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Detection and Preliminary Characterization of a New Rabbit Calicivirus Related to Rabbit Hemorrhagic Disease Virus but Nonpathogenic

LORENZO CAPUCCI, PAOLA FUSI, ANTONIO LAVAZZA, MARIA LODOVICA PACCIARINI, AND CESARE ROSSI*

Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia, 25124 Brescia, Italy

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A new rabbit calicivirus related to the rabbit hemorrhagic disease virus (RHDV) was identified. The new virus contains significant differences from the previously characterized RHDV isolates in terms of pathogenicity, viral titer, tropism, and primary sequence of the structural protein. Cross-protection experiments, antigenic data, and sequence comparisons demonstrate that the new virus is more closely related to RHDV than to the European brown hare syndrome virus, another member of the caliciviruses of the lagomorph group. The existence of a nonpathogenic calicivirus, which we propose to name rabbit calicivirus (RCV), provides an explanation for the early discrepancies found in the course of serological surveys of the rabbit population in European countries.

After the first reports of rabbit hemorrhagic disease in 1984 in China and later in Europe, the causative virus (rabbit hemorrhagic disease virus [RHDV]) was isolated and characterized by several laboratories (6, 26, 29). RHDV has been assigned to the family *Caliciviridae* on the basis of its morphology in electron microscopy, the presence of only one major structural protein of about 60 kDa (VP60), and the features of a 7.5-kb single-stranded RNA genome and a subgenomic RNA of about 2.2 kb. (21, 22). Another calicivirus, the European brown hare syndrome virus (EBHSV), is responsible for a disease of hares whose clinical progression and histopathology, particularly the high mortality rate and the presence of necrotic lesions of the liver, resemble those of RHD. The genomic organization and the viral proteins of EBHSV and RHDV are also similar in many respects (40). A peculiarity of both RHDV and EBHSV, distinguishing them from the other caliciviruses, is that the genes for the nonstructural proteins and for the capsid protein (VP60) are part of one uninterrupted open reading frame (ORF1). In spite of many similarities, the two viruses represent two clearly distinct entities, as first demonstrated by the fact that RHDV is unable to cross-infect hares and hares immunized with an RHDV vaccine are not protected against infection by EBHSV. The same is true for the reciprocal experiments with EBHSV and rabbits (5, 7, 19, 24).

The complete or partial sequences of several RHDV isolates, differing in time and geographic location of isolation, are now available (1, 21, 33). Very little sequence variability has been found between the isolates even at the level of the structural protein, which is subject to frequent mutations in other caliciviruses, e.g., the San Miguel sea lion virus and the feline calicivirus (25, 36, 37).

High morbidity, widespread histopathological lesions, and mortality rates in excess of 80% among infected animals define RHDV as an extremely pathogenic virus. The few rabbits that survive RHD develop high titers of anti-RHDV antibodies, lasting for at least 1 year after the infection. In contrast to the usual features of RHD, serological studies performed both in Italy (4) and in the Czech Republic (34) highlighted the existence of several rabbit populations with a high frequency of RHDV-seropositive individuals but no history of clinical symptoms or mortality due to RHD. Preliminary experiments involving caging seronegative rabbits from a controlled pathogen-free colony together with selected seropositive rabbits demonstrated that the seroconversion in the absence of clinical symptoms was reproducible under controlled conditions (4). In contrast, if the same kind of experiment is performed with rabbits infected by bona fide RHDV, the seronegative rabbits invariably become ill and die within a few days. Finally, a retrospective study of rabbit sera collected between 1975 and 1985, well before RHD became widespread in Europe, demonstrated the presence of anti-RHDV antibodies (34). On the basis of this evidence the existence of a nonpathogenic virus, antigenically related to RHDV, was postulated.

We report the isolation and partial characterization of a new rabbit calicivirus, with the characteristics of a putative nonpathogenic virus. Its antigenicity and the sequence of the capsid protein demonstrate that it is closely related to RHDV. In spite of these similarities, the new virus does not cause disease after infection and displays a different organ tropism, since it is preferentially detected in the intestine whereas RHDV accumulates in the liver and the spleen. To clearly distinguish the new virus from RHDV, we propose to name it rabbit calicivirus (RCV).

MATERIALS AND METHODS

Tissue preparation. Tissues were removed from the animals immediately after death. Several precautions were taken to avoid contamination between samples from different organs and between animals: a fresh set of scalpels and disposable gloves were used for dissection of each organ; surgical cloths were exchanged after use with each animal; and negative control animals were dissected at the beginning and at the end of each experimental session. Organs from the positive control animals (RHDV infected) were collected in a different room and/or at a different time.

