Specific Interaction between Human Parechovirus Nonstructural 2A Protein and Viral RNA*

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The functional properties of the nonstructural 2A protein are variable among different picornaviruses. The 2A protein of the human parechovirus 1 (HPEV1) has been shown to lack the proteolytic activity found in many other picornaviruses, but no particular function has been identified for HPEV1 2A. To obtain information about the role of HPEV1 2A in the viral life cycle, the protein was expressed in Escherichia coli. A polyclonal antibody was then raised against the protein and employed to investigate its subcellular localization in the infected cells by immunofluorescence microscopy. Typically, a diffuse cytoplasmic staining pattern, concentrated to the perinuclear area, was observed in the infected cells. However, at late stages of infection some infected cells also exhibited diffuse nuclear staining. Viral RNA, visualized by fluorescent in situ hybridization, partly colocalized with 2A in the perinuclear region. Three experimental approaches including Northernwestern blot, UV crosslinking, and gel retardation demonstrated that 2A possesses RNA binding activity. Competition experiments with various single-stranded RNA molecules addressed the specificity of 2A binding. These studies revealed that the 2A protein bound RNA corresponding to the 3'-untranslated region (UTR) of the viral genome with highest affinity. At the N- and C-terminal ends of the protein, two regions, necessary for RNA binding, were identified by mutagenesis. In addition, we demonstrated that 2A has affinity to double-stranded RNA containing 3'UTR(+) or 3'UTR(−). In conclusion, our experiments showed that HPEV1 2A binds to viral 3'UTR RNA, a feature that could be important for the function of the protein during HPEV1 replication.

Human parechovirus 1 (HPEV1) is a human pathogen associated with gastrointestinal and respiratory symptoms as well as with central nervous system infections. HPEV1 is a member of the Parechovirus genus of the family Picornaviridae. It is a small non-enveloped RNA virus with a single-stranded genome of positive polarity, ~7.4 kb in length. The genome encodes a large polyprotein, which is subsequently processed by virus-specific proteolytic activity to produce polypeptides involved in virus replication and virion assembly. Upstream of the open reading frame there is a long 5’-untranslated region (5’UTR) that is covalently linked to a small VPg protein. Downstream there is a 3’UTR and poly(A) (1).

The polyprotein processing reactions occurring in the sequences bordering the 2A polypeptide show major differences among picornaviruses. In enteroviruses and rhinoviruses, the 2A protein is a trypsin-like cysteine protease that is responsible for the cis cleavage at its own N terminus between the VP1 capsid protein and 2A. The trans-cleavage activity of 2A processes cellular factors, including eukaryotic initiation factor-4 (p220) (2–4), TATA-binding protein (5), and poly(A)-binding protein (6, 7). The cleavage of eukaryotic initiation factor-4G, and possibly poly(A)-binding protein, contributes to the shut-off of host cell protein synthesis (2, 6, 8, 9). In cardio- and aphthoviruses, 2A is involved in an unusual C-terminal proteolytic processing event between the 2A and 2B proteins (10–14).

Sequence alignment has revealed that the parechovirus 2A protein differs considerably from the corresponding proteins in other picornaviruses (15, 16). In vitro translation experiments have further shown that HPEV1 2A has no autocatalytic proteolytic activity (17). In addition, sequence analysis of 2A has revealed that the protease domain and the Asn-Pro-Gly-Pro (NPGP) peptide motif involved in protein processing are absent from the parechovirus, hepatitis A virus, and Aichi virus 2A proteins (16, 18). It has been shown recently (16) that the 2A proteins of these three picornavirus groups are related to each other and, additionally, to a recently identified family of cellular H box/NC proteins, which are possibly involved in the control of cell proliferation.

As an initial approach toward determining the function of the human parechovirus 2A, we expressed 2A in Escherichia coli, raised a polyclonal antibody reactive against the 2A protein, and studied its cellular localization relative to viral plus strand RNA in HPEV1-infected cells by conventional immunofluorescence and confocal microscopy. Analysis of the properties of 2A by Northernwestern blot, gel retardation, and UV crosslinking revealed that the protein exhibits RNA binding activity. We also analyzed the relative selectivity of the binding of 2A to RNA and mapped the regions in the polypeptide important for RNA binding. Furthermore, we found that 2A binds RNA in a cooperative manner and has affinity to double-stranded RNA. Based on its localization in the infected cells and RNA-binding properties, we suggest that 2A is involved in HPEV1 RNA replication.
Human Parechovirus 2A Protein

