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Genomic and Subgenomic RNAs of Rabbit Hemorrhagic Disease Virus Are Both Protein-Linked and Packaged into Particles

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The major subgenomic RNA of the calicivirus rabbit hemorrhagic disease virus which codes for the viral capsid protein has been cloned as cDNA. The nucleotide sequence of this mRNA was shown to be identical to the 3′ terminal region of the genomic RNA. The 5′ end of the mRNA corresponds to position 5296 of the genomic sequence; except for two differences the first 16 nucleotides of genomic and subgenomic RNAs are identical. After isolation from liver tissue viral genomic and subgenomic RNAs were found to be resistant to RNase degradation. This protection was due to RNA packaging into particles. Sucrose density gradient centrifugation of liver homogenates allowed separation of such particles containing either genomic RNA or subgenomic RNA. Genomic and subgenomic RNAs are protein-linked and for the genomic molecule this interaction is localized within the first 179 nucleotides. After radioactive labeling of purified RNA and subsequent RNase treatment a protein of 15 kDa was identified.

INTRODUCTION

The family Caliciviridae represents a poorly studied group of nonenveloped animal viruses which comprises as prototypes the feline calicivirus (FCV), the San Miguel sealion virus (SMSV), and the vesicular exanthema virus of swine (VEV). The agent causing rabbit hemorrhagic disease (RHD) which was first described 7 years ago in China (Liu *et al.*, 1984) is an additional member of this family (Šmid *et al.*, 1989; Ohlinger *et al.*, 1990; Parra and Prieto, 1990). Recently relationships between caliciviruses and the human pathogens hepatitis E virus (Reyes *et al.*, 1990) and Norwalk virus (Jiang *et al.*, 1991) have been suggested.

The calicivirus genome consists of a single-stranded RNA of positive polarity which has a size of about 8 kb (Neill and Mengeling, 1988). For VEV and SMSV it has been demonstrated that the genomic RNAs are covalently linked to proteins of 10 and 15 kDa, respectively (Black *et al.*, 1978; Burroughs and Brown, 1978; Schaffer *et al.*, 1980). In analogy to picornaviruses (Lee *et al.*, 1977) this polypeptide was termed "VPg." In addition to genome size and protein–RNA linkage, sequence comparison studies have pointed toward further similarities between picorna- and caliciviruses. (Neill, 1990; Meyers *et al.*, 1991b). One major difference between both virus families concerns the strategy of gene expression. While picornavirus-infected cells contain only genome-sized RNA which is translated into one large polyprotein (Palmenberg, 1987), caliciviruses probably use transcription and translation of subgenomic RNAs for gene expression (Neill and Mengeling, 1988; Carter, 1990). For FCV three to eight RNA species smaller than the genomic RNA have been described; according to hybridization studies these molecules form a 3′ nested set (sizes described by Neill and Mengeling (1988) are: 4.8, 4.2, and 2.4 kb; those reported by Carter (1990) are: 5.3, 4.3, 3.6, 2.7, 1.9, 1.5, and 0.55 kb). Depending upon the time after infection the relative amounts of these subgenomic RNAs vary. Interestingly negative sense RNA detected in infected cells was not only of genome size but also of 2.4 kb (Neill and Mengeling, 1988) or 5.3, 4.3, 3.6, and 2.7 kb (Carter, 1990). It has therefore been suggested that the respective subgenomic RNAs are replicated independently (Neill and Mengeling, 1988; Carter, 1990).

Characterization of rabbit hemorrhagic disease virus (RHDV) subgenomic RNAs is hampered by the fact that a tissue culture system for virus propagation is not available. RNA from liver of infected rabbits contained an RHDV-specific band of about 2.2 kb in addition to genomic RNA (Ohlinger *et al.*, 1990; Meyers *et al.*, 1991b). This subgenomic RNA most likely is analogous to the major FCV subgenomic RNA of 2.4 or 2.7 kb (Neill and Mengeling, 1988 or Carter, 1990, respectively). In the present report, analyses of the genomic as well as the major subgenomic RNAs of RHDV are presented.

MATERIALS AND METHODS

Materials

All materials were as described (Meyers *et al.*, 1991b).

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Purification of RNA from RHDV virions and Northern blotting

The initial steps of virus propagation and isolation from liver of infected animals were performed as described in the companion paper. Freon-extracted liver homogenate was centrifuged for 90 min at 38,000 rpm in a SW41Ti rotor (Beckmann). The resulting pellet was resuspended in 2 ml RNA lysis mix (Chirgwin *et al.*, 1979). The sample was cleared (5 min at 14,000 rpm, Eppendorf centrifuge) and the RNA further purified as described before (Rümenapf *et al.*, 1989).

For RNA preparation from fractions of sucrose density gradients 1 ml of liver homogenate from RHDV-infected rabbits extracted with Freon 113 (Serva, Heidelberg) was layered on top of a 15 to 30% sucrose density gradient and centrifuged for 4 hr at 25,000 rpm in a TST 28.38 rotor (Kontron) as described by Ohlinger *et al.* (1990). One hundred microliters of each 200-μl fraction was mixed with 1 vol of protease mix (20 m*M* Tris–HCl, 10 m*M* EDTA, 1% SDS, 20 μg of Proteinase K, and 200 μg Pronase, pH 7.6). After incubation at 37° for 30 min 800 μl RNAzol (Cinna/Biotex, Texas) was added and RNA prepared as recommended by the supplier.