Viral extracts and Western blotting. Liver extracts were made in 30 mM phosphate buffer (pH 7.2)–150 mM NaCl–2 mM EDTA (10%, wt/vol) by homogenization with an Ultraturrax instrument (Ika-Werk). The extracts were filtered through cheesecloth and clarified by centrifugation (5,000 × *g* for 15 min

* Corresponding author. Mailing address: Dipartimento di Virologia, Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia, via Bianchi 7, 25124 Brescia, Italy. Phone: 39 30 2290308. Fax: 39 30 225613. Electronic mail address: izsviro@master.cci.unibs.it.

at 4°C). Viral particles were concentrated 10-fold by ultracentrifugation (100,000 × *g* for 90 min) through a 20% (wt/wt) sucrose cushion. Intestinal tissue was extracted by mincing in a ceramic mortar in the presence of quartz powder and processed as described above. To detect the viral protein(s), samples were denatured for 2 min at 100°C in the presence of 60 mM Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, and 5% glycerol; separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) with a minigel system (Hoefer); and transferred to nitrocellulose filters (Schleicher & Schuell) at 0.3 A for 1 h in 25 mM Tris (pH 8.3)–192 mM glycine–20% (vol/vol) methanol (39). After transfer, the filters were saturated with 2% bovine serum albumin (BSA), dissolved in phosphate buffer (pH 7.4), and incubated for 2 h at room temperature with monoclonal antibody (MAb) 5G3 (3) at 3 μg/ml in phosphate buffer (pH 7.4)–1% BSA. The filters were washed, and MAb binding was detected by incubation with alkaline phosphatase-labeled rabbit anti-mouse IgG and the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate–Nitro Blue Tetrazolium (BCIP/NBT; Boehringer) as described by Harlow and Lane (14).

Polyclonal sera and ELISA. Competition enzyme-linked immunosorbent assay (ELISA) was performed between specific antibodies bound to the solid phase and antibodies in the liquid phase from sera of infected animals. This permitted evaluation of the presence of anti-RHDV and anti-EBHSV antibodies in the sera. Briefly, Maxisorb plates (Nunc) were coated overnight at 4°C with 50 μl of purified anti-RHDV MAb or specific polyclonal serum (3) at 2 μg/ml in 200 mM carbonate buffer (pH 9.6). The wells were washed three times for 5 min each with phosphate-buffered saline (PBS) in the presence of 0.05% Tween 20 and filled with 25 μl of PBS (pH 7.4) containing 1% yeast extract and 0.05% Tween 20 (PBS-YT). Serial fourfold serum dilutions were then added directly to the wells, followed by 25 μl of antigen diluted in PBS-YT at nonsaturating concentrations, and the mixture was incubated at 37°C for 1 h with gentle agitation. After a washing step as described above, the virus bound to the solid phase was detected by incubation with MAbs or with polyclonal serum previously conjugated to horseradish peroxidase (3). Each reaction was performed with the same MAb or polyclonal serum both for antigen capture and for detection of the bound antigen. Color development was performed in the presence of *o*-phenylenediamine (Sigma), and the A_{492} of the sample was read. The titer of specific antibodies in the serum was calculated from the dilution that reduced the A_{492} by 50% compared with the control (normal nonimmune serum).

RNA extraction. Tissue samples from the liver, spleen, lungs, intestine, nasal epithelium, and trachea were frozen in liquid nitrogen and stored at −80°C or immediately processed. Because of the relatively small amounts of nasal epithelium and trachea, these samples were pooled and treated as one sample (referred to as "upper airways"). Tissue fragments (0.2 to 1 g) were put in ceramic mortars (one for each sample) and ground in the presence of 4 M guanidinium isothiocyanate (1:5, wt/vol). Total RNA was extracted essentially as described by Chomczynski and Sacchi (8), with 500 μl of 4 M guanidinium isothiocyanate, 500 μl of water-saturated phenol, and 100 μl of chloroform added to 100 μl of sample. The phenol-chloroform extraction was repeated once and was followed by further extraction of the RNA with chloroform and precipitation with a mixture of 500 μl of cold isopropanol and 50 μl of 2 M sodium acetate (pH 4.0). After centrifugation, the pellet was washed twice with 75% ethanol, dried, and resuspended in 50 μl of diethylpyrocarbonate-treated water. The RNA concentration was measured by monitoring the UV A_{260}.

cDNA synthesis and PCR. Reverse transcription was performed on 10 μg of total RNA from each organ except for the upper airways, for which only 1 μg of RNA was used. RNA was incubated in 10 μl containing 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM KCl, 200 μM each dATP, dCTP, dTTP, dGTP, 2.5 U of avian myeloblastosis virus reverse transcriptase (Pharmacia), 0.1 μM reverse primer, and 10 U of RNasin RNase inhibitor (Promega) for 90 min at 42°C. The reagents needed to perform the PCR were then added to the same tube to give the following final concentrations in a volume of 50 μl: 10 mM Tris-HCl (pH 8.3), 1.6 mM $MgCl_2$, 75 mM KCl, 0.1 μM direct primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer). The samples were amplified through 35 cycles of primer annealing for 1 min at 55°C, elongation for 1 min at 72°C, and denaturation for 20 s at 94°C. In the last cycle, the elongation step was extended to 10 min. After amplification, a 15-μl aliquot of each sample was subjected to electrophoresis on a 1.7% agarose gel. The oligonucleotide primers used for reverse transcription and subsequent cDNA amplification of portions of the viral RNA polymerase, VP60, and ORF2 genes were designed on the basis of the complete genomic sequence of the BS89 RHDV isolate (accession n° X87607). Their sequences, positions according to the numbering of the RHDV genomic sequence, and the expected size of the amplification products are listed in Table 2.