**Table I**

| Name          | Polarity | Sequence (5'-3') |
|---------------|----------|------------------|
| 2ApGEX        | Sense    | CACCTACCGGGAGGTCCAAACCAACAGATACGT |
| 2ApQE         | Sense    | TAAATATGATCCGCTCATATGGTACAACACACAG |
| 2A            | Antisense| GCCGCGCTGATTTCGTCATATGGTACATGAG |
| 2A(10–24)     | Sense    | TATCGCTGATATGGTACATGAG |
| 2A(10–24)     | Antisense| ATTTCTGTCGATATGGTACATGAG |
| 2A(43–56)     | Sense    | TTATCGCTGATATGGTACATGAG |
| 2A(43–56)     | Antisense| GAAAGATGTCACTGATATGGTACATGAG |
| 2A(130–150)   | Antisense| AGAGATCGACCTGATATGGTACATGAG |
| 2A(108–150)   | Sense    | AGAGATCGACCTGATATGGTACATGAG |
| 5' UTR        | Antisense| CGAGTGCAAGATGTAATATTAACAAATAAT |
| 5' UTR(70)    | Antisense| AGAGATCGACCTGATATGGTACATGAG |
| 5' UTR(365)   | Antisense| TGGGTGATATGGTACATGAG |
| 3' UTR        | Sense    | AGAGATCGACCTGATATGGTACATGAG |
| 3' UTR        | Antisense| AGAGATCGACCTGATATGGTACATGAG |

**Materials and Methods**

**Cloning of HPEV1 2A**

Plasmid pHPEV1 (kindly provided by G. Stanway, University of Essex, Colchester, UK), a full-length genomic cDNA clone of HPEV1 capable of generating RNA transcripts (15), was used as the parental plasmid for the cloning experiments. To clone and subsequently express 2A in pGEX 4T-1 (Amersham Biosciences) or pQE-30 (Qiagen) plasmid vectors, PCR amplifications were performed using a common 3'-oligonucleotide primer (2A) and respective 5'-oligonucleotide primers shown in Table I. The PCR products were cloned into the vectors using standard molecular biology protocols (19) to obtain the 2ApGEX-1 and 2ApQE-30 constructs. All the constructs were verified by sequencing.

**Serial Deletions of HPEV1 2A**

PCR amplifications were carried out using DyNazyme (Finnzymes) DNA polymerase, pHPEV1 cDNA as a template, and 5'-oligonucleotide primer (2ApQE) as a common primer. Oligonucleotides 2A(10–130) and 2A(108–150) (Table I) were used as 3'-primers to produce C-terminal deletion mutants C130–150 and C108–150, respectively. The PCR products were cleaved with BamHI and SalI and cloned into the pQE-30 vector.

To introduce the deletions into the 2A sequence, the 2ApQE-30 construct was used as a template for specific PCR-based mutagenesis using Pfu Turbo DNA polymerase (Stratagene). The internal oligonucleotide primers 2A(10–24) and 2A(43–56) (Table I) were used to produce deletion mutants N10–24 and N43–56, respectively. The PCR products were treated with DpnI (New England Biolabs) and T4 polynucleotide kinase (New England Biolabs) and self-ligated using T4 DNA ligase (New England Biolabs). All the constructs were verified by sequencing.

**Expression and Purification of Fusion Proteins and Production of 2A Antibody**

E. coli strain BL21(DE3) was transformed with 2ApGEX4T-1, and strain M15 [pREP4] was transformed with 2ApQE-30 or its derivatives and grown to an optical density of 0.6 at 600 nm in Luria broth. The protein expression was induced by addition of IPTG to a final concentration of 0.5 mM, and the cells were grown for 3 h at 30 °C and collected by centrifugation (5000 x g for 10 min). The proteins were purified under native conditions on glutathione-Sepharose 4B (Amersham Biosciences) in the case of GST-2A or on Ni-NTA-agarose (Qiagen) in the case of His-2A according to the manufacturer's protocols. Polyclonal antiserum against 2A was produced by immunization of a rabbit with purified GST-2A.

**UTR Cloning**

The viral 5' UTR, 3' UTR, and the 70 nucleotide fragments of the 5' UTR, 5' UTR(70) and 5' UTR(235–365) sequences, were PCR-amplified by using the pHPEV1 cDNA clone as a template. The primers used for the construction of UTR are shown in Table I. The oligonucleotide sequences incorporated either EcoRI or SalI restriction sites (Table I underlined). The amplified sequences were cloned into pGEM3Z vector (Promega) by using standard molecular biology protocols (19). The resulting constructs allowed synthesis of positive and negative strand RNA transcripts under control of SP6 or T7 RNA polymerases.