Separation of RNA by agarose gel electrophoresis, Northern blotting, and hybridization with 5′ end-labeled oligonucleotides (Maniatis *et al.*, 1990) as probes were performed as described (Meyers *et al.*, 1989). Synthesis of oligonucleotide Ol-RHD2 (complementary to positions 5377 to 5394 of the genomic RHDV sequence) was done as described in the companion paper.

cDNA synthesis and cloning

The synthesis of double-stranded cDNA using oligo(dT) as primer and selection for molecules larger than 1 kb were performed as described (Meyers *et al.*, 1991a). RNA from pelleted virions served as starting material. Ligation of cDNA with plasmid pBluescript (Stratagene) and transformation of *Escherichia Coli* XLBlue cells (Stratagene) were done according to standard procedures (Maniatis *et al.*, 1990). Screening of the library with ^{32}P-labeled DNA (insert of clone pRHD1; Meyers *et al.*, 1991b) was done as described by Grunstein and Wallis (1979).

Primer extension

The primer extension reaction was conducted as described in the companion paper except for using RNA from pelleted virions as a template. Oligonucleotide Ol-RHD2 served as a primer.

Protease treatment of RHDV RNA

RNA samples (0.5 μg) were incubated for 15 min at 37° in 100 μl of either PK mix (10 m*M* Tris–HCl, 5 m*M* EDTA, 0.5% SDS, 5 μg yeast tRNA, 10 μg Proteinase K, pH 7.6) or PN mix (same as PK mix except for 10 m*M* EDTA and 100 μg of Pronase instead of Proteinase K). Afterward, the mixtures were extracted twice with phenol and once with chloroform prior to ethanol precipitation (Maniatis *et al.*, 1990).

RNA cleavage by RNAzyme

The reaction was performed using the components of the RNAzyme Tet 1.0 kit (United States Biochemical Corp., Heidelberg, FRG). One microgram of RHDV RNA was incubated for 1 hr at 50° in a total volume of 20 μl containing 0.2 m*M* GTP, 1.5 *M* urea, and 60 units of RNAzyme in 1× RNAzyme buffer. The reaction was stopped by removing the essential cofactor GTP by passage of the mixture through a Sephadex G-50 (Pharmacia-LKB, Freiburg, FRG) spun column (Maniatis *et al.*, 1990) and ethanol precipitation.

Iodination of RNA-bound protein

One microgram of RHDV RNA isolated from pelleted virions was used as starting material for protein labeling with Na^{125}I according to the chloramine-T method (Greenwood *et al.*, 1963). Protease treatment using Proteinase K was performed as described above.

RESULTS

cDNA cloning and sequencing of the major subgenomic RNA of RHDV

The livers from rabbits which died from rabbit hemorrhagic disease are pale and fragile and show characteristic pathologic lesions (Ohlinger *et al.*, 1990). RNA prepared from such tissue was shown to be almost completely degraded when compared with RNA from liver of noninfected rabbits (Fig. 1, lanes 1 and 2, respectively). However, in a Northern blot with an RHDV cDNA probe both genomic and major subgenomic RNAs of RHDV were detectable as dominant sharp bands; there was only a slight smear due to degraded molecules (Fig. 1, lane 1*). Obviously both RNA species are not susceptible to RNase degradation following virus-induced cell lysis. Packaging of the RNAs into viral proteins may be responsible for the observed protection. On the basis of this hypothesis RNA was isolated from pelleted particles after centrifugation of extracted liver homogenates. Denaturing agarose gel electrophoresis revealed that the resulting material was predominantly composed of genomic and 2.2-kb subgenomic RHDV RNA (Fig. 2) and thus represented a suitable starting material for analysis of the subgenomic molecule.

The subgenomic RNA was shown to bind to

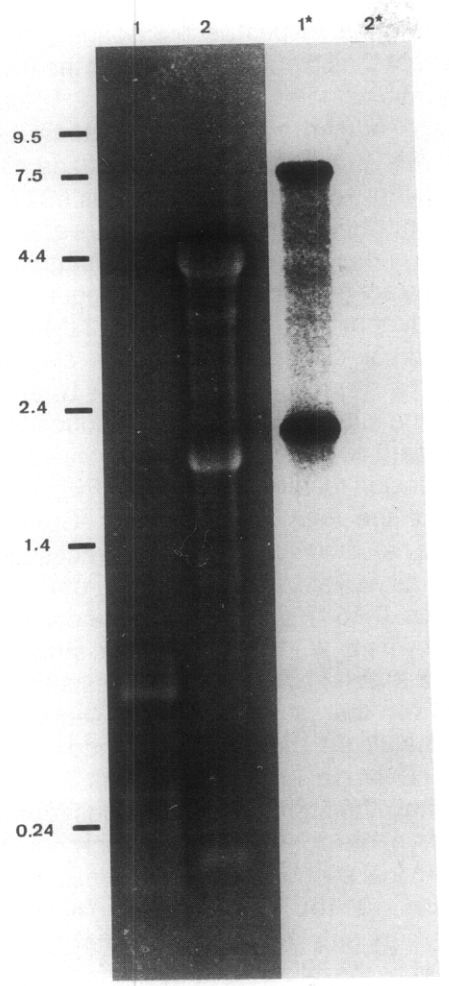

FIG. 1. RNA extracted from liver tissue of RHDV-infected (lanes 1 and 1*) and noninfected (lanes 2 and 2*) rabbits was separated by denaturing agarose gel electrophoresis. Lanes 1 and 2 show the acridine orange-stained RNA, while lanes 1* and 2* represent a Northern blot of the same samples using the 2-kb *Bam*HI fragment of clone pRHD2 as a probe. Numbers indicate the size of an RNA ladder in kb.

