In vitro translation of a subgenomic mRNA from purified virions of the Spanish field isolate AST/89 of rabbit hemorrhagic disease virus (RHDV)

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Summary

Purified preparations of the Spanish field isolate of rabbit hemorrhagic disease virus AST/89 were found to contain the plus-stranded genomic RNA of more than 7.4 kilobases (kb) and large amounts of a subgenomic mRNA of 2.4 kb. The smaller RNA was translated in vitro and shown to code for a 60 kDa protein which was immunoprecipitated using anti-RHDV as well as anti-VP60 sera.

Caliciviridae; RHDV; Subgenomic mRNA; Rabbit hemorrhagic disease

Introduction

In 1984, outbreaks of a new lethal disease of rabbits were observed in the People's Republic of China (Liu et al., 1984). The pathological signs of this disease were hemorrhages in the respiratory system, liver, spleen, etc. After experimental infection adult animals usually died within 48 to 72 h. The causative agent of rabbit hemorrhagic disease (RHD) has been purified and tentatively characterized as a *Calicivirus* (Ohlinger et al., 1990; Parra and Prieto, 1990) based on a characteristic morphology, its plus-stranded RNA genome and especially, the presence of a

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single structural polypeptide. Recently the RNA genome of a German isolate of rabbit hemorrhagic disease virus (RHDV) has been molecularly cloned and sequenced (Meyers et al., 1991a). Sequence comparison studies revealed significant homology between the non-structural proteins of feline Calicivirus (FCV) and RHDV (Meyers et al., 1991a). On the other hand there are obvious similarities to picornaviruses, in particular a long ORF which has the coding capacity for all the viral polypeptides (Meyers et al., 1991a). The published data on RHDV and FCV indicate that Caliciviruses might generate mature proteins by processing of polyproteins as well as by translation of subgenomic RNAs, a strategy which has been reported for alphaviruses (Strauss et al., 1987).

The presence of a subgenomic mRNA of 2.2 kb, packaged within virus particles, has been described for the German isolate of RHDV (Meyers et al., 1991b). This finding raises the question of whether this subgenomic RNA could have a biological function, taking into account that the same information is also represented as a part of the major ORF. It should be mentioned that in other members of the Caliciviridae such as FCV, the major structural polypeptide is coded by an independent ORF located towards the genome 3' end (Carter et al., 1992). A similar organization has also been found for human hepatitis E virus (HEV), a putative member of the Caliciviridae (Tam et al., 1991).

The study of rabbit hemorrhagic disease virus has been impeded by the lack of a permissive cell culture system, the liver, spleen etc. of dead animals being the only source of the virus. In the present report we demonstrate the presence of large amounts of a 2.4 kb subgenomic mRNA in purified preparations of the Spanish isolate AST/89 of RHDV and its biological role in the synthesis of VP60, the major RHDV capsid component.

Materials and methods

Virus purification

The virus was purified by centrifugation of liver extracts from RHDV infected animals onto 30% sucrose cushions and further analysis of pelleted virions by isopycnic equilibrium using CsCl gradients, as previously described (Parra and Prieto, 1990).

RNA extraction and Northern blotting

The RNA content of purified RHDV virions was extracted by the guanidine thiocyanate method (Chirgwin et al., 1979). The RNA was dissolved in electrophoresis buffer and run in a 1.2% agarose gel containing 0.66 M formaldehyde in MOPS buffer (Davis et al., 1986). Separated species were transferred onto nylon membranes (Hybond-N, Amersham) by capillary blotting. The filter was baked for 2 h at 80 °C. The prehybridization and hybridization were performed at 65°C in a solution containing $6 \times SSC$ (0.15 M NaCl, 0.015 M sodium citrate), $10 \times$

Denhardt's solution, 100 mg/ml Dextran sulfate, 1% SDS and 100 μg/ml of heat-denatured salmon sperm DNA. The filters were then washed 2×45 min in $0.1 \times$ SSC containing 0.1% SDS at 65°C.

The subgenomic RNA was further purified from total virus RNA preparations by zonal centrifugation at 25,000 rpm for 6 h at 25°C in a Beckman SW50 rotor using 6–20% sucrose gradients containing 30 mM *PIPES* pH 6.4, 50 mM EDTA and 0.5% sarcosyl. The fractions containing the 2.4 kb RNA were pooled and ethanol precipitated for further use.