**In Vitro Synthesis of RNA**

Nonradioactive transcription reactions were carried out in a reaction mixture (40 μl) containing 40 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine, 5 mM each NTP, 40 units of RNasin (Promega), 10 μg of linearized plasmid, and 30 units of the appropriate RNA polymerase (Promega) for 1 h at 37 °C. 20 units of RNase-free DNase (Promega) was added, and the reaction mixture was further incubated for 15 min at 37 °C. 32P-Labeled RNAs were synthesized in the presence of 50 μCi of [α-32P]UTP (Amersham Biosciences) and 0.5 mM of unlabeled UTP. The transcripts were extracted with phenol/chloroform and filtered through a Sephadex G-50 (Amersham Biosciences) spin column. The newly synthesized RNA products were analyzed by gel electrophoresis, and their sizes were compared with those of RNA markers. In the case of radiolabeled RNA probes, the gel was dried and autoradiographed. The transcripts were quantified by measuring the absorbance at 260 nm and, in the case of the radiolabeled transcripts, also by Cerenkov counting.

Plasmid pHPEV1 was used to synthesize full-length transcripts of positive or negative polarity by linearization/transcription reactions using Mulu/T7 RNA polymerase or Sacl/T3 RNA polymerase, respectively. Plasmid pGEM3Z was used to synthesize control transcripts by linearization/transcription reactions using EcoRI/T7 or Sacl/Sph RNA polymerase.

**Immunofluorescence and Fluorescent in Situ Hybridization (FISH)**

Human lung carcinoma (A549, ATCC) cells were grown on glass coverslips and infected with HPEV1 at ~5 multiplicity of infection. The cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and 0.01% glutaraldehyde, followed by permeabilization in 0.3% Triton X-100. The preparations were quenched using 0.5 M NH4Cl and, subsequently, washed three times with PBS. The coverslips (infected cells and mock-infected controls) were incubated with the primary antibody (dilution 1:150), followed by a goat anti-rabbit TRITC-conjugated secondary antibody (Jackson Laboratories) or, for colocalization experiments, a goat anti-rabbit Alexa 488-conjugated secondary antibody (Molecular Probes). The coverslips were mounted in glycerol containing 4% N-propyl gallate (Sigma) and analyzed by using a Zeiss Axioplan 2 microscope using appropriate filters.

To visualize HPEV1(+)-stranded RNA in the infected cells, an Alexa 546-labeled riboprobe representing (+)- polarity of HPEV1 RNA was synthesized by in vitro transcription reaction as described (20–22). Cells grown as monolayers on glass coverslips were infected and fixed with paraformaldehyde and hybridized with the probe at 42 °C overnight (22) or, for colocalization experiments, at 37 °C for 8 h. The coverslips were mounted in glycerol containing 2% 1,4-diазабицилыхан (2,2,2)-пропан (Sigma).

Confocal microscopy was done with a confocal laser scanning microscope (TCS4D; Leica Lasertechnik). For colocalization, pictures were recorded sequentially. Images were processed with Adobe Photoshop software.

**RNA Binding Experiments**

Northwestern Assay—RNA binding assay with the membrane-bound proteins was performed as described previously (23). Briefly, ~1 μg of...
the protein was run in a 12.5% SDS-PAGE and then transferred to a nitrocellulose membrane (Schleicher & Schuell). After incubation in denaturation buffer (6 x urea, 0.01% Tween 20), the membrane was renatured at room temperature in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1× Denhardt’s reagent. Next, the membrane was probed with radiolabeled RNA corresponding to the full-length (+)-HPEV1 RNA. After five washes, the membrane was dried and subjected to autoradiography.

**UV Cross-linking/Label Transfer Assay—**Radiolabeled RNA probe (25 ng) was incubated with 2A (0.75 µg) in binding buffer A (10 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1.5 mM MgCl₂, 10 mM dithiothreitol, 5% glycerol) in a total volume of 15 µl at room temperature for 45 min. UV cross-linking was carried out as described previously (24). Briefly, the mixtures were placed as drops on a microscope slide and irradiated on ice with UV light (254 nm, 0.8 J/cm²) using Stratalinker 2400 (Stratagene). Nonprotected RNA was digested for 30 min at 37 °C by addition of 10 µg of RNase A (Promega). In a control reaction, the RNase treatment was omitted. The protein-RNA complexes were resolved on 12.5% SDS-PAGE, and the gels were dried and subjected to PhosphorImager analysis (Fuji) or autoradiography.

For competition binding experiments, radiolabeled pGEM3Z RNA probe (56 nt) was incubated with 2A protein without or in the presence of single-stranded unlabeled RNA competitors in various concentrations (as indicated in Fig. 5D). The unlabeled RNA competitors used for competitions are as follows: the fragment containing first 70 nucleotides from the 5’UTR of HPEV1 RNA genome, 5’UTR (70); the 70-nucleotide fragment containing the 295–385 region from the 5’UTR of HPEV1 RNA, 5’UTR (295–365); 3’UTR; polyA (Amersham Biosciences); 69 nucleotides from the multiple cloning region of the pGEM3Z vector, which did not contain HPEV1 sequences. The double-stranded (ds) competitors were generated by annealing of the (+) and (−) polarity strands of 5’UTR and 3’UTR RNA in hybridization buffer (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl) at 80 °C for 5 min and cooled to room temperature. The concentrations of various unlabeled dsRNA competitors versus radiolabeled single-stranded (ss) RNA used in the competition binding assay are indicated in Fig. 6A. In competition binding assay radiolabeled RNA was incubated with 2A protein in the presence of the unlabeled complementary RNA competitor (as indicated in Fig. 6B) in 8-fold molar excess to the radiolabeled RNA.