oligo(dT) cellulose (data not shown); therefore a cDNA library was established in pBluescript using oligo(dT) as first strand primer. Positive clones were identified by colony hybridization with the 3′ terminal *Bam*HI fragment of cDNA clone pRHD2 (Meyers *et al.*, 1991b). Several positive clones were further analyzed by restriction site mapping. For 15 of 19 analyzed plasmids a typical 0.45-kb fragment was observed among the *Eco*RI cleavage products which indicated a 3′ end coterminal with the viral genome. Six of these 15 clones contained cDNA inserts of about 2.1 kb and were therefore chosen for determination of the terminal nucleotide sequences. With respect to the genomic 3′ end these 6 clones started within the poly(A) tail (a maximum of seven A residues was detected) or within the last 23 nucleotides preceding the poly(A) stretch. The 5′ terminal sequences of the cDNA clones corresponded to a region of 13 nucleotides located at position 5331 to 5343 of the genomic sequence. Three cDNA fragments differing with respect to their 3′ ends started at exactly the same nucleotide (position 5331 of the genome). These findings strongly suggested that the majority of the cDNA clones with 2.1-kb inserts should be derived from the 2.2-kb mRNA and should not represent genomic clones. Nucleotide sequencing

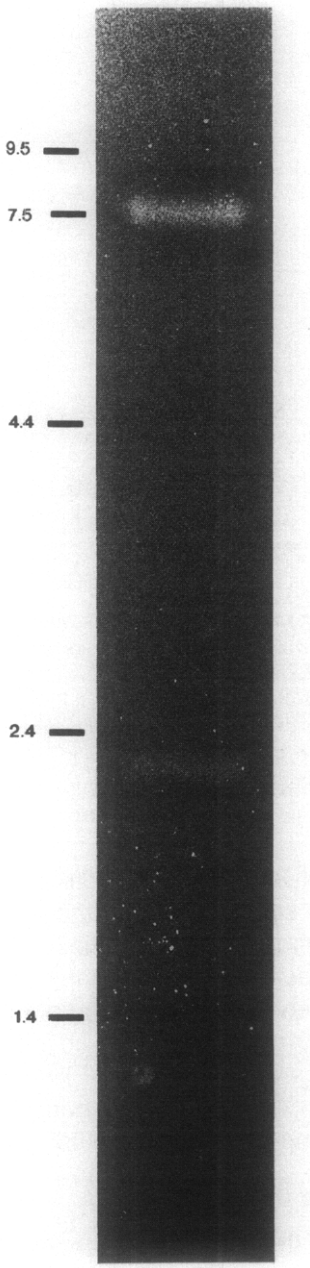

FIG. 2. Acridine orange-stained denaturing agarose gel with RNA isolated after pelleting of particles from Freon-extracted liver homogenate of RHDV-infected rabbits. The numbers indicate the size of an RNA ladder in kb.

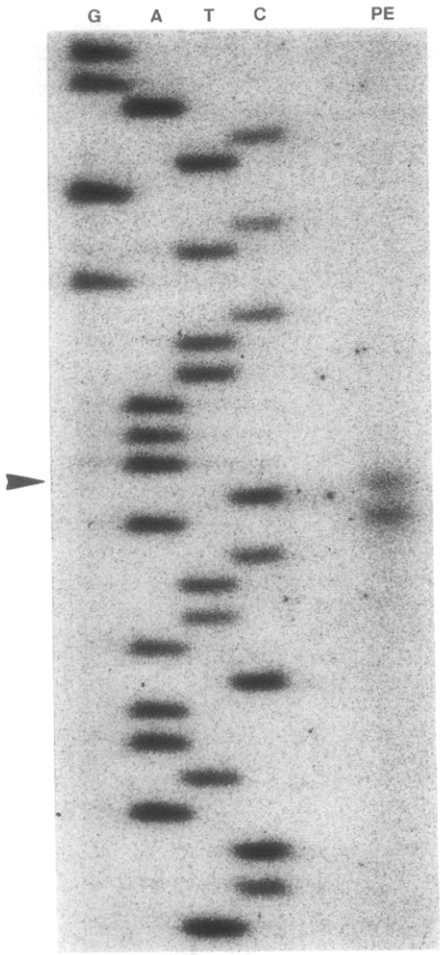

FIG. 3. Primer extension reaction for determination of the 5′ end from the RHDV 2.2-kb RNA. The extension products are separated in the lane denoted PE next to a sequencing reaction with clone pRHD2 as template. Oligonucleotide OI-RHD2 served as primer for both the extension and the sequencing reaction. The band corresponding to the last nucleotide derived from the 2.2-kb RNA is marked by an arrowhead.

of clone pRHDm1 revealed 100% identity with the genomic RHDV sequence. Since the respective genomic region was shown to encode the RHDV capsid protein (Meyers *et al.*, 1991b) the major subgenomic RNA most likely serves for expression of the viral structural protein. The 3′ region of the 2.2-kb mRNA also includes a small open reading frame (ORF) which was shown to be present in the genomes of both RHDV (Meyers *et al.*, 1991b) and FCV (Carter, 1990).