The DNase-free RNase A treatment was made using 0.1 mg/ml of enzyme in $2 \times$ SSC for 15 min at 25°C.

cDNA cloning

RHDV cDNA was produced from the AST/89 isolate RNA using a commercial kit from Pharmacia, following the supplier's instructions and oligo dT as primer. *Eco*RI/*Not*I adaptors were added to the cDNA which was subsequently cloned into lambda gt11 and packaged in vitro using a commercial kit from Boehringer Mannheim. The phage library was screened by plaque hybridization using convalescent anti-RHDV serum. The clone T3 used in subsequent experiments was sequenced and shown to be 96.7% homologous to the previously published sequence for the German isolate of RHDV (nucleotides 6050–7437). The fragment (1.1 kbp), derived from the T3 cDNA clone, used as a probe in the Northern blot experiments, represented nucleotides 6050–7125 of the German isolate of RHDV.

In vitro translation and immunoprecipitation

Purified 2.4 kb RNA was translated in vitro for 1 h at 30°C using a commercial rabbit reticulocyte lysate (Amersham, code N.90) in the presence of 2 mCi/ml [^{35}S]methionine (NEG-009H). The resulting incubation mixtures were analyzed by SDS-PAGE directly or after immunoprecipitation using convalescent anti-RHDV or anti-VP60 sera and protein A-sepharose. The precipitates were extensively washed prior to being dissolved in electrophoresis sample buffer. After boiling for 3 min the samples were spun for 2 min and the supernatants analyzed by SDS-PAGE.

SDS-PAGE

Purified RHDV virions, in vitro translation reactions or their derived immuno-precipitates, were fractionated using 12% SDS-polyacrylamide gels (Laemmli, 1970). After electrophoresis, the gels were stained using Coomassie Brilliant Blue-R. In order to detect the radioactively labelled proteins by fluorography, the gels were impregnated with 1 M sodium salicylate. Dried gels were exposed to AGFA Curix RP-2 films at -80°C for 18 h.

Antisera

Convalescent-phase sera were obtained from surviving rabbits at a farm on which the rest of the animals died of hemorrhagic disease (Parra and Prieto, 1990). The anti-VP60 serum was obtained after immunization of rabbits using the β-galactosidase-VP60 fusion protein, produced by a lambda gt11-T3 lysogenized *Escherichia coli* strain. The fusion protein was purified by affinity chromatography using *p*-aminobenzyl-1-thio β-D-galactopyranoside-agarose.

Results

To obtain RHDV nucleic acid for cloning purposes, highly purified virus preparations were made from the livers of experimentally infected rabbits which died showing clinical signs of the hemorrhagic disease (Argüello et al., 1988; Liu et al., 1984). The homogenates used had a high hemagglutination titer on group 0 human erythrocytes, a distinctive property of the causative agent of RHD (Pu et al., 1985). The resulting purified RHDV virions were homogeneous in terms of their buoyant density which was 1.365 g/ml. Prior to RNA extraction further purity tests were performed and RHDV virions were examined under the electron microscope to find out if the size and morphology of the virions were homogeneous. A single type of virus particles of about 35 nm in diameter were found, these being approximately round, and without an envelope (Fig 1a). The high degree of purity obtained was also confirmed by the results of an SDS-poly-acrylamide gel electrophoresis of the virus preparation which showed only a single
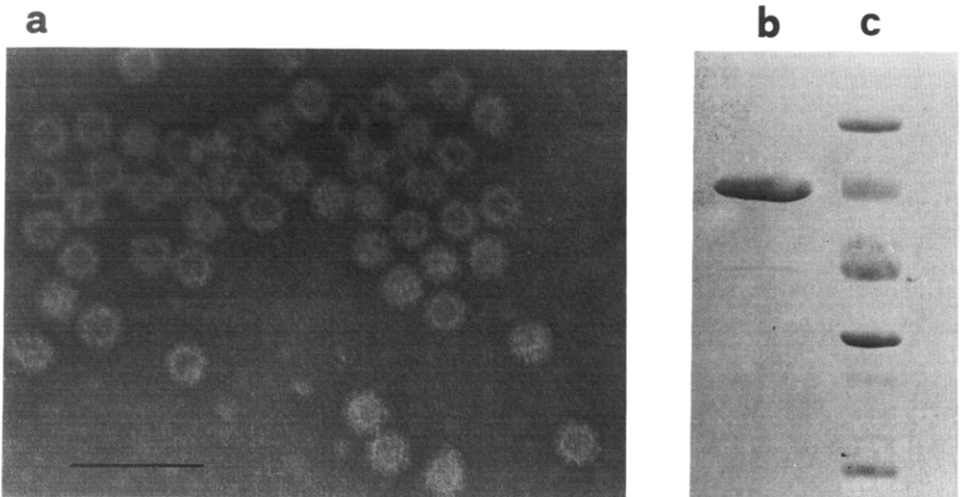