**Gel Retardation Assay/Gel Mobility Shift Assay—**The radiolabeled RNA transcript (0.54 nt) was incubated with 2A in 16 µl of binding buffer A for 45 min at room temperature. In control experiments the 2A-RNA complexes and free RNA were subsequently incubated in buffer SB containing 1% SDS and 100 mM β-mercaptoethanol. Some samples were additionally treated with 1 µg of RNase A (Promega) prior to incubation in buffer SB. The reaction products were analyzed by electrophoresis in 1% agarose gels in TAE buffer. The gels were then dried and subjected to analysis using PhosphorImager or autoradiography.

The experimental binding curve was statistically fit to data using Sigma Plot program (SSPS Inc.). In control experiments the radiolaabeled 3’UTR RNA was incubated in the presence of 0.75 µg of BSA or ΔN25 (kindly provided by N. O. Kalinina, Moscow State University, Russia) proteins or without a protein.

**RESULTS**

**Expression and Purification of the Fusion 2A Protein Expressed in E. coli—**As a first step toward analyzing the biochemical properties of the HPEV1 2A protein, the genomic region coding for 2A was cloned into IPTG-inducible bacterial expression vectors pGEX4T-1 and pQE-30. These vectors allowed expression of 2A as fusion proteins containing either GST or oligohistidine affinity tags at the N terminus. IPTG induction resulted in high expression levels of soluble 2A in E. coli. Chromatography on glutathione-Sepharose or Ni-NTA-agarose was used to purify GST-2A and His-2A, respectively. The proteins were purified to near-homogeneity as evaluated by SDS-PAGE and Coomassie Blue staining (Fig. 1).

**2A Distribution in the Infected Cells—**To determine the intracellular localization of the 2A protein during HPEV1 infection, A549 cells infected with HPEV1 and fixed with paraformaldehyde were examined by indirect immunofluorescence using the 2A antibody at different times post-infection (p.i.). In infected cells, a positive signal was first observed at 4 h p.i., whereas no significant immunofluorescence was detected in mock-infected cells. No immunostaining was detected in HPEV1-infected cells when preimmune serum, the control GST-antiserum, or the TRITC-conjugated secondary antibody alone were employed, demonstrating the specificity of the antibodies (Fig. 2A, f–h).

At 4 h p.i., the 2A protein exhibited a rather diffuse cytoplasmic staining localized mainly in the perinuclear area (Fig. 2A, a). At 6 h p.i., the 2A signal became more intense, and it was evenly distributed throughout the cytoplasm. In addition, there appeared a weak, diffuse nuclear staining that blurred the border between the cytoplasm and the nucleus (Fig. 2A, b). At late stages of infection (8 and 10 h p.i.), two distinct types of staining patterns were observed. Some cells had the same type of diffuse cytoplasmic staining seen early in infection, whereas others displayed a stronger nuclear staining and weaker cytoplasmic staining (Fig. 2A, c and d). The proportion of cells displaying nuclear staining grew over time. The nuclear localization of the 2A protein at late stages of infection was confirmed by confocal microscopy (data not shown).

In parallel, we employed FISH to visualize viral RNA in HPEV1-infected cells. As shown in Fig. 2A, c, RNA could be detected as granules covering the cytoplasm and also as larger aggregates that could be found mainly in the perinuclear area.

The observation that 2A was found mainly in the perinuclear area prompted us to test a possible colocalization of 2A with viral RNA. We examined the colocalization of 2A and viral RNA at 6 h p.i., at the peak of viral RNA synthesis (22). A549 cells were infected with HPEV1, stained with 2A specific antibody, and subsequently subjected to FISH. The specimens were analyzed by confocal microscopy (Fig. 2B, a and b). Merging the two confocal images of Fig. 2B, a and b) revealed that 2A partially colocalized with viral RNA (Fig. 2B, c), indicating that the 2A protein is present at the perinuclear sites of viral replication.

**Detection of the RNA Binding Activity of 2A and Its Specificity—**It was demonstrated recently (22) that the replication complex of HPEV1 is localized mainly in the perinuclear area of the infected cells. Following our finding that 2A is partially colocalized with viral RNA, we next studied the possible interactions between 2A and viral RNA.

