells were washed three times in PBS and pelleted. The cell pellets were resuspended in an equal volume of lysis buffer (50 mM Tris-HCl [pH 8], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride) and incubated for 20 min on ice. The suspension was centrifuged for 30 s at 12,000 × g at 4°C. The supernatant was recovered and stored at -70°C.

Identification of the 36-kDa protein isolated from cytoplasmic extracts prepared from PV-infected HeLa cells. Cytoplasmic extracts from PV-infected HeLa cells were prepared as described above. Proteins contained within the supernatant recovered from NP-40 lysis were precipitated with 60% ammonium sulfate. The resulting pellet was resuspended in dialysis buffer (25 mM KCl, 5 mM HEPES-KOH [pH 7.8], 2 mM MgCl₂, 0.1 mM EDTA [pH 8.0], 3.8% glycerol, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) and dialyzed overnight against buffer containing an additional 10% glycerol. A 200- μ l aliquot of the concentrated extract was resolved on a 10 to 18% gradient acrylamide gel for 20.5 h. The resolved bands were compared to the products of a UV cross-linking experiment. The protein band in the Coomassie blue-stained gel corresponding to the 36-kDa protein present in the gel from the UV cross-linking was excised and in-gel digested with trypsin, and the resulting peptide mixture was subjected to liquid chromatography tandem mass spectrometry analysis (LC-MS/MS) by using a microscale reversed-phase high-pressure liquid chromatography system built at the City of Hope (14, 15, 26).

Expression of recombinant protein and purification. The plasmid, pHCl2, encoding hnRNP C1, was obtained from Gideon Dreyfuss (13). To generate pGEX-4T.HC12 (to express GST/hnRNP C1), the following synthetic oligonucleotides were used to PCR amplify the HC12 insert fragment from pHCl2 for cloning into the pQE-30 expression vector: 5'-AAAGGATCCATGGCCAGCAACGTTACCAACAAG-3' and 5'-GACAGAGACAGCGCCAATGGAGGATGAAAGCTT-3'. The amplified insert was digested with BamHI and HindIII, as was the pQE-30 vector. The two fragments were ligated with T4 DNA ligase to create pQE-30.HC12. pQE-30.HC12 was digested with HindIII and filled in with Klenow fragment. pGEX-4T was digested with XhoI and blunt ended with Klenow fragment. Both vectors were digested with BamHI, and the two fragments were ligated with T4 DNA ligase to create pGEX-4T.HC12.

The following synthetic oligonucleotides were used to create pGEX-4T.HC12 K50Q (expresses GST/hnRNP C1 K50Q) by QuikChange (Stratagene) site-directed mutagenesis of pGEX-4T.HC12: 5'-GGCTGCTCTGTTTCATCAGGGCTTTGCTTCGTTTCAG-3' and 5'-CTGAACGAAGCAAAGCCCTGATG AACAGAGCAGCC-3'.

To create pGEX-4T.HC12 ΔC (expresses GST/hnRNP C1ΔC), pGEX-4T.HC12 was first linearized with NotI, blunt ended with Klenow fragment, and then digested with BamHI and treated with calf intestinal alkaline phosphatase. The resulting ~5-kb fragment was ligated to the 726-bp fragment created by MlyI digestion of pGEX-4T.HC12, followed by recovery and BamHI digestion of the 2,455-bp fragment. In GST/hnRNP C1ΔC, amino acids 241 through 290 have been removed from the auxiliary (acidic) domain at the C-terminal end of hnRNP C1. To create pGEX-4T.HC12 K50QΔC [which expresses GST/hnRNP C1 (K50Q)ΔC], pGEX-4T.HC12 K50Q was treated exactly as described above for pGEX-4T.HC12.

GST/hnRNP C fusion proteins were expressed in JM109(DE3) (American Type Culture Collection) cells by induction, for 3 h, initiated with IPTG (isopropyl- β -D-thiogalactopyranoside) at an optical density of 0.5. The cells were pelleted, washed three times in PBS, and ruptured with a French press. The expressed proteins were bound to glutathione-Sepharose 4B (Amersham Biosciences), washed, eluted, dialyzed against protein buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM dithiothreitol), and stored at -70°C.

UV cross-linking assay. UV cross-linking assays were carried out as previously described (48) with the following exception: α -³²P-labeled RNA probe was added to either recombinant protein (equimolar amounts) in binding buffer (5 mM HEPES-KOH [pH 7.8], 25 mM KCl, 10 mM MgCl₂, 150 mM NaCl, 0.1 mM EDTA, and 3.8% [vol/vol] glycerol) or to HeLa S10 cytoplasmic extracts from PV-infected cells which had been preincubated with poly(rI-rC) in binding buffer to reduce nonspecific protein binding.

GST pull-down assays. [³⁵S]methionine-labeled PV proteins were synthesized *in vitro* in HeLa cytoplasmic extracts as described below. PV ΔNS RNA, at a concentration of 100 ng/ μ l, was used as the translation template. This RNA was transcribed from a construct with a large deletion (nucleotides 1172 to 2954) in P1 designed to eliminate most of the structural protein coding region.

Equimolar amounts of recombinant fusion protein or GST in TBST buffer (10 mM Tris-HCl [pH 7.5], 0.2 M NaCl, 0.5% Tween 20) with 0.2% bovine serum albumin was preincubated with the [³⁵S]methionine-labeled PV proteins with constant rocking for 30 min at room temperature (45). Glutathione-Sepharose 4B, 50:50 in PBS, was added, and the samples were incubated with constant rocking for 60 min more at room temperature. Samples were washed three times (10 min each wash, with constant rocking) with TBST buffer at 4°C. The bound proteins were eluted by boiling in Laemmli (32) sample buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Coupled *in vitro* translation-replication assays. For each translation-replication experiment, 50- μ l reactions were assembled with the following components:

65% (vol/vol) HeLa S10 cytoplasmic extract, 10% (vol/vol) HeLa ribosomal salt wash, 10% (vol/vol) 10 \times "minus" CTP replication buffer (10 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 125 μ M CTP, 600 mM potassium acetate, 300 mM creatine phosphate, 4 mg of creatine kinase/ml, 155 mM HEPES-KOH [pH 7.4], and 2 mM guanidine hydrochloride), and 1.3 μ g of PV virion RNA. HeLa ribosomal salt wash was prepared as described by Brown and Ehrenfeld (12). For the analysis of translation products, 10 μ l (of the 50- μ l reaction) was added to 10 μ Ci of [³⁵S]methionine (>1,000 Ci/mmol; Amersham Pharmacia Biotech). After incubation at 30°C for 5 h, each 10- μ l reaction was added to 10 μ l of 2 \times Laemmli sample buffer and boiled, and the translation products were resolved by SDS-PAGE (12.5% resolving gel, 5% stacking gel). The gel was fluorographed, dried, and subjected to autoradiography on X-MR film (Kodak).

The residual 40 μ l was used for analysis of RNA replication products. After incubation at 30°C for 5 h, each 40- μ l reaction was centrifuged for 20 min at 13,000 \times g at 4°C. Supernatants were removed, and the replication complexes were resuspended in replication mix (65% HeLa S10 cytoplasmic extract, 10% [vol/vol] 10 \times "minus" CTP replication buffer, 25 μ Ci of [α -³²P]CTP; 3,000 Ci/mmol; Amersham Pharmacia Biotech) with or without added 66-RNA or 66- Δ 5-10 RNA and with added recombinant protein or protein buffer. After incubation for 90 min at 34°C, reactions were terminated with SDS stop buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% SDS, 1 mM EDTA). Total RNA in each reaction was subjected to two phenol-chloroform extractions and ethanol precipitation. RNA pellets were washed in 70% ethanol, dried, and resuspended in 15 μ l of diethyl pyrocarbonate-treated water and 15 μ l of 2 \times RNA gel loading buffer (Ambion). RNA products were subjected to gel electrophoresis on a native 1.1% agarose Tris-borate-EDTA gel containing ethidium bromide. The amounts of 18S and 28S rRNA in each lane, as determined by examination under UV light, were used to confirm equal loading of samples in the gel. The gel was then dried and subjected to phosphorimager analysis on a Personal Molecular Imager FX (Bio-Rad).