Internal controls for reverse transcription-PCR. Positive and negative controls were performed with 10 ng of purified RHDV RNA and 10 μg of RNA extracted from the organs of serologically negative rabbits, respectively. The reverse transcription-PCR mixtures were normalized by the addition, prior to the RNA extraction and retrotranscription steps, of known amounts of viral particles or genomic RNA (10 pg to 1 ng) from the swine vesicular disease virus (SVDV). The reverse transcription-PCR was performed as described above in the presence of primers complementary to the 2A (5′ TCAAATGTGACTGGATAGT GCTT 3′) and VP1 (5′ ACCCACTGGTGTGACTGAGGGTA 3′) regions of the swine vesicular disease virus genome. The size of the amplified product from swine vesicular disease virus was 281 bp.

Southern blot and hybridization. After being stained with ethidium bromide, the gels were photographed and blotted to activated nylon membranes (Hybond N; Amersham) by capillary or vacuum transfer (VacuGene; Pharmacia). The membranes were hybridized under high-stringency conditions (50% formamide at 42°C), as described previously (27), to a probe labeled with [α-^{32}P]dCTP by random priming (Amersham). The probe consisted of the sequence of the RHDV protease, polymerase, and VP60 genes cloned into plasmid p672 (2).

Cycle sequencing. DNA fragments amplified by PCR were separated on 1.4% agarose gels and purified with the Geneclean kit (BIO 101). The purified DNAs were sequenced by the chain termination method with [α-^{35}S]dATP (Amersham) and the Amplicycle sequencing kit (Perkin-Elmer) as specified by the manufacturers.

Nucleotide sequence accession number. The RCV capsid protein sequence has been submitted to the EMBL database as accession number X96868.

RESULTS

Identification of animals infected by the putative virus. The presence of anti-RHDV antibodies in a large number of rabbits from several Italian regions was analyzed. The survey showed that at a number of sites, a high frequency of seropositive animals did not correlate with the severe clinical picture normally associated with RHDV infection. This situation offered an opportunity to try to isolate a putative RHDV-related nonpathogenic virus whose existence had been previously hypothesized (4, 34). We selected a rabbit farm in which the disease had never been reported but the vast majority of the animals were RHDV seropositive (6b). In particular, all the breeding females had titers ranging from 1:30 to 1:1,280 and, as expected, newborn animals showed a low but detectable anti-RHDV titer (range, 1:10 to 1:80) due to transfer of maternal antibodies. This was evident from the strict correlation between antibody titers in the newborns and in their mothers and from the fact that the antibodies belonged to the IgG class. Furthermore, in the majority of the young animals, the anti-RHDV titers sharply decreased after weaning at the age of 5 weeks. On the other hand, when the same rabbits were moved to different cages for fattening, a rapid conversion back to positive titers (range, 1:160 to 1:640) occurred in over 90% of the animals (now 6 to 8 weeks old). Seroconversion was associated with an initial predominance of IgM antibodies, indicative of a newly contracted infection. Animals from this pool of young rabbits were chosen randomly for the experiments described below.

Antigenic relationships among the caliciviruses of lagomorphs. The degree of antigenic relatedness of the putative new virus (from now on called RCV) to RHDV and EBHSV was evaluated by competition ELISA. Serum antibodies from seven RCV-infected animals were tested in competition with either reference polyclonal sera or MAbs against RHDV and EBHSV, respectively. For comparison, similar reactions were also performed with sera from animals that had survived RHDV or EBHSV infection. These results are summarized in Table 1; all sera from RCV-infected rabbits were able to compete with MAbs 1H8, 3H2, and 6F9 (3) and with the IgG-RS polyclonal serum for binding to RHDV. On the other hand, the competition with anti-EBHSV antibodies (IgG-HS, MAb 5F5) for binding to EBHSV demonstrated null or very low titers. The antibody titers in different animals appeared to be rather consistent, not exceeding a fourfold difference between the highest and lowest values of the range. This pattern of reactivity was paralleled by sera from RHDV-convalescent rabbits (Table 1), although antibody titers in these animals appeared to be 10- to 20-fold higher than in RCV-infected ones, possibly as a consequence of the much higher level of replication attained by RHDV and/or its different tropism. The low level of competition of RHDV-convalescent-phase sera with IgG-HS and MAb 5F5 was expected in view of the previously described limited cross-reactivity against EBHSV (3).