The 5′ end of the 2.2-kb mRNA

Analysis of the terminal sequences of different cDNA clones revealed that the 5′ end of the RHDV major subgenomic RNA most likely corresponds to a region located around nucleotide 5331 of the genomic sequence. Even though three clones started exactly at this position, the respective nucleotide probably did not represent the 5′ terminal residue of the RNA which is usually not included in cDNA clones. To determine the exact 5′ end of the 2.2-kb RNA a primer extension reaction was performed. Oligonucleotide OI-RHD2, which is complementary to nucleotides 5377 to 5394 of the genomic RHDV sequence (Meyers *et al.*, 1991b), served as a primer. The extension products were run in a denaturing acrylamide gel next to a sequencing reaction of the genomic cDNA clone pRHD2 (Meyers *et al.*, 1991b) (Fig. 3). Since OI-RHD2 was also used as primer for the sequencing reaction, the 5′ terminal sequence of the mRNA can be directly deduced from Fig. 3. With regard to the upper band obtained after the primer extension reaction (Fig. 3, lane PE), the first nucleotides of the RNA sequence are $^{5'}$GUGAAUGUU . . . $^{3'}$. This result was confirmed by direct sequencing of the RNA as well as by nuclease S1 protection analysis (S1 probe: 0.45-kb *Bgl*II/*Bam*HI fragment from clone pRHD1 (Meyers *et al.*, 1991b)) (data not shown). The 5′ end of the 2.2-kb mRNA therefore corresponds to position 5296 of the genomic RHDV sequence, and thereby exceeds the utmost 5′ nucleotide of the above described cDNA clones by 35 residues.

Interestingly the stretch of nucleotides representing the 5′ region of the 2.2-kb mRNA is highly homologous to the 5′ terminal sequence of the genomic RHDV RNA (Meyers *et al.*, 1991b). When the first 16 nucleotides of both RNAs are compared only two differences can be detected, while the following sequences exhibit no significant homology (Fig. 4).

The first translational start codon located at position 10–12 of the mRNA is in frame with the ORF coding for the RHDV capsid protein (Meyers *et al.*, 1991b). A second in-frame AUG follows 29 codons later. For both start codons the −3 and +4 positions are occupied by G-residues, a nucleotide context which favors translation initiation (Kozak, 1987). When compared with other eukaryotic mRNAs the distance between the RNA 5′ end and the first AUG is shorter than in most analyzed cases (Kozak, 1987). Initiation at this start codon would, however, result in translation of a RHDV capsid protein with a calculated size identical to the apparent molecular weight of 60 kDa observed after SDS–PAGE.

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genomic RNA:  GUGAAaGUUAUGGcGG cuAuguCgCGccuuaCuggcaugacuac
mRNA:         GUGAAuGUUAUGGaGG gcAaagCcCGugcagCgccgcaaggcga
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FIG. 4. Comparison of the 5′ terminal sequences of the genomic and 2.2-kb subgenomic RHDV RNAs. Nucleotides identical in both sequences are capitalized. The region of high homology between the two RNAs is marked by a box.