Coimmunoprecipitation. Cytoplasmic extracts from PV-infected HeLa (S3) cells for use in coimmunoprecipitation assays (39) were prepared at various times postinfection as described above. Lysates were precleared by incubation on ice for 1 h with protein A-agarose (50% in lysis buffer) bound to nonspecific antibody. Nonspecific complexes were pelleted by centrifugation at 10,000 \times g at 4°C for 10 min. The supernatants were removed and used in the immunoprecipitation assay.

Next, 50 μ l of precleared lysate was diluted with 450 μ l of lysis buffer and then added to 15 μ l of polyclonal antibody, followed by incubation on ice for 2 h. Precleared protein A-agarose (100 μ l in PBS [50:50]) was added to each sample, followed by incubation on ice for an additional hour. Immune complexes were pelleted by centrifugation at 1,000 \times g at 4°C for 5 min and washed three times with lysis buffer. Each pellet (or 50 μ l of precleared lysate [total RNA]) was resuspended in 400 μ l of proteinase K buffer (100 mM Tris-HCl [pH 7.5], 12.5 mM EDTA, 150 mM NaCl, 1% SDS) and incubated with 100 μ g of predigested proteinase K for 30 min at 37°C.

RNA was extracted twice with phenol-chloroform, precipitated in ethanol at -20°C for 1 h, washed in 70% ethanol, dried, and resuspended in 60 μ l of hybridization buffer (40 mM PIPES [pH 6.4], 1 mM EDTA [pH 8.0], 400 mM NaCl, 80% formamide).

RNase protection assay. RNA probes were prepared as described above. Half of each 60 μ l of RNA sample recovered from coimmunoprecipitation was used for a single-cycle RNase protection assay (to detect PV positive-strand RNA), and the other half was used for a two-cycle RNase protection assay (to detect PV negative-strand RNA) (40).

For the single-cycle assay, 75 fmol of an [α -³²P]UTP-labeled RNA probe for positive-strand RNA, containing nucleotides complementary to positive-sense sequences 5601 to 5809 of PV viral RNA, as well as 49 nucleotides of vector sequence, was incubated with 30 μ l of the RNA in hybridization buffer at 85°C for 10 min. For the two-cycle assay, the remaining 30- μ l samples were incubated without labeled probe. Samples were incubated for 12 h at 55°C.

Samples were cooled to room temperature and digested with an RNase digestion mixture (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 5 mM EDTA [pH 7.5], 350 U of RNase T₁/ml, 4.5 μ g of RNase A/ml) at 7°C for 1 h. Samples were then treated with 100 μ g of predigested proteinase K and 20 μ l of 10% SDS, followed by incubation at 37°C for 30 min. RNA was recovered by phenol-chloroform extraction and ethanol precipitation with the addition of 20 μ g of tRNA (from baker's yeast). After washing with 70% ethanol and drying, the RNAs from the single-cycle assay were resuspended in 15 μ l of diethyl pyrocarbonate-treated water and 15 μ l of 2 \times RNA gel loading buffer and resolved on a polyacrylamide-7 M urea gel in Tris-borate-EDTA buffer. The gel was then subjected to phosphorimager analysis on a Personal Molecular Imager FX (Bio-Rad). The RNAs for the two-cycle assay were resuspended in 30 μ l of hybrid-

ization buffer and incubated at 85°C for 10 min with 25 fmol of an [α -³²P]UTP-labeled RNA probe for negative-strand RNA, complementary to nucleotides 5601 to 5809 of the negative-strand PV viral RNA with 49 nucleotides of vector sequence. These RNAs were then subjected to the same procedure used to detect positive-strand viral RNA.

RESULTS

Identification of 36-kDa HeLa protein as hnRNP C1/C2.

The 36-kDa protein previously determined to interact with the 3' end of PV negative-strand RNA was isolated in preparation for MS analysis. Cytoplasmic extracts from PV-infected HeLa cells were prepared and fractionated by precipitation with 60% ammonium sulfate. We had determined that the pellet generated by this treatment was enriched in the 36-kDa protein (49). An aliquot of the concentrated extract was subjected to electrophoresis on an SDS-containing gradient polyacrylamide gel, and the resolved bands were compared to the products of a UV cross-linking experiment. The protein band corresponding to the 36-kDa protein was excised and in-gel digested with trypsin, and the resulting peptide mixture was subjected to LC-MS/MS (10). Sequence assignments for the eluting peptides were made based on their corresponding MS/MS data. Software search algorithms, provided by the manufacturer of the mass spectrometer, were applied to interpret the spectra and to search protein and nucleotide databases for the identification of protein. All spectra were evaluated by hand to confirm the assignments made. The analysis resulted in six peptides being positively identified to be derived from the protein human hnRNP C1/C2. Collectively, the peptides corresponded to sequence coverage of ca. 24% of the protein. The peptides that were identified are common to both isoforms of hnRNP C (Fig. 1) and, therefore, it cannot be decidedly concluded that either the C1 or C2 isoform was exclusively present in the sample.

hnRNP C1 proteins interact with PV 66-RNA probe. We had previously shown that the interaction between the 36-kDa protein from cytoplasmic extracts of PV-infected HeLa cells and the 3' end of PV negative-strand RNA was specific and dose dependent (49). To confirm that hnRNP C would correspondingly form an RNP complex with the 3' end of PV negative-strand RNA, UV cross-linking experiments were performed by using recombinant hnRNP C proteins and radiolabeled RNA (66-) encompassing nucleotides 2 through 66 (positive-strand numbering) from the 3' terminus of PV negative-strand RNA. This RNA was, by MFOLD analyses (47), predicted to form a portion of stem a, stem-loop b, and stem-loop c of the PV negative-strand cloverleaf (Fig. 2A). Recombinant GST/hnRNP C1 fusion protein (65 kDa) and mature recombinant hnRNP C1 protein (generated by thrombin cleavage of the GST fusion protein) both UV cross-linked to the PV 66-RNA probe (Fig. 2B, lanes 5 and 6). Moreover, mature (thrombin-cleaved) recombinant hnRNP C1 protein comigrated precisely with the 36-kDa protein from cytoplasmic extracts prepared from PV-infected HeLa cells (Fig. 2B, lanes 3, 4, and 6).

RRM mediates interaction between hnRNP C1 and PV 66-RNA. To determine whether the interaction with PV negative-strand RNA is mediated by the RRM of hnRNP C1, we mutated an amino acid highly conserved among vertebrates (K50Q) in the RRM of GST/hnRNP C1 (53). This lysine is the most amino-terminal residue of the RNP1 octamer