Fig. 1. Negative-stained electron micrograph (a) and protein pattern of purified RHDV (lane b). Molecular size markers (94, 67, 43, 30 and 20 kDa) were run in lane c. Bar = 100 nm.
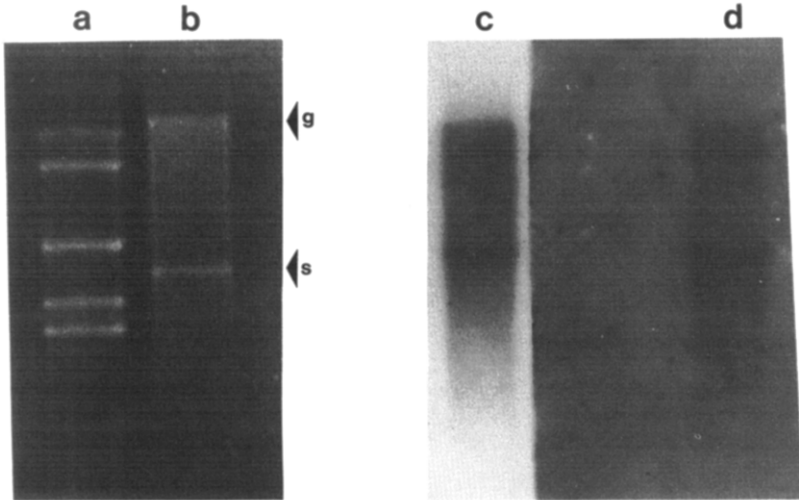
Fig. 2. Electrophoretic and Northern blot analysis of RNA species extracted from purified RHDV virions. Lane a: RNA molecular weight markers (7.4/5.3/2.8/1.9 and 1.6 kb); lane b: 2 μg virus RNA stained with ethidium bromide. The arrows indicate the mobility of genome (g) and subgenomic (s) RNAs. Lane c: Northern blot using a ^{32}P-labeled probe derived from the 2.4 kb subgenomic RNA (s); lane d: Northern blot using a cDNA probe.

polypeptide, Mr ≈ 60,000 (Fig. 1b). This polypeptide was identified as VP60, the major RHDV capsid component, by Western blotting using a convalescent rabbit serum (Parra and Prieto, 1990).

The RNA content of RHDV virions was extracted and analyzed in agarose-formaldehyde gels. Surprisingly a two-band pattern was obtained (Fig. 2b), the genomic RNA (labeled g), migrating close to the 7.4 kb RNA marker, and a subgenomic RNA (labeled s) of about 2.4 kb. The two RNA species were protected against RNase A treatment before guanidine thiocyanate extraction but sensitive after viral protein removal. These results clearly show that the two RNA species were single-stranded and encapsidated in virus particles. The two RNA species were found to be encapsidated in a molar ratio of 1:1 by densitometric measurement of their band intensities after agarose gel electrophoresis and calibration with respect to known amounts of the corresponding RNA marker. It should be stressed at this point that encapsidation of RHDV genome and subgenomic mRNA into virus particles has recently been described (Meyers et al., 1991b) also indicating a significant variation of the RNA molar ratios between independent experiments. In clear contrast we have consistently obtained molar ratios of approximately 1.0 in more than 20 independent RHDV purifications using the isolate AST/89.

In order to investigate the polarity of the two encapsidated RNA species a radioactive probe of the 2.4 kb RNA was made after purification of this RNA by sucrose density gradients and further reverse transcription using random primers. The probe identified both RNA species (Fig. 2c) in Northern blot experiments,
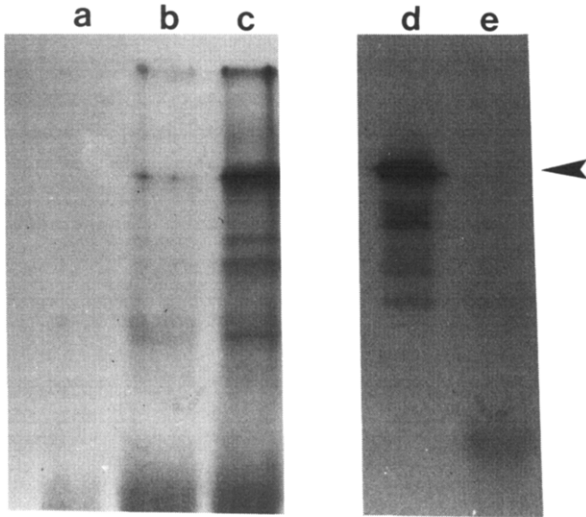
Fig. 3. SDS-PAGE analysis of products synthesized from RHDV 2.4 kb mRNA in a rabbit reticulocyte cell-free system. Lane a: no RNA added; lane b: 0.2 μg of RHDV 2.4 kb RNA; lane c: 2 μg of RHDV 2.4 kb RNA; lane d: immunoprecipitate of translation products of 2.4 kb subgenomic mRNA (1 μg) using a convalescent rabbit serum or anti-VP60 serum; lane e, immunoprecipitate of translation products of 2.4 kb subgenomic mRNA (1 μg) using a preimmune rabbit serum. Lanes d and e were run in a different gel in which an adjacent track contained purified RHDV. The arrow indicates the electrophoretic mobility of rabbit hemorrhagic disease virus VP60.