TABLE 1. Reactivity of sera from RCV-, RHDV-, and EBHSV-infected animals toward RHDV and EBHSV

Serum[a]	Anti-RHDV titers[b] of:				Anti-EBHSV titers[b] of:	
	IgG-RS	1H8	3H2	6F9	IgG-HS	5F5
RCV (rabbits)	1/160–1/640	1/40–1/320	1/160–1/320	1/160–1/640	<1/5–1/10	<1/5–1/10
RHDV (rabbits)	1/2,560–1/5,120	1/1,280–1/2,560	1/1,280–1/5,120	1/2,560–1/5,120	1/10–1/80	1/10–1/40
EBHSV (hares)	1/10–1/80	<1/5–1/80	1/10–1/320	1/10–1/320	1/2,560–1/5,120	1/1,280–1/2,560

[a] Sera were taken between 21 and 27 days p.i. Virus-host combinations are indicated.
[b] Titers expressed as the serum dilution that inhibits the absorbance by 50% after background subtraction.

In contrast, sera from EBHSV-infected hares (Table 1) yielded a clearly different pattern from the previous ones, particularly after competition with the anti-RHDV MAb 1H8 and IgG-RS and, of course, with IgG-HS and MAb 5F5. In the case of MAbs 3H2 and 6F9, competition was achieved at similar dilutions with sera from either RCV- or EBHSV-infected animals. It must be remembered, though, that a much stronger immune response is generated after EBHSV infection than after RCV infection and that a partial cross-reactivity of MAb 6F9 toward EBHSV has been previously demonstrated (3). Therefore, the RCV sera have a greater specificity than the EBHSV sera for the 3H2 and 6F9 epitopes. Finally the anti-RHDV MAb 5G3 recognizes a linear epitope that is well conserved in all three viruses (see below).

One can conclude that RCV and RHDV share a number of similar or identical epitopes. As a result, the humoral immune response to RCV can be distinguished only on a quantitative basis from the response to RHDV. By analogy to RHDV, RCV probably presents a few epitopes similar to EBHSV, but the overall antigenic profiles of the two viruses are clearly very different.

Antibody titers, tested at 2-week intervals, remained constant in RCV-infected rabbits for at least 5 months. During this time, none of the rabbits showed the typical clinical symptoms of RHD. Furthermore, ELISAs for direct virus detection in the blood or in tissue extracts from different organs were negative, in agreement with the hypothesis that a low-titer, nonpathogenic virus was involved.

Viral detection by PCR and Western blotting. To try to isolate RCV, five rabbits were sacrificed at the presumed time of de novo infection, about 10 days after weaning. Total RNA was extracted from the liver, spleen, small intestine, lungs, and upper airways of each animal. We reasoned that if infection by a virus related to RHDV was responsible for the seroconversion, it should be possible to detect the viral genome by RT-PCR with primers that yield efficient amplification of the RHDV cDNA. The initial experiments were carried out with primers P1 and P2 (Table 2), specific for the RHDV RNA polymerase gene, because the RNA polymerase sequence is known to be well conserved among different viruses. After RT-PCR, two of five rabbits showed a clearly positive signal corresponding to a band of 340 bp in an agarose gel (Fig. 1A). Interestingly, detection of the amplified fragment was restricted to the intestine, in clear contrast to the typical RHDV infection, which results in virus accumulation in the liver and spleen. The sensitivity and specificity of the assay were further increased by subjecting the same material to Southern blot analysis (Fig. 1C); under these conditions, the positivity of animals 2 and 4 was confirmed. In addition, positive signals were detected from the intestine of animal 1, the liver of animal 2, the spleens of animals 3 and 4, and the upper airways of all the animals except for animal 1. The specific band amplified from the airways was rather weak: it was detected in animal 3 and was detected in animals 2, 4, and 5 after prolonged exposure (result not shown).

The chances of cross-contamination were minimized as described in Materials and Methods. Furthermore, RNA samples extracted from the organs of negative control animals (Fig. 1, lanes I, L, S, and A) were included in all the experiments. Finally, all the samples were normalized for the efficiency of RT-PCR by optimizing the extraction procedure. Figure 1B shows that bands of comparable intensities could be amplified from the SVDV control added to the various tissues. The presence of an RHDV-related RNA was confirmed by detection of specific bands after RT-PCR with primers complementary to the VP60 gene of RHDV (see below).

Aliquots of the tissue homogenates from the two clearly positive animals were also analyzed by Western blot analysis with MAb 5G3; this antibody recognizes a linear epitope on