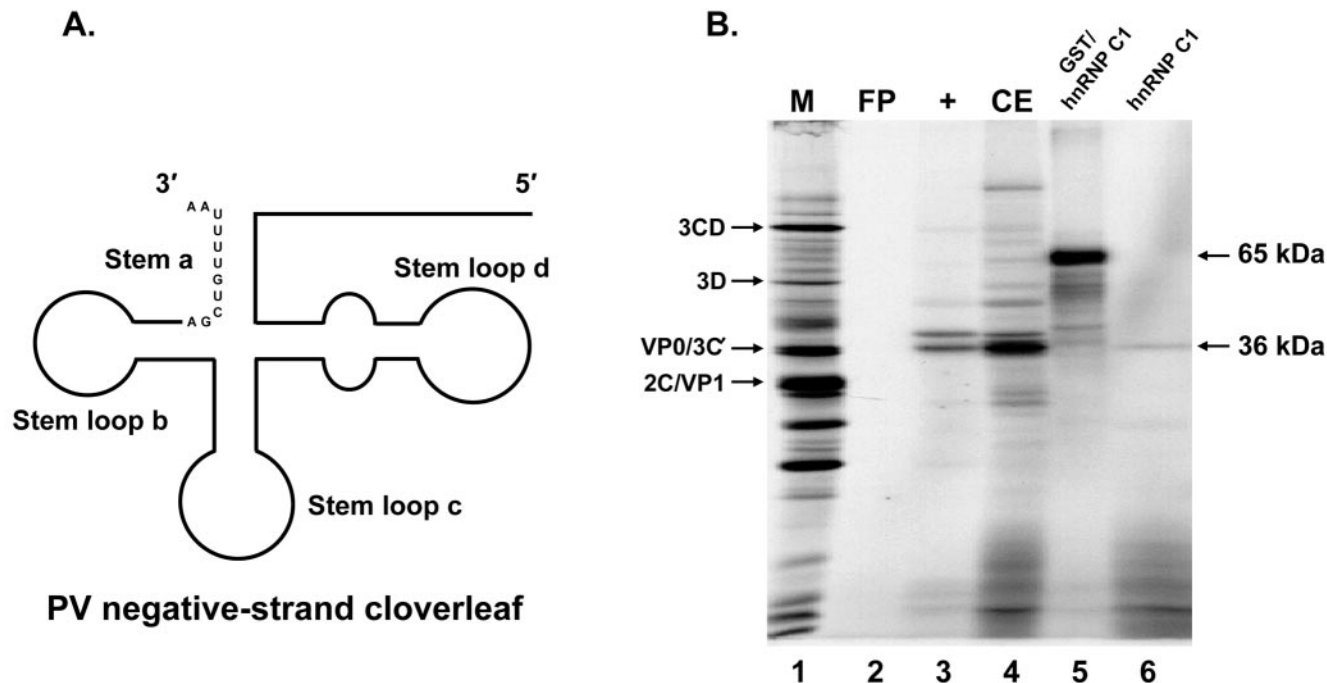


FIG. 2. Recombinant hnRNP C1 interacts with a secondary structure element predicted to form at the 3' end of PV negative-strand RNA. (A) Schematic representation of the computer-predicted PV negative-strand RNA cloverleaf structure. The first 11 nucleotides from the 3' end are shown and may represent a (partial) binding site for hnRNP C (50). (B) UV cross-linking assays with PV negative-strand 66-RNA probe and either extracts from PV-infected HeLa cells or recombinant hnRNP C1. Cytoplasmic extracts prepared from PV-infected HeLa cells (lanes 3 and 4), or recombinant proteins (lanes 5 and 6) were preincubated with poly(rI-rC) in binding buffer. [α - 32 P]UTP-labeled 3' negative-strand probe (nucleotides 2 to 66, positive-strand numbering) was added to each sample. Proteins bound to the RNA were UV cross-linked, and samples were treated with an RNase cocktail. Proteins that interacted with the RNA probe were resolved by SDS-PAGE and visualized due to the [α - 32 P]UTP labeling of the cross-linked RNA. Lanes: FP, free probe without added extract or protein; +, partially purified extract enriched in the 36- and 38-kDa proteins; CE, crude extract; M, marker proteins ([35 S]methionine labeled from PV-infected HeLa cells).

(KGFAFVQY), one of the hallmark consensus sequences of the RNP motif (17). The first residue of the RNP1 motif is either R or K in the majority of RNP domains. Within the RNP domain of the U1 small nuclear ribonucleoprotein A, an R52Q mutation in the RNP1 segment abolished U1 RNA binding, whereas R52K only slightly affected RNA binding (30). Since these data suggested that a salt bridge may be formed between a basic amino acid in this position and the phosphates of the cognate RNA, we chose this site to make a charge to neutral mutation which could potentially abolish RNA binding by the RNP1 segment of hnRNP C1. In addition to K50Q, two other mutated proteins, generated by carboxy-terminal truncations (residues 241 through 290) of wild-type GST/hnRNP C1 protein (GST/hnRNP C1 Δ C) and mutant K50Q protein (GST/hnRNP C1 K50Q Δ C), were generated to test the requirement for the auxiliary domain (residues 168 through 290) in interactions of hnRNP C1 with other proteins. The four GST/hnRNP C1 proteins were predicted to have the phenotypes listed in Table 1. To test the RNA-binding phenotypes of the four GST/hnRNP C proteins, UV cross-linking with the PV 66-RNA probe representing the 3' end of the PV negative-strand RNA was performed (Fig. 3). GST/hnRNP K50Q and GST/hnRNP K50Q Δ C, both containing the RRM mutation, demonstrated significant defects in their abilities to interact with the PV 66-RNA probe compared to the GST/hnRNP C1 wild-type and GST/hnRNP C1 Δ C (Fig. 3, compare lanes 6 and

8 to lanes 5 and 7). These data suggest that the interaction between the viral RNA and hnRNP C is mediated by the RRM of the protein. In contrast, truncation of the auxiliary domain in hnRNP C1 did not impair RNA binding. Such a highly acidic region would not be expected to interact with the RNA directly. Nevertheless, residues in the CID of hnRNP C1, amino acids 180 to 208, have been shown to influence C1 oligomerization and RNA binding mediated by the RRM (54). However, recombinant hnRNP C1 proteins Δ C and K50Q Δ C retain residues forming the CID.

GST/hnRNP C1 interacts with PV replication polypeptides.

To determine whether hnRNP C interacts with PV nonstructural proteins involved in RNA synthesis, GST pull-down assays (Fig. 4) were performed with the four GST/hnRNP C1 proteins described in Table 1 and [35 S]methionine-labeled PV nonstructural precursors and mature proteins generated by in

TABLE 1. Predicted phenotypes of four GST/hnRNP C1 proteins

Protein	Size (kDa)	RNA binding	Protein-protein interactions
GST/hnRNP C1 wild type	~65	Wild type	Wild type
GST/hnRNP C1 Δ C	~59	Wild type	Deficient
GST/hnRNP C1 K50Q	~65	Deficient	Wild type
GST/hnRNP C1 (K50Q) Δ C	~59	Deficient	Deficient

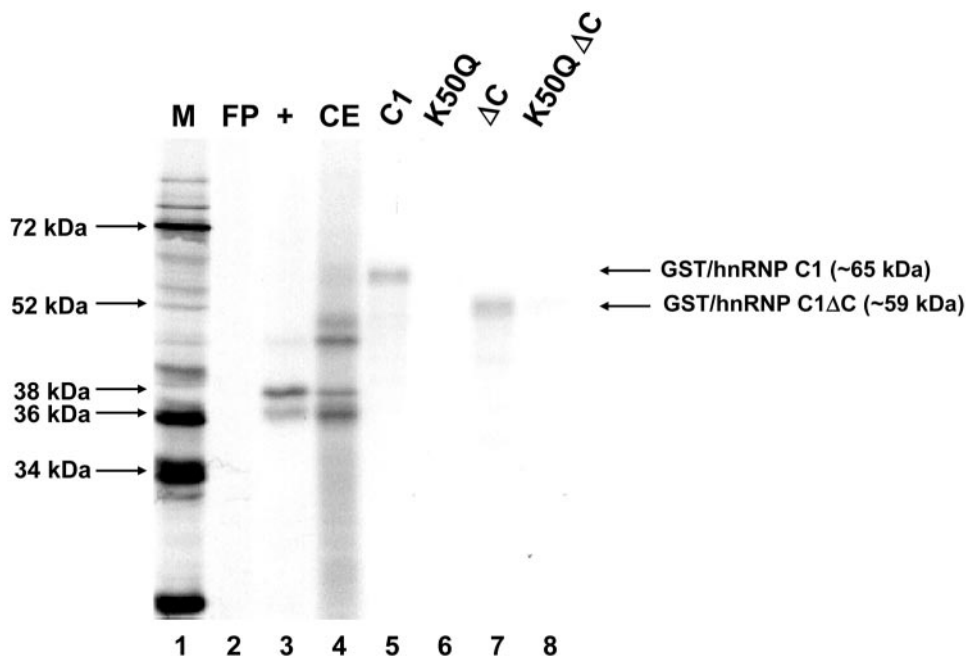


FIG. 3. UV cross-linking assay using extracts from PV-infected HeLa cells or recombinant forms of hnRNP C1. Shown are GST/hnRNP C1 wild type (lane 5), GST/hnRNP C1 K50Q (lane 6), GST/hnRNP C1 Δ C (lane 7), or GST/hnRNP C1 K50Q Δ C (lane 8) UV cross-linked to 66-negative-strand probe. Lanes: FP, free probe without added extract or protein; +, partially purified extract enriched in the 36- and 38-kDa proteins; CE, crude extract; M, marker proteins (35 S)methionine labeled from PV-infected HeLa cells).