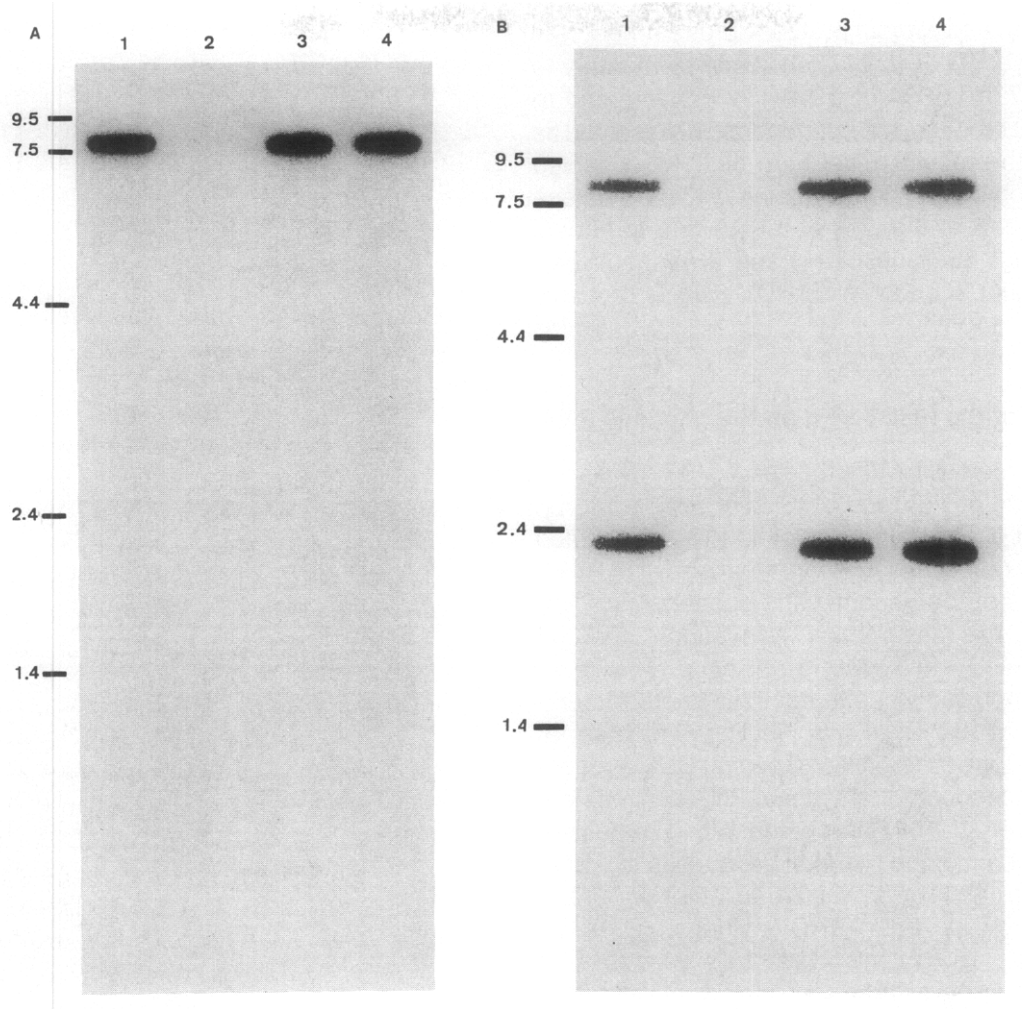

FIG. 5. Northern blot with RNA from pelleted RHDV virions. Lane 1: RNA from pelleted virions; lanes 2, 3, and 4: RNA after phenol extraction. RNA in lanes 3 and 4 was treated with Proteinase K (3) and Pronase (4) prior to phenol extraction. Oligonucleotide OI-RHD9 served as a genomic probe (A), while OI-RHD2 was used for detection of both genomic and subgenomic RNAs (B). Numbers refer to the size of an RNA ladder in kb.

Identification of a protein linked to RHDV RNA

Pilot experiments directed toward isolation of RHDV RNA led to the observation that phenol extraction resulted in a loss of most of the RNA. It has been reported that the genomic RNAs of other caliciviruses, VEV and SMSV, are covalently linked to proteins of 10000 and 15000 Da, respectively (Black *et al.*, 1978; Burroughs and Brown, 1978; Schaffer *et al.*, 1980). Accordingly, the RHDV RNA might disappear from the aqueous phase because a covalently linked protein acts like an anchor which keeps the RNA in the interphase. To verify this hypothesis RNA from pelleted RHDV virions was treated prior to phenol extraction with Proteinase K or Pronase. In a Northern blot with oligonucleotide OI-RHD9 as a probe (complementary to nucleotides 57 to 74 of the RHDV genome) the phenol-induced loss of RHDV genomic RNA is demonstrated (Fig. 5A, lanes 2 and 1, respectively). However, treatment of RNA with either Proteinase K or Pronase before phenol extraction led to complete recovery of RHDV RNA in the aqueous phase (Fig. 5A, lanes 3 and 4, respectively). These data strongly supported the assumption that RHDV RNA is also linked to a protein. Since the employed RNA extraction procedure depends on protein denaturation by guanidine isothiocyanate with subsequent separation in a cesium chloride density gradient (Chirgwin *et al.*, 1979), the protein/RNA interaction must rely on a tight linkage, most likely a covalent bond. In analogy to picornaviruses the RNA-bound protein of two other caliciviruses has already been termed VPg (Black *et al.*, 1978; Burroughs and Brown, 1978; Schaffer *et al.*, 1980).

With regard to the high homology between the 5′ terminal sequences of the 2.2-kb mRNA and the genomic RNA, it was tempting to speculate that the subgenomic molecule is also protein-linked. To investigate this possibility of an mRNA/protein interaction, phenol

extraction with and without protease pretreatment was again employed. OI-RHD2 which is complementary to positions 5377 to 5394 of the RHDV genome and to nucleotides 81 to 98 of the subgenomic RNA served as a probe for the resulting Northern blot. As observed with the genomic RNA both Proteinase K and Pronase are able to prevent the loss of mRNA due to phenol extraction (Fig. 5B, lanes 3, 4, and 2, respectively). Thus, the major subgenomic RHDV RNA is probably also linked to a VPg.

Localization of the RHDV VPg on the genomic RNA

As for the picornaviral VPg (Lee *et al.*, 1977) the analogous proteins of VEV and SMSV are believed to be bound to the 5′ end of the genomic RNA. It seemed likely that the respective RHDV protein(s) is also linked to the 5′ ends of the genomic and subgenomic RNA. Evidence for this assumption was sought through cleavage of RHDV RNA with a ribozyme. This technique was employed since in contrast to other methods it does not rely on *in vivo* labeling, which so far cannot be performed for RHDV. The first CUCU-recognition sequence for a Tetrahymena rRNA-derived ribozyme (Kruger *et al.*, 1982) is located 176–179 nucleotides downstream of the 5′ end of genomic RHDV RNA (Meyers *et al.*, 1991b). In a Northern blot with ribozyme-treated RNA from RHDV virions, OI-RHD9 (Meyers *et al.*, 1991b) hybridized to a predominant band migrating slower than the 0.24-kb marker RNA (Fig. 6, lane 1). The respective signal could not be obtained when ribozyme-digested RNA was extracted with phenol (Fig. 6, lane 2). Treatment of ribozyme cleaved RHDV RNA with a mixture of Proteinase K and Pronase prior to phenol extraction, however, led again to detectable bands in the Northern blot. The predominant band migrated faster than the 0.24-kb marker molecule (Fig. 6, lane 3). This shift in size in addition to the phenol-induced loss of the hybridization signal from the sample without protease treatment indicates linkage of the VPg to one of the first 179 nucleotides and thereby supports the assumption of a protein/RNA interaction at the 5′ end of the RNA.