To rule out the possibility that the large GST tag would interfere with potential RNA binding activity of 2A, we used the His₆-tagged protein in the subsequent experiments. Initial experiments were designed to determine whether the HPEV1 2A protein possesses RNA binding activity in a Northwestern blot assay. The same amount of another His₆-tagged protein, ΔN25 (26), and BSA were used as negative controls (Fig. 3A). After electrophoresis, the proteins were transferred onto a
membrane and renatured. The membrane was then incubated with radiolabeled RNA transcript corresponding to the full-length (+) HPEV1 RNA. Further washings removed unbound RNA, and the filter was analyzed by autoradiography. As shown in Fig. 3B, autoradiography revealed that the 2A protein bound RNA, whereas no binding of RNA to N25 or BSA was observed.

To study further the RNA binding activity of 2A, we performed UV cross-linking assays. The protein-RNA complexes were cross-linked by UV light; the unbound RNA was removed by RNase A digestion, and the cross-linked complexes were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3C, a strong radiolabeled band could be seen in the position expected for the 2A protein. A weaker band migrating at the position of 32 kDa, which probably corresponds to a cross-linked dimer of 2A, was also detected (Fig. 3C). The results in Fig. 3C also show that binding of 2A resulted in the formation of high molecular weight complexes. The upper band (>175 kDa) probably represents complexes of more than 10 monomers of 2A bound to the one RNA molecule. Formation of such large cross-linked complexes indicates that the protein molecules are bound to RNA in close association with each other. Contiguous arrangement of bound protein molecules is characteristic for a cooperative binding mode. When no RNase was added, the band disappeared into a smeared background with most of the labeled RNA on the top of the gel (Fig. 3C). No band was observed when incubation was carried out without UV irradiation (data not shown) or when the 2A protein was substituted with BSA or ΔN25 (Fig. 3C).

Our next goal was to find out whether the 2A protein has any sequence specificity or preference in binding to RNA. The specificity of the 2A-RNA interaction was assessed by comparing the abilities of different unlabeled RNA substrates to compete with the radiolabeled probe for binding to 2A. Fig. 3D shows the results of an experiment in which pGEM3Z radiolabeled RNA was used to evaluate the affinity of 2A for various competing substrates. The unlabeled competitors were used in 10-, 15-, 45-, and 95-fold molar excess to the single-stranded radiolabeled RNA probe. The competitors included poly(A), the fragments of 5′-untranslated region, 5′UTR(70) and 5′UTR(295–365), 3′-untranslated terminal end of HPEV1 RNA of both positive and negative polarity, and non-parechovirus sequence...
FIG. 3. Analysis of the RNA binding activity of the 2A protein. A and B, the Northwestern blot assay. A, the proteins were transferred onto a membrane after electrophoresis in SDS-PAGE and stained with Ponceau S. B, RNA-protein blot. The membrane was then denatured in 6 M urea, renatured, and probed with a radiolabeled RNA-transcript corresponding to HPEV1(+) RNA and subjected to autoradiography. C, UV cross-linking assay of the 2A RNA binding activity using radiolabeled HPEV1(+) RNA probe. Probes were subjected to UV cross-linking with the 2A protein, BSA, or ΔN25. The reaction products were incubated in the presence or absence of RNase A and then analyzed by 12.5% SDS-PAGE and autoradiography. The arrow indicates the position of a complex between 2A and RNA. The positions of molecular mass markers (shown in kilodaltons) are indicated on the right. D, analysis of binding preference of 2A to single-stranded RNAs. The 2A protein was incubated with the radiolabeled pGEM3Z RNA either in the absence or in the presence of increasing amounts of the unlabeled competitor RNAs. The reactions were analyzed by UV cross-linking assay and autoradiography. The competitors were used at 10-, 15-, 45-, and 95-fold excess (from left to right in the graph) compared with the radiolabeled RNA. The autoradiograph of the protein-RNA complexes at 10-fold excess of the competitors compared with the radiolabeled RNA is shown below the graph. The percentage of RNA bound was calculated by PhosphorImager software. E, α and β, increasing amounts of the 2A protein were incubated with the radiolabeled RNA transcript corresponding to the 3'UTR(+) (α) or 5'UTR(+) HPEV1 RNA (β) and analyzed by gel retardation in nondenaturing agarose/TAE gel. The amounts of the 2A protein used in the assay are indicated. α, plot of the percentage of RNA bound versus molar concentration of 2A (solid circles correspond to 3'UTR bound 2A; open circles correspond to 5'UTR bound RNA).
derived from pGEM3Z. The RNA-protein complexes were analyzed by UV cross-linking assay (Fig. 3D, lower panel). In comparison to competition in the presence of 3′UTR RNA, 2A binding to the radiolabeled RNA probe was more effective at the same unlabeled: labeled molar ratios of the other RNA competitors. When the radioactivity associated with RNA-2A complexes was quantitated by PhosphorImaging and compared, the difference between the viral 3′UTR and other RNA competitors became apparent (Fig. 3D, upper panel). For example, addition of a 10-fold excess of unlabeled 3′UTR (+) RNA resulted in a 45 ± 5% reduction in binding of 2A to radiolabeled RNA. 3′UTR (-) RNA competitor produced almost the same effect, with an ~43% reduction in binding. At the same molar ratio, other competitors produced only an ~25% reduction in binding. Binding differences between the 3′UTR RNA and other RNAs were enhanced at higher concentrations of the competitors. Maximum excess of the 3′UTR RNA competitor essentially prevented complex formation with the radiolabeled RNA, whereas at the same concentration of other competitors formation of complexes between 2A and the radiolabeled RNA was still observed.