showing that the genomic RNA had the same polarity and contained the informa-tion coded in the smaller 2.4 kb RNA. To further support this conclusion and discard the hypothesis of probe contamination with sequences derived from the genomic RNA, a labelled cDNA clone (1.1 kbp), corresponding to nucleotides 6050–7125 of the previously published sequence for the German RHDV isolate, was used as probe in Northern blot experiments. The positive reaction of both bands (Fig. 2d) confirmed the partial identity of the two encapsidated RNA species.

The presence of a 2.4 kb subgenomic RNA in RHDV virions allowed us to speculate about its putative role as the VP60 mRNA. This hypothesis has been drawn taking into account that a similar size RNA was detected in RHDV-infected rabbit livers (Ohlinger et al., 1990) using a cDNA probe prepared to the virion RNA. Moreover as demonstrated in vesicular exanthema virus (VEV), a member of the Caliciviridae, a subgenomic mRNA isolated from infected cell cultures was translated into a single polypeptide of the same electrophoretic mobility as the major VEV capsid component (Black et al., 1978). Support for our hypothesis comes from in vitro translation of 2.4 kb RNA in a reticulocyte cell-free system. Rabbit reticulocyte lysates were incubated in the absence of added RNA (Fig. 3a) or with increasing amounts of the 2.4 kb RNA extracted from RHDV virions (Fig. 3b,c). The purified 2.4 kb species was translated into a major polypeptide which migrated to the same position as VP60 purified from RHDV virions and run in an

adjacent track of the gel. The role of the smaller RHDV RNA species as the mRNA for VP60 was further demonstrated by immunoprecipitation of the translation product of purified 2.4 kb mRNA, using an RHD-convalescent rabbit serum, or a specific anti-VP60 serum (Fig. 3d). The use of a normal or preimmune rabbit serum (Fig. 3e) did not precipitate the main translation product of 60 kDa.

Discussion

Rabbit hemorrhagic disease virus gene expression is a poorly understood process which appears to be rather complex considering the identification of a long open reading frame (ORF) covering the majority of the genome length (Meyers et al., 1991a) and the existence of at least a subgenomic mRNA with coding capacity for the structural polypeptide VP60. From the data in this paper it can be deduced that the 2.4 kb subgenomic message is fully functional and yielded a translation product of identical size and antigenic properties as VP60. Considering the size of the translation product and the theoretical coding capacity of this subgenome message, it appears that this viral polypeptide does not seem to require post-translational modifications and is not produced from a polyprotein precursor. It is nevertheless difficult to explain the need of a larger ORF (Meyers et al., 1991a), which also includes the coding sequence of VP60 at its 3' end, in relation to the synthesis of this viral polypeptide. Recently the nucleotide sequence of the 3'-terminal 2,486 bases of the feline Calicivirus (FCV) genome has been published (Neill et al., 1991). In contrast to the data published for RHDV, in-frame stop codons can be found upstream of the AUG initiation codon for the structural polypeptide of FCV, clearly indicating that in this case there is not a continuous ORF from the 5' end of the genome. As in RHDV a subgenomic 2.4 kb mRNA has been also found encoding the FCV capsid protein. Recently published data (Tam et al., 1991) also demonstrate that the hepatitis E virus (HEV) genome does not code for a unique ORF and shows that its expression strategy involves the use of different open reading frames and at least three different transcripts. Further sequence data on different RHDV isolates will be necessary to find out if this virus has a unique genome organization among the Caliciviridae or if this is a property of a particular isolate.

Several authors have described encapsidation of genome and subgenomic RNAs (Hofmann et al., 1990; Sethna et al., 1991) in other plus-stranded RNA viruses. It will be of interest to find out if the encapsidation of subgenomic mRNA is relevant for RHDV biology. One can easily think that the presence of this message in the virions could be essential for the virus replication if VP60 has a function as a replication or transcription factor, as well as being a structural component. On the other hand, it is tempting to correlate different degrees of mRNA encapsidation with virulence. If this were to be true, then one could predict that strains producing severe or mild symptoms would have different amounts of encapsidated subgenomic mRNA. The lack of an in vitro system for RHDV propagation will

make it more difficult to find the answers to these and other questions relative to RHDV biology.

Acknowledgements

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