The detection of additional bands in both lanes 1 and 3 of Fig. 6 is probably due to nonspecific activity of the ribozyme.

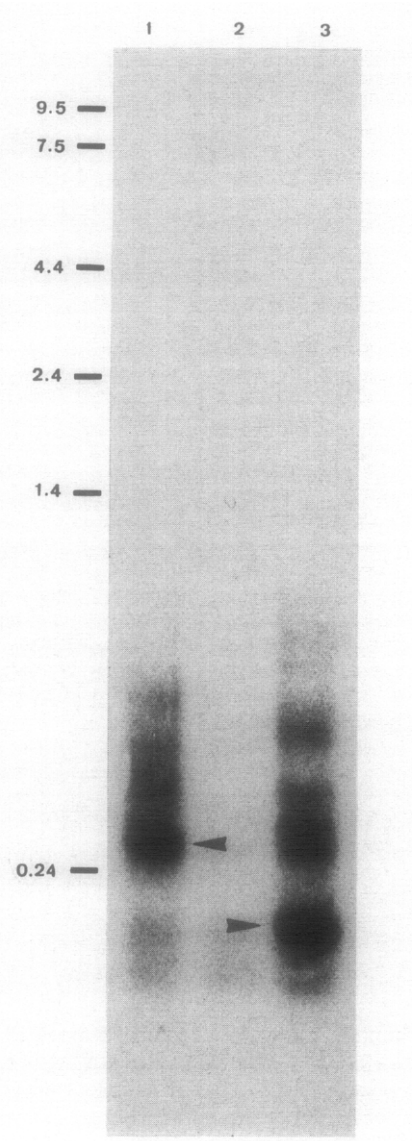

FIG. 6. Northern blot with RNA from pelleted RHDV virions after cleavage with a ribozyme derived from Tetrahymena rRNA. Lane 1 shows ribozyme-treated RNA, lanes 2 and 3 contain RNA after ribozyme treatment and phenol extraction with (3) or without (2) protease pretreatment. Oligonucleotide OI-RHD9 served as probe. Arrowheads point at the assumed 5′ terminal cleavage products. Numbers indicate sizes in kb of an RNA marker.

Demonstration of RHDV VPg

In order to visualize protein(s) bound to RHDV RNA purified RNA was subjected to ^{125}I-labeling. After separation of the reaction products in a denaturing agarose gel two bands corresponding to the genomic and subgenomic RNAs of RHDV could be detected by autoradiography of the dried gel (Fig. 7A). For demonstration of the RNA-bound protein the labeled material was subjected to RNase treatment and SDS–PAGE. A strong single band of about 15 kDa was visible on the resulting autoradiography (Fig. 7B, lane 1). The labeled molecule represents a protein since it was susceptible to proteinase K (Fig. 7B, lane 2). The demonstration of a single protein band in this experiment suggests that both analyzed RHDV RNAs are bound to the same VPg.