in vitro translation of PV1 Δ NS RNA. PV1 Δ NS RNA contains a large deletion within PV precursor P1 (nucleotide 1172 to 2954) removing most of the coding region for the structural proteins. We had previously determined that the PV capsid proteins interact nonspecifically with GST/hnRNP C1 (data not shown). GST/hnRNP C1 wild type and GST/hnRNP C1 K50Q mutant, which contain intact auxiliary domains, interacted with PV precursors P2 and P3 and with the PV replication protein, 3CD (Fig. 4A, lanes 4 and 6). Figure 4B is a representation of the PV polyprotein processing scheme diagramming the generation of the precursors and mature proteins. P1 is the primary precursor to the structural proteins, whereas P2 and P3 are the primary precursors to the nonstructural proteins. Truncation of the auxiliary domain diminished the ability of GST/hnRNP C1 to interact with P2, P3, and 3CD (Fig. 4A, compare lanes 5 and 7 to lanes 4 and 6). These data demonstrate that hnRNP C1 interacts with PV replication proteins and that the auxiliary domain is important for these interactions. However, we note that hnRNP C may not interact directly with both P2 and P3. It is possible that hnRNP C interacts directly with P3 (or its cleavage products) and then that polypeptide interacts with P2 (or its cleavage products) or visa versa.

GST/hnRNP C1 Δ C inhibits PV RNA synthesis in vitro. In vitro PV translation-replication assays were carried out to study the effects of RNA-binding deficiency (GST/hnRNP C1 K50Q) or defects in protein-protein interactions (GST/hnRNP C1 Δ C) on RNA synthesis (Fig. 5). We had previously determined by Western blot analysis that the concentration of hnRNP C in HeLa S10 cytoplasmic extract supplemented with 10% HeLa ribosomal salt wash is ca. 100 nM (data not shown).

Nondepleted HeLa extracts used for replication reactions were supplemented with a fivefold molar excess of recombinant proteins versus endogenous hnRNP C protein. Each of the four GST/hnRNP C proteins was added to a separate replication reaction at a concentration of 500 nM. Although hnRNP C has been reported to be primarily a nuclear protein (17), there are significant quantities of the protein in our HeLa S10 cytoplasmic extracts. Such levels of hnRNP C in the extracts are likely to arise from a population of C that is a bona fide cytoplasmic "resident" and from the Dounce homogenization process used to prepare our cytoplasmic extracts which may rupture some nuclei, resulting in the release of nuclear proteins into the extracts.

The addition of GST/hnRNP C1 Δ C to the RNA replication reaction (Fig. 5A, lane 3) decreased single-stranded RNA synthesis by \sim 50% compared to reactions to which no protein or GST/hnRNP C1 was added (Fig. 5A, lanes 1 and 2). Examination of Fig. 5B, showing equivalent translation products available for all replication reactions, verifies that these differences were due to RNA synthesis and not due to differences in the levels of viral replication proteins. One possible interpretation of these results is that GST/hnRNP C1 Δ C was able to bind to viral RNA but was deficient in interacting with viral proteins or cellular proteins involved in RNA synthesis. The fivefold molar excess of GST/hnRNP C1 Δ C versus endogenous hnRNP C would permit the mutated protein to compete with the wild-type protein for binding to the viral RNA. Further support for this explanation is provided by the finding that GST/hnRNP C1 K50Q Δ C does not inhibit replication (Fig. 5A, lane 5), presumably due to deficiencies in both RNA binding and protein-protein interactions. The fact that hnRNP C1

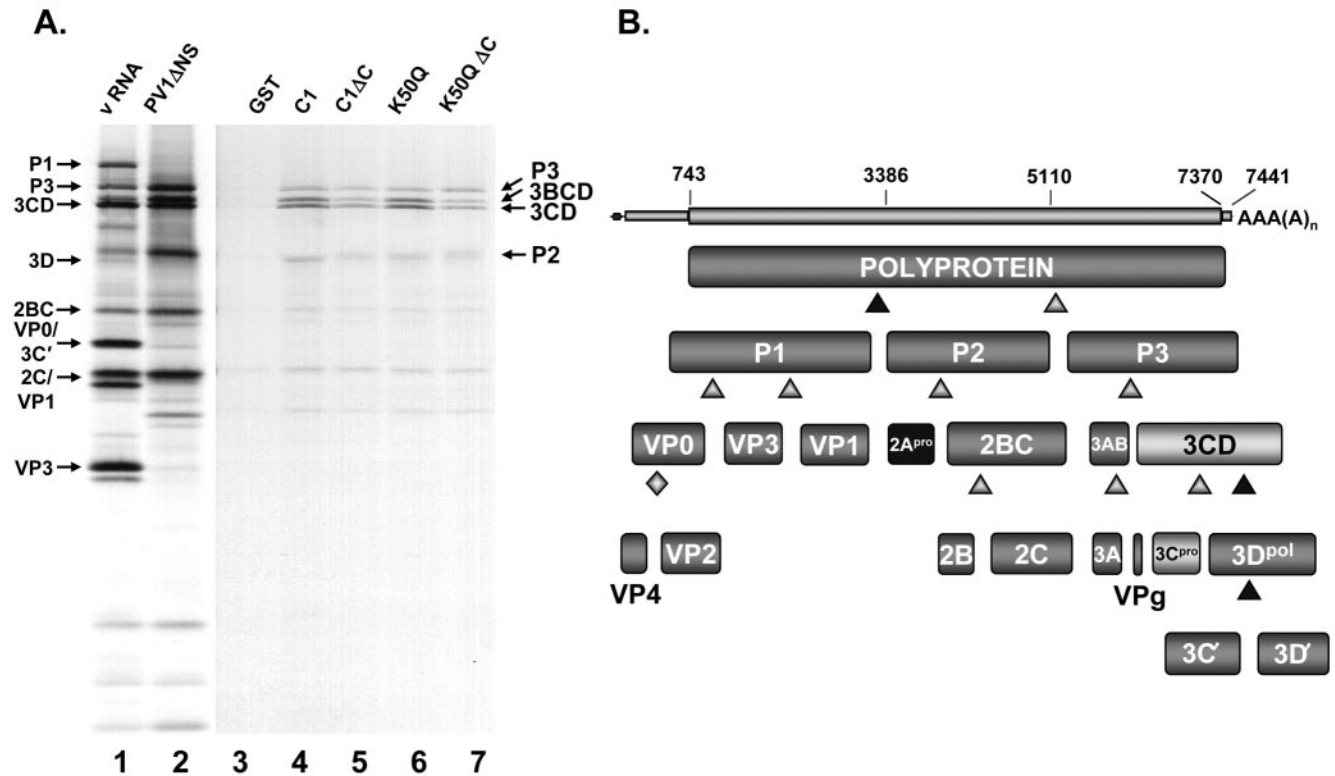


FIG. 4. GST pull-down of PV proteins with four GST/hnRNP C1 fusion proteins. (A) Recombinant hnRNP C1 proteins; C1 (lane 4), Δ C (lane 5), K50Q (lane 6), K50Q Δ C (lane 7), or GST (lane 3) in TBST buffer with 0.2% bovine serum albumin were incubated with in vitro synthesized [35 S]methionine-labeled PV proteins and precursors and protein-protein interactions were detected as described in Materials and Methods. Lane 1 shows [35 S]methionine-labeled PV proteins synthesized in vitro from virion RNA, and lane 2 shows a sample of the [35 S]methionine-labeled PV proteins, used in the assay, that were synthesized in vitro from PV Δ NS RNA (a construct with a deletion [nucleotides 1172 to 2954] in P1 to eliminate capsid protein). (B) Representation of the PV polyprotein processing scheme. Precursor P1 is processed to form the structural proteins, and precursors P2 and P3 are processed to form the nonstructural proteins. The viral proteinases, 2A and 3C/3CD, are highlighted with differential shading, and the cleavages for which each is responsible are shown with correspondingly shaded triangles. The diamond indicates the VP0 cleavage site that, when cleaved during virion morphogenesis, results in the production of VP4 and VP2. The identity of the activity responsible for this cleavage is unknown.