TABLE 2. Oligonucleotide primers and PCR products

Primer name	Polarity	5′-end position	Primer sequence, 5′ to 3′	Product size (bp)
P1	+	4510	GACTACTCAAAGTGGGACTCC	340
P2	−	4850	TCGGAGTCATGGCATACACG	
P9	+	4802	CTCCATTCTACACGTATGG	567
P10	−	5369	ATGCCATCGGTTGTGG	
P3	+	5311	GGCAAAGCCCGTACAGCGCCGCAA	805
P4	−	6116	AACCCTCCAGGTACTGGTTG	
P5	+	6069	CAGGTGGAACGGCCAAATAG	429
P11	−	6498	CTGTGCACCTGAAGTGTTGGT	
P5	+	6069	CAGGTGGAACGGCCAAATAG	670
P6	−	6739	ACCCAGCTGTGGCATTGACGT	
P15	+	6300	CCAGATGGCTTTCCTGACATG	439
P6	−	6739	ACCCAGCTGTGGCATTGACGT	
P15	+	6300	CCAGATGGCTTTCCTGACATG	594
P13	−	6894	CACACTTAAACCAATCTCCAT	
P14	+	6358	GCGGGGTGGGTCGGATTTGG	381
P6	−	6739	ACCCAGCTGTGGCATTGACGT	
P12	+	6607	ACATACACACCTCAACCAGA	287
P13	−	6894	CACACTTAAACCAATCTCCAT	
P7	+	6685	ATCATGTTCGCGTCTGTCGTCAGG	386
P8	−	7071	GCGCCTGCAAGTCCCAATCC	
P21	+	7025	ATGGCTTTTCTTATGTCTG	353
P22	−	7378	TTAAACACTGGACTCGCCAG	

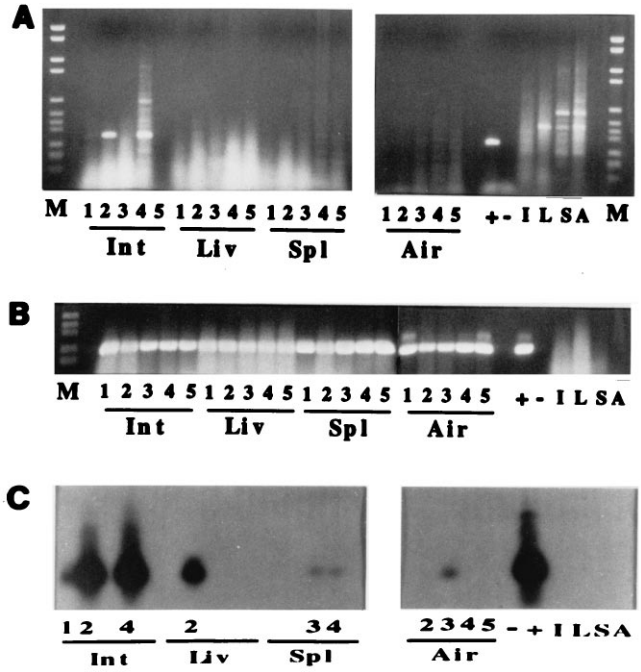

FIG. 1. Detection of viral RNA by RT-PCR. (A) RT-PCR and analysis by agarose gel electrophoresis of RNA extracted from five animals exposed to infection by RCV. The expected 340-bp amplified fragment is visible in samples from the intestines of animals 2 and 4. Results for the intestine (Int), liver (Liv), spleen (Spl), and upper airways (Air) are shown. The + and − signs indicate positive (RHDV RNA) and negative (no substrate) PCR controls. I, L, S, and A, RT-PCR on extracts from the intestine, liver, spleen, and upper airways, respectively, of a noninfected rabbit. M, molecular weight markers (Boehringer marker VI; sizes [in base pairs from the top]: 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, and 220). (B) Amplification of the SVDV-specific band from aliquots of the samples listed in the panel above. +, SVDV genomic RNA alone. Negative controls: −, no RNA; I, L, S, and A, total RNA from different organs without previous addition of SVDV. (C) Southern blot analysis. Aliquots of the samples shown in panel A were subjected to Southern blotting and hybridized with the p672 probe corresponding to the sequence of the RHDV-BS89 isolate from nucleotides 3077 to 7033.

VP60 and cross-reacts with the EBHSV structural protein. A positive band with a size similar to the RHDV VP60 was seen in samples collected from the intestine of animals 2 and 4 but not from the negative controls (Fig. 2). The 5G3 MAb also recognized a protein of about 30 kDa from the intestine of animal 4 (Fig. 2, lane 4). A protein of this size (lane 5) is detected in samples of RHDV that display an unusual morphology in electron micrographs, namely, a smooth capsid surface (5, 6a). The mechanism by which such smooth RHDV particles are generated is still unclear. It is not known whether the 30-kDa band visualized from some RCV samples is also associated with a distinctive morphology of the virions. Other less prominent bands (lanes 4 and 5) probably represent additional degradation products of the capsid protein. The results obtained with the 5G3 MAb were confirmed by Western blotting with polyclonal sera from an RHDV-convalescent rabbit and from a rabbit immunized with recombinant RHDV VP60 (data not shown).