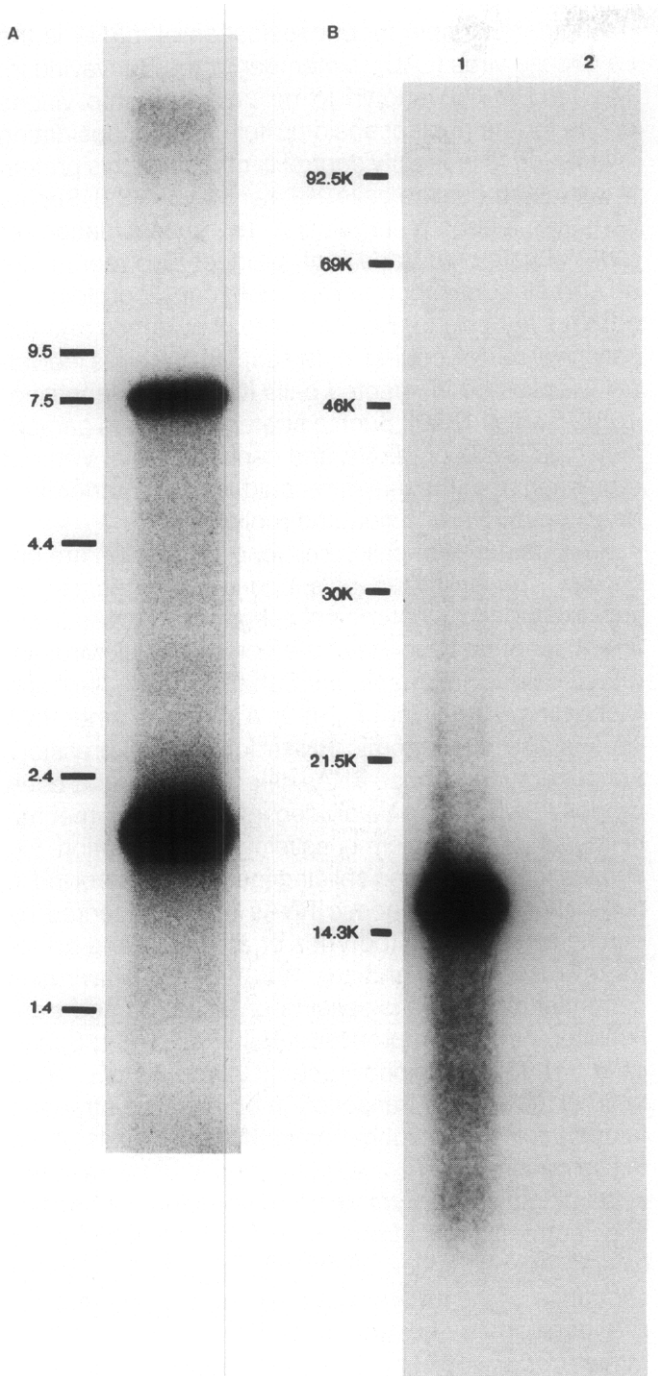

FIG. 7. ^{125}I-labeling of RNA from RHDV particles. (A) The reaction products were glyoxylated in the presence of 1% SDS and separated in a 1% agarose gel containing formaldehyde. Numbers indicate the size of an RNA ladder in kb. (B) After treatment with RNase the sample was applied to 12% SDS–PAGE (lane 1). The same material was incubated with Proteinase K prior to loading (lane 2). Numbers indicate sizes in kDa.

Separation of RHDV virions in sucrose density gradients

According to the data presented in the first paragraph under Results both the genomic and the major subgenomic RHDV RNAs found in liver tissue of infected rabbits are not susceptible to RNases. We hypothesized that protection of the RNA is due to its interaction with viral protein(s). Since both RNAs can be isolated from material pelleted to 100 S (Fig. 2), not only genomic but also subgenomic RHDV RNA might be efficiently packaged into particles. In this case both RNAs are expected to be either found in one virus particle or packaged separately. To investigate this question, liver homogenate from infected rabbits was separated by sucrose density gradient centrifugation. It has been reported that three bands can be visualized in such gradients (bands 1, 2, and 3) with estimated sedimentation coefficients of 175 S, 136 S, and 100 S, respectively (Ohlinger *et al.*, 1990). When tested for viral antigen two peaks were found. The first one from the bottom of the tube (peak I) correlated with visible band 1 and the second one (peak II) correlated with bands 2 and 3 (Ohlinger *et al.*, 1990). Analysis of RNA prepared from individual fractions collected from such a gradient revealed that viral RNA could almost exclusively be detected in fractions from band 1; thus both viral antigen and RNA are present within peak I (data not shown). On Northern blots with RNA from peak I gradient fractions, separation of genomic and subgenomic RHDV RNA was demonstrated (Fig. 8). Since the fractions with about equimolar amounts of both RHDV RNAs were located between those containing either genomic or subgenomic RNA, the former fractions most likely resulted from overlapping of two individual

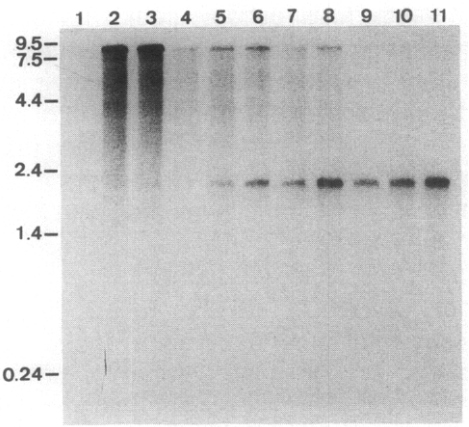

FIG. 8. Northern blot with RNA extracted from individual fractions which were collected after sucrose density gradient centrifugation of liver homogenate from RHDV-infected rabbits. The fractions shown here comprise the part of the gradient containing visible band 1 (Ohlinger *et al.*, 1990). Numbering of fractions was from bottom to top. The insert of clone pRHD2 served as a probe. The sizes of an RNA marker are indicated in kb. When tested for viral antigen either by hemagglutination assay or by Western blotting with a serum directed against the viral capsid protein all fractions were found to be positive (data not shown).

peaks (Fig. 8). It can be concluded that RHDV genomic and 2.2-kb subgenomic RNA are separately packaged into particles.

DISCUSSION

Cells infected with caliciviruses contain in addition to genomic RNA also subgenomic viral RNAs (Ehresmann and Schaffer, 1977; Black *et al.*, 1978; Neill and Mengeling, 1988; Carter, 1990; Ohlinger *et al.*, 1990). Only limited information is available on the structure of these molecules. For FCV it has been reported that all RNAs of positive polarity are polyadenylated and 3′ coterminal (Neill and Mengeling, 1988; Carter, 1990). The genomes of VEV and SMSV were shown to be covalently linked to proteins called VPg (Black *et al.*, 1978; Burroughs and Brown, 1978; Schaffer *et al.*, 1980). The characterization of the genomic and subgenomic RNAs from the RHDV presented in this report adds important new information to this subject. The genomic and subgenomic RNAs are colinear for the complete distance between the mRNA 5′ end and the poly(A) tails. In addition, the 5′ terminal 16 nucleotides of the 2.2-kb RNA are highly similar to the utmost 5′ sequence of the genomic molecule (Fig. 4). Moreover, not only genomic but also subgenomic RHDV RNA is linked to VPg.