Δ C appears to be a *trans*-dominant inhibitor in the in vitro assay for PV RNA synthesis indicates that interactions between hnRNP C and other protein(s) are important for efficient RNA replication. The addition of GST/hnRNP C1 K50Q slightly reduced RNA synthesis (Fig. 5A, lane 4), perhaps due to the sequestration of binding partners necessary for replication.

GST/hnRNP C1 rescues positive-strand RNA synthesis in hnRNP C-depleted HeLa cytoplasmic extracts. hnRNP C proteins bind poly(U) and oligo(U) stretches found in naturally occurring RNAs. The apparent dissociation constant (k_d) of hnRNP C1 for an RNA ligand with at least five contiguous uridylate residues is ca. 170 nM (20). Consequently, in preparation for carrying out in vitro RNA replication experiments in depleted extracts, we used poly(U) Sepharose to deplete HeLa S10 cytoplasmic extracts and HeLa RSW of hnRNP C. This approach proved ineffective since the depletion was not specific, as evidenced by removal of other factors important for translation and replication, and incomplete, as demonstrated by Western blot analysis (data not shown). Similarly, immunodepletion of HeLa cytoplasmic extracts with magnetic beads coated with protein A bound to polyclonal antibody specific for

hnRNP C decreased translation and replication but failed to completely deplete the extracts of hnRNP C (data not shown).

As previously described, short RNAs corresponding to the 3' end of PV negative-strand RNA interact with hnRNP C found in HeLa cytoplasmic extracts (Fig. 2B) (49). The formation of these RNP complexes represented another, albeit indirect, depletion strategy. To sequester endogenous hnRNP C in extracts while performing in vitro RNA replication assays, these short RNAs were added to the replication reactions (Fig. 6). For depletion, increasing molar excesses of the 66-RNA were added (Fig. 6A, lanes 4 to 6) compared to the virion RNA used as an RNA template in the reactions. Similarly for mock depletion, increasing molar excesses of 66- Δ 5-10 deletion RNA were added (Fig. 6A, lanes 9 to 11), since we had shown that this RNA does not detectably bind hnRNP C from HeLa cytoplasmic extracts in a UV cross-linking assay (47). In 66- Δ 5-10 RNA, nucleotides 5 to 10 (positive-strand numbering) from the 3' end of the PV negative-strand RNA were deleted, but the negative-strand cloverleaf (Fig. 2A) stem-loop b and stem-loop c structures are still predicted to form by MFOLD analyses (47). The addition of a 200 times molar excess of 66-RNA over virion RNA to the in vitro replication reaction

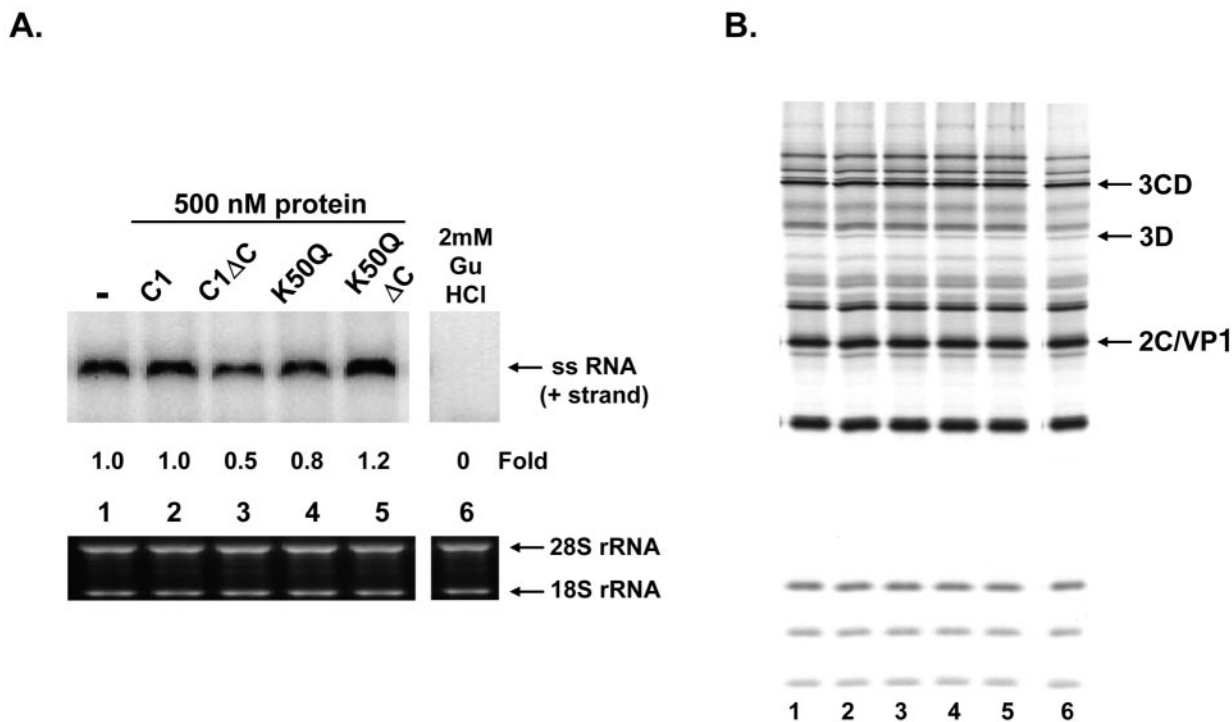


FIG. 5. In vitro translation-replication of virion RNA in HeLa S10 cytoplasmic extract-ribosomal salt wash with or without the addition of recombinant GST/hnRNP C1 proteins. Recombinant hnRNP C1 proteins (in panel A) hnRNP C1 wild type (lane 2), hnRNP C1ΔC (lane 3), hnRNP C1 K50Q (lane 4), hnRNP C1 K50QΔC (lane 5), or protein buffer alone (lane 1) were used. Components of a cell extract were assembled, mixed with PV virion RNA, and split into two fractions. (A) The replication fraction was incubated, for synthesis of nonstructural proteins, without the addition of [³⁵S]methionine. This fraction was centrifuged to pellet RNA replication complexes that were then resuspended in buffer with a labeling mix that may have included recombinant protein. Guanidine hydrochloride (lane 6), an inhibitor of negative-strand RNA synthesis, was added to one of the reactions which served as a negative control. After incubation for RNA synthesis, the RNA was resolved on a 1.1% agarose gel in Tris-borate-EDTA buffer containing ethidium bromide. The amounts of 18S and 28S rRNA in each lane were used to confirm equal loading of samples in the gel. Comparative density values (counts [arbitrary units] per square millimeter) of the signals for single-stranded RNA are shown below the autoradiograph of the agarose gel. (B) The translation fraction was incubated with [³⁵S]methionine and synthesized proteins were subjected to electrophoresis on an SDS-containing polyacrylamide gel. This figure shows representative data from two independent experiments.

(Fig. 6A, lane 6) decreased single-stranded RNA synthesis to ~40% of the no-protein sample and the mock sample (Fig. 6A, compare lane 6 to lanes 1 and 11, respectively). When recombinant GST/hnRNP C1 was added to a concentration of 100 nM, a rescue of single-stranded RNA synthesis (Fig. 6A, lane 8) was observed at levels comparable to the equivalent mock-depleted/add-back sample (Fig. 6A, lane 13).