These results suggest that 2A has a preference for binding to the 3′UTR HPEV1 RNA. The notion that 2A has higher affinity to the 3′UTR RNA was further supported in electrophoretic retardation experiments in nondenaturating agarose gels. These experiments allowed us to determine the dissociation constants ($K_d$) of the 2A-3′UTR and 2A-5′UTR complexes. Increasing amounts of the 2A protein were incubated with the radiolabeled RNA, which corresponded to 3′UTR or 5′UTR, and then subjected to electrophoresis (Fig. 3E, a and b). Most interestingly, ~20% of the 3′UTR RNA was bound to 2A at a protein concentration of 10 nM (Fig. 3E, c). However, the 5′UTR-2A complex was not detected at the same protein concentration (Fig. 3E, c). Saturation of the 3′UTR RNA binding was achieved at about 0.1 μM of the 2A protein, whereas the saturation of the 5′UTR RNA binding was achieved at significantly higher protein concentration, 0.4 μM.

The percentage of the retained radioactivity was represented as a function of the 2A concentration, and the apparent $K_d$ value for the 2A-RNA complexes was estimated as the 2A concentration, at which half of the RNA is bound (Fig. 3E, c). The $K_d$ values for the 2A-3′UTR and 2A-5′UTR complexes were determined to be 0.03 and 0.1 μM, respectively. Taken together, these results allowed us to conclude that the 2A protein exhibits a preference for binding to the 3′UTR HPEV1 RNA rather than to other RNA sequences.

The above-mentioned findings together with a suggestion that proteins interacting with RNA structures at the 3′UTR region of picornaviruses are important during viral RNA replication (27) prompted us to investigate the influence of the secondary structure of 3′UTR (+) RNA on binding to 2A. This was studied by preparing equal amounts of RNA in various states and observing their interaction with 2A. The RNA probe was first heat-denatured at 95 °C for 5 min and then rapidly cooled on ice to maintain the denatured state. Alternatively, the probe was slowly cooled to room temperature to allow the RNA secondary structure to reform. Fig. 3F shows that the ability of the denatured probe to interact with the 2A protein was reduced suggesting that the secondary structure of the 3′UTR has an influence on its ability to bind the 2A protein.

**Binding Cooperativity**—Additional gel retardation experi-
N/H9004 43–56, C/H9004 130–150, and C/H9004 108–150, which had N- or C-terminal deletions of 15, 14, 20, and 42 amino acids, respectively (Fig. 5A). Each of the expressed deletion mutant proteins was purified and examined for the level of purity by SDS-PAGE (Fig. 5B).

The RNA binding activities of the 2A mutants were analyzed by UV cross-linking. As can be seen in Fig. 5C, the N/H9004 43–56 mutant completely lost its ability to bind to RNA, whereas deletion of residues 10–24 at the N terminus did not affect RNA binding. Most interestingly, the C/H9004 130–150 mutant still...
demonstrated ability to bind RNA but could bind less RNA than intact 2A. However, the CΔ108–150 mutant protein could not bind RNA, suggesting that the C terminus of the protein could play an important role in the RNA interaction. In conclusion, these data provide evidence that the basic N-terminal 43–56 region is essential for interaction with RNA and that the C terminus also appears to play a pivotal role in the RNA binding activity of 2A.

The 2A Protein Has Affinity to dsRNA—RNA-RNA duplex regions are generated in the course of picornavirus replication, and therefore, it was of interest to test whether 2A could recognize double-stranded RNA in addition to single-stranded RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. The protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. In this assay, the protein was incubated with radiolabeled pGEM3Z RNA and unlabeled dsRNAs corresponding to RNA. The results shown in Fig. 6A, b, demonstrate that the ssRNA binding activity of 2A was gradually reduced in the presence of increasing concentrations of the 3′ UTR (+)-3′ UTR (−) duplex, and no significant effect was detected when the 5′ UTR (+)-5′ UTR (−) duplex was used as a competitor. Most interestingly, comparison of the effects of the competitors on the ssRNA binding activity of the 2A protein (Figs. 3D and 6A, b) revealed that 2A has a preference in binding in the following order: 3′ UTR > other ssRNA > 3′ UTR (+)-3′ UTR (−) duplex.