Interestingly, the RHDV 2.2-kb subgenomic RNA is efficiently packaged into particles. In sucrose density gradients the mRNA is found within so-called peak I close to particles containing genomic RNA. When analyzed by electron microscopy, peak I fractions were shown to contain viral particles (Ohlinger *et al.*, 1990). It is therefore likely that the mRNA is not simply complexed by proteins but encapsidated into particles. This assumption is supported by the finding that genomic as well as subgenomic RNAs are protected from degradation in liver of rabbits infected with RHDV. Accordingly the packaging signal must be present in both molecules. It is not known whether subgenomic RNAs from other caliciviruses are also encapsidated. However, packaging of mRNAs has been reported for other RNA viruses. In particular coronaviral mRNAs are found within virions. While porcine transmissible gastroenteritis virus (TGEV) encapsidates mRNAs only in a molar ratio of 0.07 to 0.2 with respect to genomic RNA, the bovine coronaviral mRNAs for the N and M proteins are more abundant than the genome (Hoffmann *et al.*, 1990; Sethna *et al.*, 1989, 1991). It has not been determined whether these subgenomic RNAs and the genome are contained in one virion. For RHDV the molar ratio between 2.2-kb subgenomic and genomic RNAs varied significantly between independent experiments.

Another example for encapsidation of mRNA is the La Crosse virus (LAC), a member of the Bunyaviridae. The LAC mRNA shown to be packaged into virions codes for the nucleocapsid protein and encapsidation is believed to indirectly control synthesis of this protein in infected mosquito cells (Hacker *et al.*, 1989). Such a negative feedback regulation by encapsidation of mRNA by its own translation product also represents an attractive idea for control of both capsid protein and mRNA synthesis for RHDV. There is evidence that the caliciviral mRNA coding for the capsid protein is individually replicated in infected cells (Neill and Mengeling, 1988; Carter, 1990). Such a strategy results in particularly high levels of mRNA and capsid protein. Without regulation, this mechanism could lead to interference between mRNA and genome replication.

Basic features of caliciviral gene expression are unknown. The utilization of subgenomic RNAs seems very likely since 3′ coterminal subgenomic RNAs of different lengths have been detected in calicivirus-infected cells (Ehresmann and Schaffer, 1977; Neill and Mengeling, 1988; Carter, 1990). A variety of other RNA viruses use subgenomic RNAs for gene expression. For generation of these RNAs different ways have been proposed which can be divided into two basic mechanisms dependent on either splicing or transcription. Examples for an assumed splicing mechanism involved in generation of subgenomic RNAs are represented by equine arteritis virus (de Vries *et al.*, 1990) and orthomyxoviruses (Lamb and Lai, 1982). For coronaviruses cotranscriptional *trans*-splicing or leader-primed transcription have been proposed (Lai *et al.*, 1984; Spaan *et al.*, 1988). For alphaviruses (Grakoui *et al.*, 1990) internal initiation of transcription on negative-stranded templates leads to subgenomic RNAs. Analysis of the RHDV genomic and 2.2-kb subgenomic RNAs resulted in identification of a stretch of 16 homologous nucleotides at the 5′ ends of both molecules; the sequences exhibit two nucleotide differences and the 5′ terminal sequence of the mRNA was also found at the respective position in the genomic RNA (5296 to 5311, Meyers *et al.*, 1991b). Therefore leader-primed transcription or splicing most likely do not represent the mechanism for generation of this subgenomic RNA unless the leader or spliced fragment is shorter than six residues at which position the first nucleotide exchange was found. Since the RHDV 2.2-kb RNA is protein-linked, the VPg or a complex of VPg with several bound nucleotides can be imagined as transcription primer. Alternatively primerless initiation of transcription could occur at the sequence homologous to the 3′ terminal sequence of the genomic minus strand. In the latter case co- or post-transcriptional addition of VPg would occur. After screening of the RHDV sequence

(Meyers *et al.*, 1991b) further regions of high homology to the 5′ terminal 16 nucleotides could not be identified. Therefore the initiation mechanism(s) for transcription of other putative RHDV mRNAs analogous to those described for FCV remains obscure.

For RHDV so far only two RNA species, namely genomic RNA and 2.2-kb subgenomic RNA, have been clearly identified. Weak additional bands were, however, detected on some Northern blots (data not shown). It therefore seems likely that in RHDV-infected cells several species of subgenomic RNA are generated which, however, are difficult to demonstrate in the material obtained from rabbit liver. Taking into account the high number of mRNA species reported for FCV (Neill and Mengeling, 1988; Carter, 1990), the strategy of caliciviral gene expression appears to be quite complex. On the other hand, identification of long open reading frames, probably comprising the information for several proteins, together with detection of a putative protease motif in the sequences of FCV and RHDV (Neill, 1990; Meyers *et al.*, 1991b) suggests translation and proteolytic processing of polyproteins as an alternative way to mature viral proteins. Further investigation of caliciviral molecular biology will most likely add new aspects to our knowledge on the strategies, mechanisms, and regulation of RNA virus gene expression.

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