Sequence analysis of the RCV structural protein. Small differences in the primary sequence of viral proteins may bring about profound changes in viral tropism and pathology (9, 11, 20, 28, 30, 35). To begin to address this issue for RCV, the entire sequence of the structural protein gene was determined. Several pairs of PCR primers were designed on the basis of the available sequences of the RHDV VP60 gene. Primers that yielded good PCR products from the RCV cDNA are listed in Table 2 (P3 through P15), together with information about their polarity relative to the genomic RNA, sequence, position with respect to the RHDV BS89 complete genome sequence, and expected size of the amplified product. Several overlapping DNA fragments were subjected to cycle sequencing after their size and identity were verified by electrophoretic analysis and Southern blotting (data not shown). The complete sequence of the RCV capsid protein (length, 576 amino acids; calculated molecular mass, 60,151) is presented in Fig. 3. The alignment of the RCV capsid protein with VP60 from four RHDV isolates at different times and locations and with the EBHSV capsid protein is also displayed. As expected from the serological data, the outcome of the cross-protection experiments (see below), and the relative ease of obtaining efficient PCR primers, RCV and the various RHDV isolates show an average amino acid identity of 91.5%. However, this high degree of identity is significantly lower than the average identity (98%) among bona fide members of the RHDV group. The identity between RCV and EBHSV (75%) is comparable to the average value between EBHSV and the RHDV isolates (76.4%). If the computation is restricted to a region within the C-terminal half of the capsid protein from amino acids 301 to 434, the identity decreases to 83.4%, further emphasizing the differences between RCV and RHDV. This region corresponds to capsid protein domains c, d, and e according to the nomenclature introduced by Neill (25) on the basis of sequence comparison studies between different caliciviruses. In RCV, the c domain (from amino acids 301 to 324) presents a 3-amino-acid deletion, corresponding to N-308, A-309, and T-310 of the RHDV sequence. This deletion represents the main distinctive feature of the RCV capsid protein. It is noteworthy that 31 of 46 amino acid changes (67%) between RCV and RHDV occupy the same position as changes detected when EBHSV is compared with RHDV. The cysteine residue at position 274 of the RHDV sequence is conserved in RCV; the latter contains two additional cysteine residues at position 259 and 329, respectively. In principle, these residues could

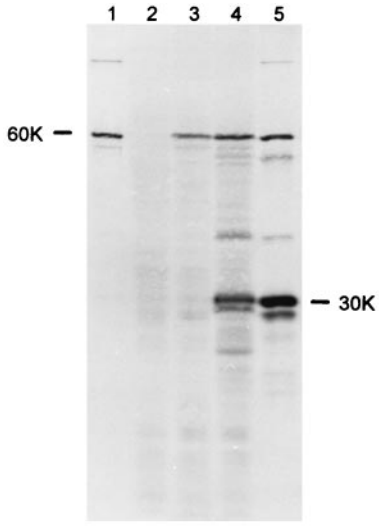

FIG. 2. Detection of the capsid protein by Western blotting. Lanes: 1, liver extract (1:100 dilution) from an RHDV-infected rabbit; 2, intestine from a seronegative control rabbit; 3 and 4, extracts from the intestines of PCR-positive rabbits infected by RCV; 5, liver extract (1:6 dilution) containing smooth RHDV particles. The positions of the full-length capsid protein and of the 30-kDa product are indicated.