In subsequent experiments, 2A was incubated with radiolabeled probe and complementary unlabeled RNA and subjected to UV cross-linking. We used 5′ UTR and 3′ UTR of both (+) and (−) polarity as RNA probes. As can be seen in Fig. 6B (lanes 3 and 4), two bands were observed corresponding to 2A bound to ss3′ UTR (asterisk) and to the duplex 3′ UTR (+)-3′ UTR (−) (double asterisks). However, in case of 5′ UTR RNA (+) and (−), only one band corresponding to 2A bound to ss5′ UTR could be detected (Fig. 6B, lanes 1 and 2). These results indicate that 2A possesses affinity for both ssRNA and RNA duplex containing 3′ UTR (+)-3′ UTR (−) sequences.

DISCUSSION

The replication of RNA viruses is a complex process, requiring multiple protein-RNA and protein-protein interactions. Nonstructural proteins play key roles in the replication of viruses. In addition to being involved in viral RNA replication (34, 35), the picornaviral nonstructural proteins are associated with the rearrangement of intracellular membranes and the induction of the membranous viral replication complexes during virus infection (22, 36, 37). The picornavirus nonstructural 2A protein has been proposed to exert its function on viral RNA replication either directly or indirectly (38–42); however, the exact mechanisms of its action are not known.

To our knowledge, this study represents the first analysis of the biochemical properties and cellular localization of the parechovirus 2A protein. This analysis was facilitated by the expression of HPEV1 2A as a fusion protein, successful purification of the soluble protein, and production of a specific, polyclonal antibody against 2A. By using this antibody, we showed that at early stages of HPEV1 infection the 2A protein can be found diffusely distributed throughout the cytoplasm, concentrated to the perinuclear region. Later, however, 2A could also be detected in the nucleus of some infected cells. Despite the presence of the HPEV1 2A protein in the nucleus, no canonical nuclear localization signal in the 2A sequence was identified. Partial nuclear localization of 2A could have resulted from a property of the host cell, e.g. increased permeability of the nuclear membrane or the stage of cell cycle. The nuclear localization of 2A may be required for a specific modification of the protein or recruitment of a cellular factor to the viral complex. On the other hand, the nuclear localization of the 2A protein may be part of a strategy to modulate host cell functions.

The presence of viral proteins in the nucleus appears to be uncharacteristic for proteins of cytoplasmic viruses. However, some positive- and negative-stranded RNA viruses, which replicate in the cytoplasm, have been described to sequester nu-
clear factors in order to facilitate virus replication and, by altering nuclear-cytoplasmic trafficking, to disrupt host cell functions and cellular responses to viruses (43–50). Therefore, interaction with the nucleus does not appear to be restricted to those viruses that use the nucleus as sites of replication. Because the 2A protein is only 16 kDa, passive diffusion could account for its nuclear entry. However, the stretches of basic residues of 2A may act as cis elements, e.g. nuclear localization signals for active nuclear import. This was experimentally proved for recombinant α3 protein, which exhibited a staining pattern similar to 2A (51, 52) and Rev protein of human immunodeficiency virus (53). Furthermore, a diffuse cytoplasmic distribution and association of the 2A protein with the nucleus was recently demonstrated for mengo- and encephalomyocarditis (EMCV) viruses (54). Even at the earliest times of post-infection, the 2A protein of EMCV was detected in the nucleus of the infected cells. Most interestingly, the nuclear localization was not unique to the EMCV 2A protein; the viral polymerase, the 3D protein, as well as the viral protease and VPg were also found in the nucleus (54).

Previous electron microscopic data have shown that in HPEV1-infected cells, vesicles carrying viral RNA can be found in small groups distributed throughout the cytoplasm and in larger clusters in the perinuclear region (22). As the 2A protein exhibited a diffuse cytoplasmic staining pattern, the exact subcellular compartment targeted by it could not be identified. However, colocalization analysis of 2A and viral RNA revealed that the 2A protein was concentrated to the perinuclear area and partially colocalized with HPEV1 RNA. This observation implies a possible association of 2A with the viral replication complex. Furthermore, results showing that antibody against viral RNA polymerase, the 3D protein, revealed a staining pattern very similar to that of 2A further supported this hypothesis.