PV RNA coimmunoprecipitates with hnRNP C in PV-infected HeLa cells. To determine whether hnRNP C and PV RNA interact in PV-infected cells, immunoprecipitation of hnRNP C from extracts prepared from PV-infected HeLa cells was performed (39), and the pelleted complexes were examined for coimmunoprecipitation of PV RNA (Fig. 7). RNase protection assays, one cycle for PV positive-strand RNA (Fig. 7A) and two cycles for PV negative-strand RNA (Fig. 7B) (40), were used to detect PV RNA of each polarity. The extra cycle of the two-cycle RNase protection assay increases the detection sensitivity for the much less abundant negative-strand RNA. We also purified total RNA (no coimmunoprecipitation) from our cytoplasmic extracts from PV-infected HeLa cells for use as a positive control in both the single-cycle and two-cycle RNase protection assays. In both cases, total RNA exceeded the amount of RNA detected after coimmunopre-

cipitation with any of the antibodies. We used tRNA as a negative control for confirming specificity of our probes. As shown in Fig. 7A (lanes 3 to 6), PV positive-strand RNA coimmunoprecipitated with hnRNP C from infected HeLa cells. The amount of positive-strand RNA recovered increased over the course of the infection. PV negative-strand RNA also coimmunoprecipitated with hnRNP C from infected HeLa cells (Fig. 7B, lanes 3 to 6), and the amount of this RNA increased over time postinfection.

Our selection of antibody utilized as the positive control for coimmunoprecipitation of PV positive-strand or negative-strand RNA was based on previous reports of proteins interacting with each specific RNA. Antibody to the cellular protein PCBP was used as a positive control for coimmunoprecipitation of PV positive-strand RNA (Fig. 7A, lanes 7 to 10) since this protein has been shown to interact with the stem-loop I (3, 18, 41) and the stem-loop IV (10, 18) secondary structures of that RNA. The amount of PV positive-strand RNA that coimmunoprecipitated with antibody to hnRNP C was significantly greater than the amount that coimmunoprecipitated with antibody to PCBP (Fig. 7A, compare lanes 3 to 6 to lanes 7 to 10). The amount of PV negative-strand RNA that coimmunoprecipitated with hnRNP C exceeded the amount that coimmunopre-

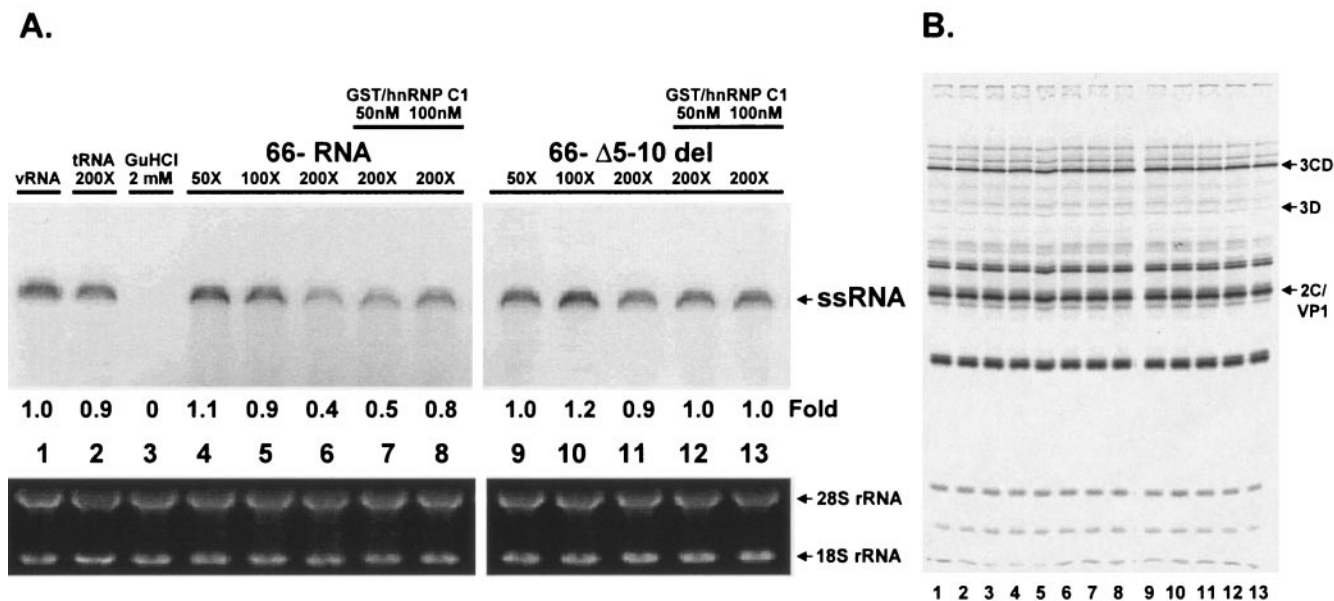


FIG. 6. In vitro translation-replication of virion RNA in HeLa S10 cytoplasmic extract-ribosomal salt wash with or without the addition of short RNAs representing the 3' end of PV negative-strand RNA. RNAs utilized were 66-RNA (lanes 4 to 8 in panel A) and 66-Δ5-10 RNA (lanes 9 to 13 in panel A). (A) The replication fractions were incubated, for synthesis of nonstructural proteins, without the addition of [³⁵S]methionine. These fractions were centrifuged to pellet RNA replication complexes, which were then resuspended in buffer with a labeling mix that may have included short RNAs, with increasing concentrations of (lanes 7 to 8 and lanes 12 to 13) or without GST/hnRNP C1 protein. Samples and controls were further prepared as described in the legend to Fig. 5. Lanes: 1, vRNA (virion RNA only without additional short RNA or protein); 2, 200× tRNA (heterologous short RNA was added as a negative control); 3, 2 mM GuHCl (an inhibitor of negative-strand RNA synthesis added to one of the reactions which served as a negative control). (B) The translation fractions were prepared as described in the legend to Fig. 5. This figure shows representative data from two independent experiments.

noprecipitated with PV 2C (Fig. 7B, compare lanes 3 to 6 and lanes 7 to 10), a viral protein shown to bind to negative-strand RNA (5, 6).

DISCUSSION

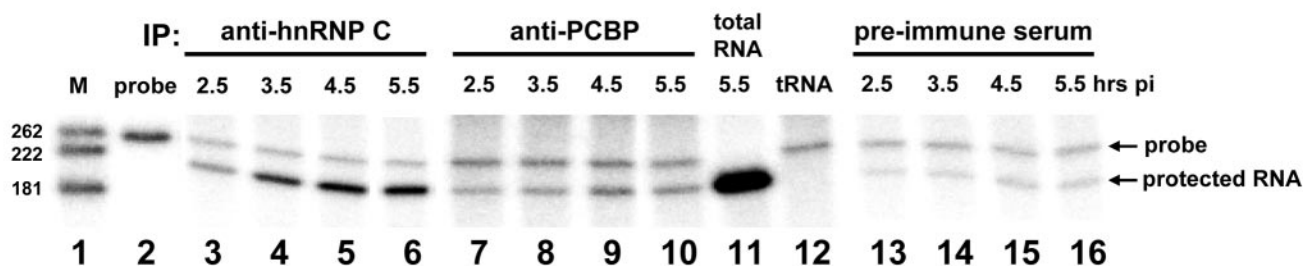
Cellular factors involved in the picornavirus replicative cycle are likely to be components of RNA processing and transport apparatuses or cytoplasmic translation machineries recruited to function in the replication of viral RNA. The most frequently observed cellular proteins found to be involved in viral RNA synthesis are the hnRNP complex proteins (33). Several RNA-binding proteins, including members of the hnRNP complex, have been proposed to play a role in the life cycles of different viruses by interacting with viral RNAs in functional complexes (1, 2, 11, 18, 19, 24, 28, 29, 41). In the present study, we investigated the possible role of the cellular protein hnRNP C in the process of PV RNA synthesis. During PV infection, this nuclear RNA-binding protein relocates to the cytoplasm due to alterations in nucleocytoplasmic trafficking (9, 22, 23). Interference with protein trafficking, through the disruption of import and induced efflux of nuclear proteins, may be an essential part of the picornavirus replicative cycle by providing abundant nuclear proteins in the cytoplasm that could function in translation, RNA synthesis, or packaging. Moreover, previous studies have indicated that an RNA-binding protein normally localized to the nucleus may exhibit different RNA-binding properties when in the cytoplasm (25, 27).