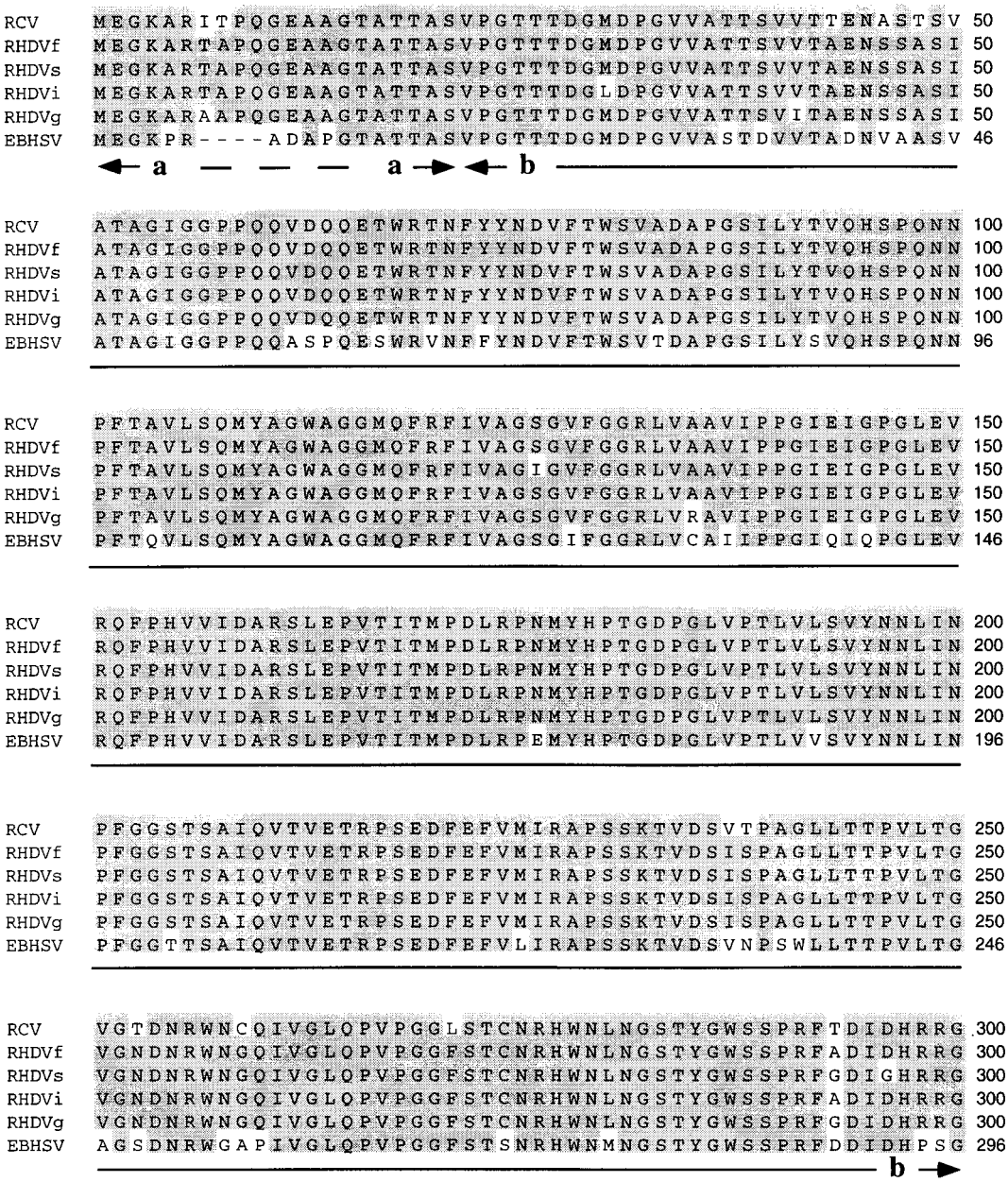

FIG. 3. Sequence of the RCV capsid protein and multiple-sequence alignment. The alignment was performed with the PileUp program of the Genetics Computer Group package with the following settings: GapWeight, 3.0; GapLengthWeight, 0.10. The sequences of the RHDV isolates and of EBHSV are available from the EMBL sequence database under the following accession numbers: RHDVf, French (33) RHDV isolate Z29514; RHDVs, Spanish (1) RHDV isolate Z24757; RHDVi, Italian RHDV isolate X87607; RHDVg, German (21) RHDV isolate M67473; EBHSV (40), European brown hare syndrome virus U09199. The bottom row shows the subdivision of the capsid protein into different domains (see the text).

participate in disulfide bridges with a precise structural function in capsid assembly. This possibility must take into account that the position of the single cysteine within the EBHSV sequence is not conserved. The RCV capsid protein contains two closely spaced, negatively charged amino acids (E-473 and D-477) that are absent in the RHDV protein and contribute to a relatively low calculated isoelectric point of 4.54. The genetic distances between RCV, EBHSV, and the four RHDV isolates are depicted in a dendrogram (Fig. 4), which was obtained by entering the data of the alignment into the program Growtree of the Genetics Computer Group package.

Organization of the 3′-terminal region of the RCV genome. We wanted to determine if RCV maintained one of the most important properties of the genome organization of the caliciviruses of lagomorphs, i.e., the presence of only two ORFs, as opposed to three in the other caliciviruses. Figure 5A shows the sequence of the boundary between the RNA polymerase gene and the VP60 gene of RCV and its alignment with the corresponding region from the Italian RHDV isolate. Note that the continuous reading frame is conserved in RCV. The site specifically cut by the RHDV 3C-like protease (41), Glu at position P1 and Gly at position P1′, is also present in RCV.