In this work, we further demonstrated that the HPEV1 2A protein possesses RNA binding activity. The competition binding experiments showed that neither unrelated nor related RNA was as effective as 3’UTR RNA in forming the RNA-protein complex. Our observations indicate that 2A exhibits two types of RNA-binding properties, sequence-specific and nonspecific. There are some known proteins, including viral proteins, that exhibit such dual characteristics (55–60). Heterogeneous nucleic ribonucleoproteins are capable of both sequence-specific binding to certain sequences and less sequence-specific binding at high concentrations, a function probably serving to hinder the formation of secondary structures in the RNA (61, 62). How then could 2A discriminate between target and non-target RNAs? We suggest that 2A discriminates between different RNAs on the basis of both nucleotide sequence and RNA structures. In agreement with this possibility, 2A bound the denatured 3’UTR(+), RNA with lower affinity than native RNA. The secondary structure of the 3’UTR could, therefore, be a regulatory factor in the interaction with 2A. This notion is also supported by the fact that polio- and rhinovirus 3’UTR contains certain structural elements, which have been shown to control viral RNA synthesis (63–65). It is also possible that in infected cells other viral or host proteins in the replication complex increase the RNA binding specificity of 2A.

RNA binding in the presence of increasing concentrations of 2A, followed by native TAE-agarose gel electrophoretic mobility-shift assay, resulted in two forms of RNA, free and retarded, indicating all or none behavior, which is consistent with cooperative binding (25, 28–33). The observation that in the presence of 2A discrete lengths of RNA, but not the entire RNA molecule, were protected against digestion with RNase indicates that the RNA is coated with 2A protein as a result of cooperative binding. Furthermore, the binding of 2A to 3’UTR(+) was clearly dependent on the native conformation of the protein. 2A presented a specific 3’UTR(+) RNA binding activity with an approximate Kd of 0.03 μM.

It is generally believed that conserved sequences and structures at the 3’ terminus of viral genomic RNA function as cis-acting signals that interact with viral proteins to initiate minus strand RNA synthesis during viral replication. Based on the results of the current study showing that 2A preferably binds to positive sense 3’UTR RNA and on the subcellular distribution of 2A in the infected cells, we hypothesize that 2A is involved in recognition of specific sequences during the RNA synthesis. Because the initiation of RNA synthesis starts at the 3’ terminus of positive- and negative-strand RNA, it can be suggested that the interaction presented here is important for viral RNA replication possibly by keeping the 3’-end of RNA immobilized so that other viral and/or host proteins could complex together for initiation of RNA synthesis. The results of poliovirus 2A mutagenesis and experiments using dicistronic poliovirus genomes have demonstrated that this protein is involved in RNA replication (38, 39). Furthermore, recent experiments (66) using mutant chimeric viruses have revealed that poliovirus 2A contains a motif important for viral replication. Deletion of this motif leads to a decrease in the levels of viral replication. Moreover, it has been shown that this motif is not necessary for the proteolytic activity of the protein (66).

Although 2A is an RNA-binding protein, it does not contain any obvious nucleic acid-binding sequences. To identify the RNA-binding region we prepared and examined various deletion constructs. We found that the N-terminal basic-rich region (amino acids region 43–56) as well as the C terminus is important for the RNA-binding properties of the protein. It seems unlikely that the protein lost its affinity to RNA simply because of an alteration in the conformation of the deletion mutants, but this possibility cannot be completely ruled out.

In addition to the ssRNA binding activity of 2A, we found that the protein possesses affinity to dsRNA. dsRNA-binding proteins are found in diverse organisms, including viruses, bacteria, and lower and higher eukaryotes. Most of these proteins are known to bind dsRNA without obvious RNA sequence specificity (67). In contrast, the 2A protein has the ability to bind duplex RNA containing 3’UTR(+)–3’UTR(−), but not other dsRNA molecules studied. This finding could be explained by sequence and structural factors recognized by 2A.

In conclusion, we found that during parechovirus infection, the 2A protein could typically be found diffusely in the cytoplasm, with the bulk of the protein localized to the perinuclear area, where partial colocalization with viral RNA was detected. In an attempt to elucidate the function of the protein, we showed that 2A is an ssRNA-binding protein with preferential binding to 3’UTR HPEV1 RNA. In addition, we demonstrated that 2A has the ability to bind to the duplex containing 3’UTR(+)–3’UTR(−). However, the affinity to the duplex was significantly lower than to ssRNA. The localization of the protein in the infected cells and its abilities to interact with RNA suggest that 2A participates in viral replication events. Furthermore, we defined the N-terminal 43–56-amino acid region as well as the C terminus of 2A as being important for the RNA binding of 2A. Mutational analysis of these regions in the viral genome would be of great interest for our future work.

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2 O. Samuilova, C. Krogerus, T. Pöyry, and T. Hyypia, unpublished data

3 Denis Kainov is acknowledged for helpful discussions.