We had previously demonstrated that a 36-kDa protein from

HeLa cytoplasmic extracts interacts with short RNAs (66-, 108-, 180-, and 224-) corresponding to the 3' end of PV negative-strand RNA (49). LC-MS/MS was used to identify this protein as human cellular hnRNP C1/C2. Subsequently, we showed that recombinant hnRNP C1 interacts with these same RNAs. Sequences and/or structures, such as the negative-strand cloverleaf, found within RNAs corresponding to the 3' ends of PV negative strands would presumably contain the determinants for formation of a complex to initiate positive-strand RNA synthesis. Mutation of a conserved residue in the RRM (K50Q) of hnRNP C1, replacing an amino acid with a positively charged side chain with one having an uncharged side chain, severely disrupted the interaction between hnRNP C1 and the 3' end of PV negative-strand RNA. This suggests that the RRM present in hnRNP C mediates the interaction with PV RNA.

GST/hnRNP C1 interacted with the PV precursor polypeptides P2 and P3, as well as with the PV replication protein 3CD. Interactions with the precursors may be biologically significant in and of themselves or may suggest a functional interaction with one of the mature replication proteins whose amino acid sequences are contained within P2, such as 2BC or 2C, or within P3, such as 3CD. GST/hnRNP C1ΔC, with a truncated auxiliary domain, demonstrated decreased ability to interact with PV 3CD and replication precursors and significantly reduced positive-strand RNA synthesis compared to the wild type when added to in vitro replication assays. This suggests that hnRNP C1ΔC may be acting as a *trans*-dominant-negative inhibitor of positive-strand RNA synthesis by binding

A.



B.

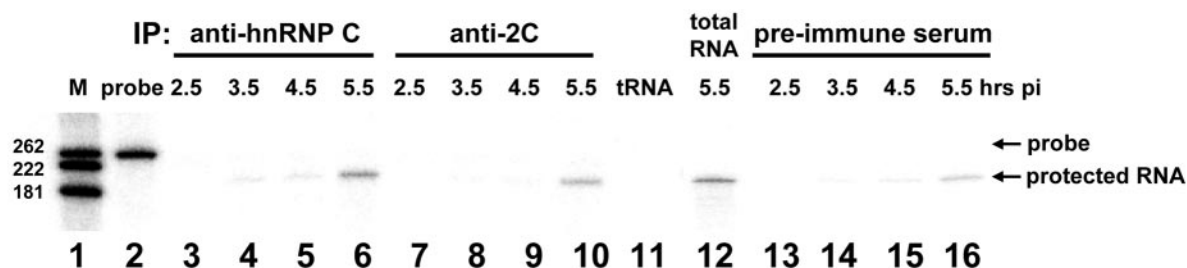


FIG. 7. Coimmunoprecipitation time course assay with cytoplasmic extracts prepared from PV-infected HeLa cells at various times postinfection (hours postinfection [hrs p.i.]). Extracts were precleared and incubated with polyclonal antisera or preimmune serum in NP-40 lysis buffer. Immune complexes were pelleted, washed, and treated with proteinase K. RNA was then phenol-chloroform extracted, ethanol precipitated, and detected by RNase protection assay. (A) RNA was hybridized with α - 32 P-labeled probe specific for PV positive-strand RNA. RNAs were treated with RNase A and RNase T₁. RNA products and excess probe were separated on a urea acrylamide denaturing gel. Antibody specific for PCBP was used to coimmunoprecipitate PV positive-strand RNA as a positive control for the single-cycle RNase protection assay. (B) For the two-cycle assay, positive-strand and negative-strand RNAs were hybridized in the first cycle and then RNase treated to eliminate excess positive-strand RNA. In the second cycle, the RNAs recovered after the first cycle were hybridized to α - 32 P-labeled probe specific for PV negative-strand RNA, RNase treated, and resolved on a urea acrylamide denaturing gel. Antibody specific for PV protein 2C was used to coimmunoprecipitate PV negative-strand RNA as a positive control for the two-cycle RNase protection assay. Lanes: total RNA, all of the RNA recovered from the extract (from time point noted) without carrying out the coimmunoprecipitation step; tRNA, 5 μ g of tRNA was added to labeled probe as a negative control.

to PV RNA but lacking the ability to efficiently interact with viral replication proteins or cellular proteins involved in replication.

Short RNAs (PV 66-RNA) that efficiently bind endogenous hnRNP C in HeLa cytoplasmic extracts decreased single-strand RNA synthesis when added to *in vitro* RNA replication assays. When recombinant GST/hnRNP C1 protein was also added, RNA synthesis was rescued to 80% of mock-“depleted” levels. Interestingly, an RNA representing the first 66 nucleotides from the 3' end of PV negative-strand RNA, with a deletion of nucleotides 5 to 10 (positive-strand numbering), does not bind hnRNP C efficiently nor does it interfere with RNA synthesis in the *in vitro* assay. The terminal 11 nucleotides starting from the 3' end (3' to 5') of PV negative-strand RNA (AAUUUUGUCGA) show a remarkable sequence similarity to the SELEX-identified oligonucleotide for highest-affinity binding to the hnRNP C native protein (rAGUAUUU UUGUGGA) (50). These nucleotides could constitute the site, or partial site, where hnRNP C binds to the PV negative-strand RNA. Nonviral sequences present at the 3' end of the PV negative-strand RNA significantly reduce positive-strand

RNA synthesis, indicating that authentic 3'-terminal nucleotides are important for initiation from that site (7, 8). Sequences forming loop b of the negative-strand cloverleaf affect the interaction of hnRNP C with the cloverleaf structure (49). We hypothesize that viral proteins or other cellular proteins may bind at this site and interact with hnRNP C, thus stabilizing its binding to the RNA and allowing its participation in a PV RNA synthesis initiation complex.

hnRNP C interacts with the 3' end of PV negative-strand RNA *in vitro*. In addition, we have data from preliminary UV cross-linking experiments demonstrating that recombinant hnRNP C also interacts with the 5' end of PV positive-strand RNA, albeit with decreased affinity compared to its interaction with PV negative-strand RNA (J. E. Brunner and B. L. Semler, unpublished results). Both positive- and negative-strand PV RNA coimmunoprecipitated with hnRNP C in extracts prepared from PV-infected HeLa cells. It is probable that PV negative-strand RNA may only exist, during the course of an infection, in the context of either the replicative form (RF) or the replicative intermediate (RI) (for a review, see reference 42). Therefore, we hypothesize that hnRNP C-specific anti-

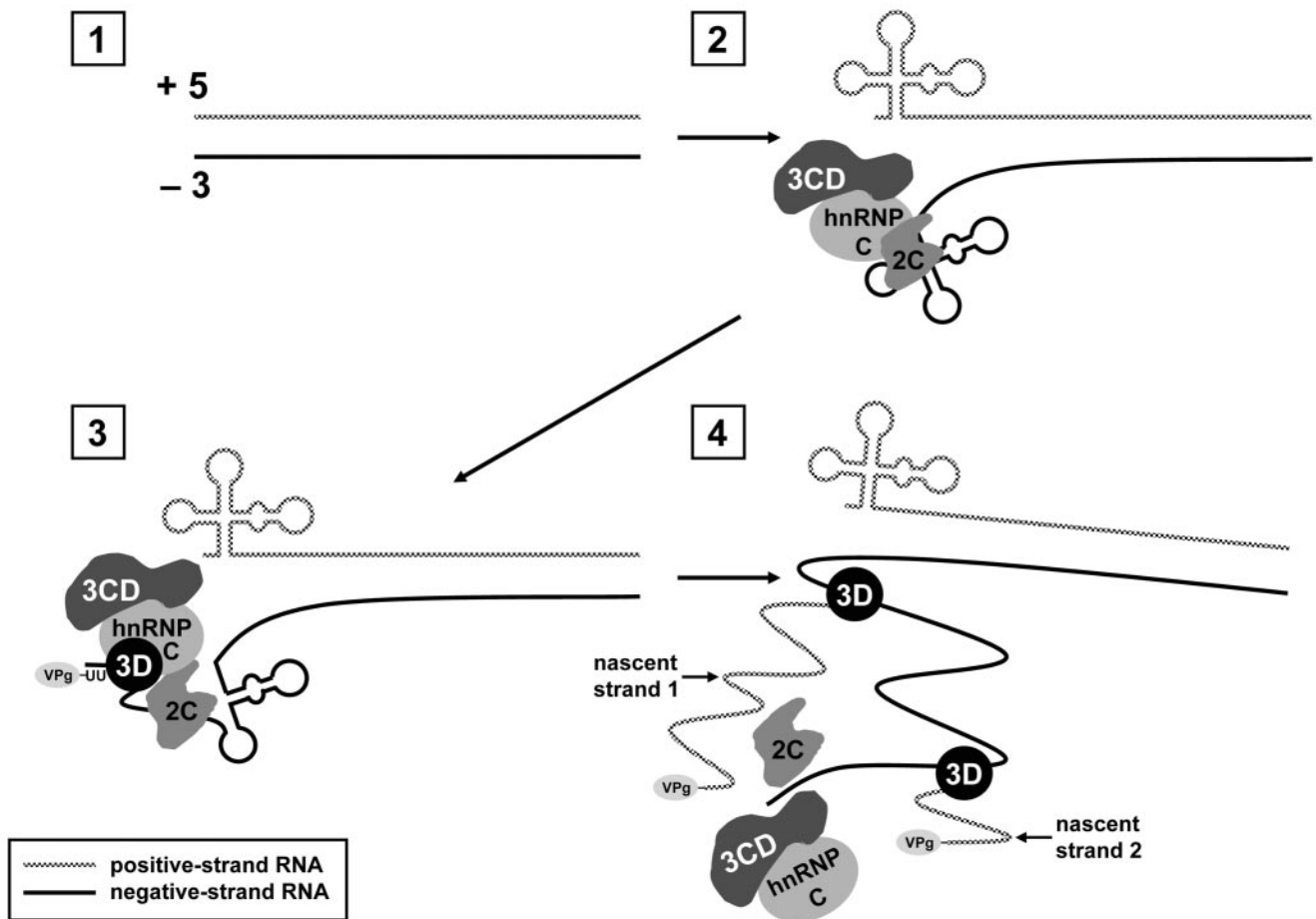


FIG. 8. Proposed model for initiation of PV positive-strand RNA synthesis. (Diagram 1) PV genomic (positive-strand) RNA is used as a template for synthesis of the negative-strand intermediate RNA. The two full-length RNAs form a duplex referred to as the RF. (Diagram 2) As the RNA duplex breathes, cloverleaf structures form at the ends of both the positive- and negative-strand RNAs. PV protein 2C, or 2BC, and hnRNP C bind to the negative-strand cloverleaf and stabilize the separation of the RNA strands. (Initial binding of hnRNP C to the RF may involve sequences and/or structures contained within the 3' end of negative-strand RNA and the 5' end of positive-strand RNA.) 3CD may be bound to hnRNP C. (Diagram 3) PV protein 2C, a putative viral helicase, relaxes the secondary structure of the negative-strand cloverleaf. hnRNP C binds near the terminus of the negative-strand RNA to expose the two terminal adenines for binding of VPgUpU. (Diagram 4) The viral RdRp, 3D^{pol}, elongates the positive-strand RNA on the negative-strand template. (3D^{pol} may be recruited to the initiation complex independently or may be present as the result of inter- or intramolecular cleavage of 3CD.) Multiple initiations of RNA synthesis occur on the same negative-strand RNA template. hnRNP C and PV protein 2C may be temporarily cleared from the template by elongating 3D but most likely stay loosely associated in the replication complex.

body pulled down RNAs of the PV RI form due primarily to the interaction of hnRNP C with the negative-strand RNA.

The data presented here do not directly demonstrate that hnRNP C plays a role in PV positive-strand RNA synthesis. However, based upon its binding to the 3' ends of PV negative-strand RNAs, we have proposed a model for the initiation of positive-strand RNA synthesis in PV-infected cells that includes the functional involvement for hnRNP C (Fig. 8). Synthesis of the negative-strand RNA from the positive-strand RNA template produces the full-length RF. This full-length RNA duplex is dynamic, and RNA breathing at the 5' positive-strand/3' negative-strand end allows the positive-strand and negative-strand cloverleaf structures to form by transient, thermodynamically favored (i.e., intramolecular) folding of the RNA. Proteins binding to the RNA structures, as they form, would stabilize the separation of the termini. It has been shown

that PV protein 2C, an NTPase and putative helicase (35, 43), as well as its direct precursor, 2BC, bind to the PV negative-strand cloverleaf (5, 6). Binding is mediated by two regions in 2C with RNA-binding activity that are distinct from the region that demonstrates NTPase activity (46). The binding of 2C is dependent on the intact sequence UGUUUU (5' to 3') of stem a of the negative-strand cloverleaf within the context of a double-stranded structure (5). The binding of 2BC is dependent on intact stem b of the negative-strand cloverleaf and its spatial orientation to stem a (6). PV 2C and/or 2BC bind to the negative-strand cloverleaf, in the stem a/stem-loop b region, possibly interacting with hnRNP C, which binds the negative-strand cloverleaf in the stem a region. 2C already present or released from 2BC by 3C^{pro} or 3CD binds to stem a of the negative-strand cloverleaf and exerts its proposed helicase activity to unwind the intramolecular RNA duplexes (or possibly,

intermolecular duplexes formed by RF RNAs). hnRNP C may facilitate this process, with its putative NTP-binding domain (17), which ultimately leads to exposure of the two terminal adenines on the 3' end of the negative-strand RNA. Interestingly, hnRNP C preferentially binds to single-stranded oligo(U) or poly(U) (17), although contiguous uridines are not obligatory for the generation of a high-affinity ligand (31). It has been suggested that high affinity may depend on context or structure of the RNA and that hnRNP C functions as a sequence-independent RNA chaperonin to maintain long lengths of RNA topologically single-stranded and accessible to splicing factors (51). Accordingly, it may be necessary for PV 2C and/or 2BC to bind the negative-strand cloverleaf first, when RNA structures and duplexes are intact, and then hnRNP C may bind with high affinity once the four adjacent uridines are single stranded and exposed. Of all of the PV 3' negative-strand RNAs tested *in vitro*, we found that hnRNP C has the highest affinity for the 66-RNA (data not shown). This may be explained by the fact that the complete stem a duplex is not predicted to form in the 66-RNA which would leave the uridines more accessible for binding.

In our model, hnRNP C could interact with 3CD and recruit it into the initiation complex. VPgpUpU, in high concentration as the result of uridylation of VPg on the 2C *cis*-acting replication element (*cre*) of the positive-strand RNA (37, 38), binds to the terminal adenines and primes elongation of the positive-strand RNA by the PV RdRp, 3D^{pol}, either bound directly or released from its precursor 3CD. hnRNP C, due to its preference for binding single-stranded oligo(U), may bind to the four consecutive uridines near the 3' terminus of the negative-strand RNA to keep the two terminal adenines exposed for multiple VPgpUpU-primed reinitiations of RNA synthesis by the viral polymerase.

Considerable information has emerged about RNA sequences, *trans*-acting factors, and mechanisms involved in initiation of picornavirus negative-strand RNA synthesis (for a review, see reference 42). However, the models for the initiation of positive-strand RNA synthesis are much less developed. The ratio of positive-strand RNA to negative-strand RNA in infected cells is ca. 40:1. Presumably, this ratio is optimal for efficient virus replication. Perhaps cellular factors, such as hnRNP C, increase the efficiency of positive-strand synthesis to ensure the rapid amplification of translation templates and genomic RNA. The relocalization of this nuclear RNA-binding protein to the cytoplasm may occur concomitant with an increase in the levels of negative-strand intermediate RNA (in RF) and viral replication proteins. By interacting with the 3' end of PV negative-strand RNA, hnRNP C could recruit viral proteins to an initiation complex for positive-strand RNA synthesis, thus enhancing the efficiency of the amplification of genomic RNA. Alternatively, hnRNP C may stabilize the single-stranded 3' end of the PV negative-strand RNA partially duplexed in the RF in preparation for binding of uridylylated VPg necessary to prime elongation by the 3D polymerase on the intermediate negative-strand RNA template for positive-strand RNA synthesis.

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