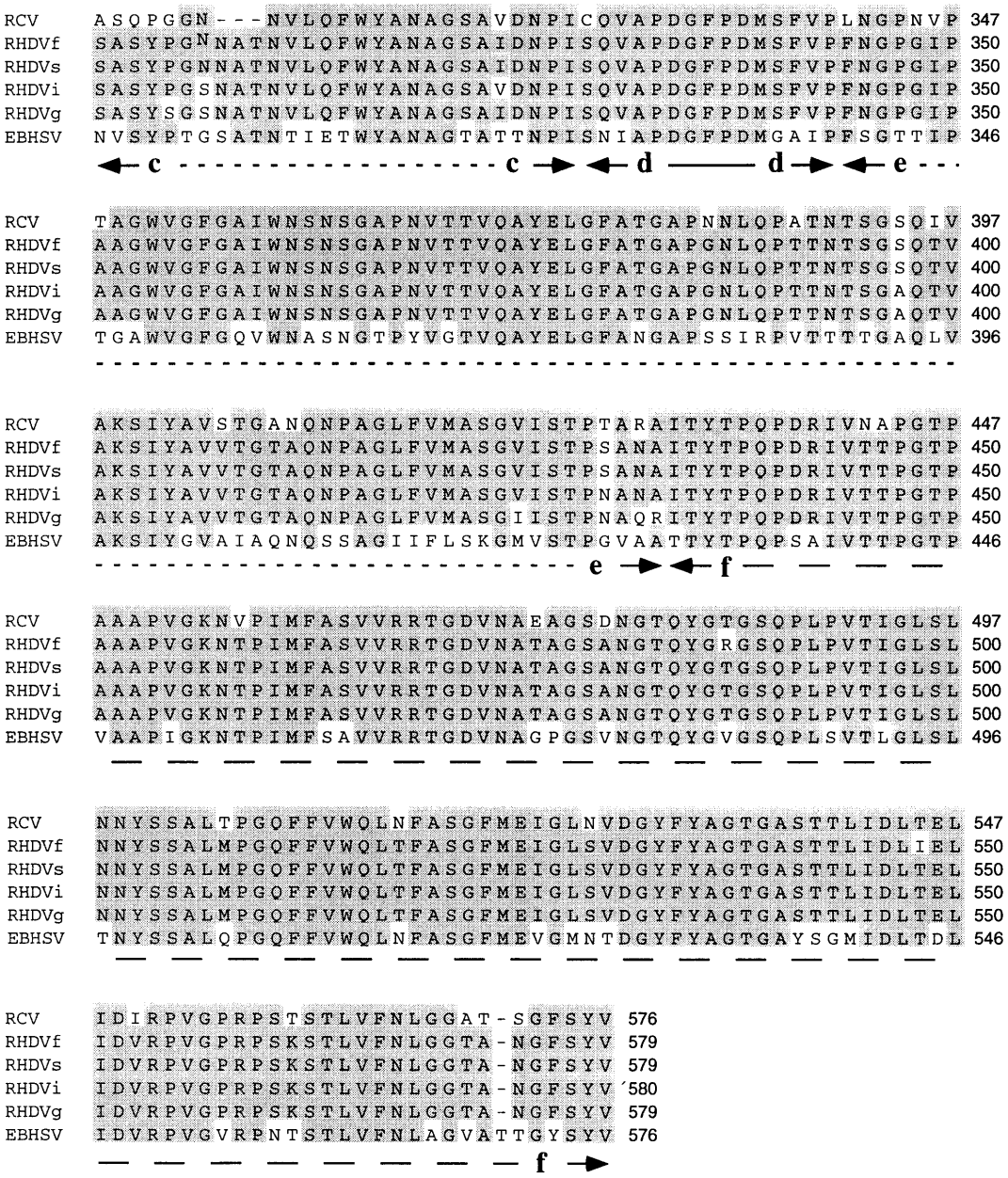

FIG. 3—*Continued.*

Therefore, it is likely that processing of the polyprotein between the RNA polymerase and the VP60 domains is identical in the two viruses. Figure 5B shows the alignment of the region downstream of the capsid protein gene after amplification (primers P21 and P22 [Table 2]) and the sequence of the ORF2 gene. Interestingly, the putative ORF2 initiation codon, originally identified in RHDV (21), is absent in the RCV sequence. Instead, the next in-frame AUG codon of RHDV (9 nucleotides downstream) appears to correspond to the initiation codon in RCV. The RCV ORF2 encodes a polypeptide of 112 amino acids; not taking into account the first 4 amino acids of the putative RHDV ORF2, the RCV and RHDV-BS89 ORF2s share a 92% identity.

Taken together, these data demonstrate that the RCV genome organization is indeed the same as that of RHDV.

Experimental reproduction of infection. Reproduction of infection, under controlled experimental conditions, was attempted to further characterize RCV. Tissue homogenates from the intestine of animals 2 and 4 were pooled and administered to seronegative rabbits by the oronasal route. A total of eight rabbits (group A) were subjected to the experimental infection and put in four separate cages. Each cage also housed one seronegative, noninfected animal (group B) to verify the possibility of transmission through contact. In addition, a third cage with two seronegative animals (group C) was introduced into the same room but at a distance sufficient to avoid direct contact and contamination by body fluids from the other rabbits. Starting at days 5 postinfection (p.i.), serological tests for anti-RHDV antibodies were positive for all animals from group A (Table 3, rabbits 3 to 6, 9, and 10) and the levels of

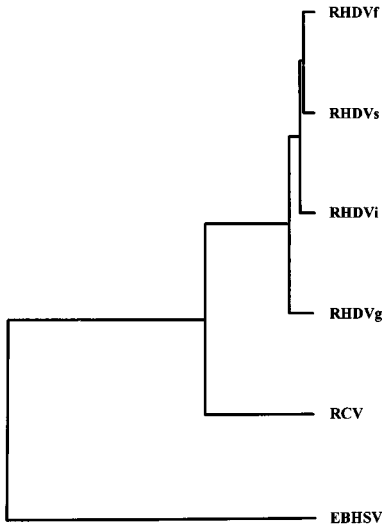

FIG. 4. Dendrogram of predicted genetic relatedness among the caliciviruses of lagomorphs. The amino acid sequences of the entire capsid protein gene were used to generate the tree. Branch lengths are inversely proportional to the similarities computed by the PileUp program for the multiple-sequence alignment.

antibody increased over the following days. Animals from group B (Table 3, rabbits 7, 8, 11, and 12) also became seropositive with a slight delay (1 or 2 days) and at lower titers compared with group A. In contrast, group C animals (Table 3, rabbits 13 and 14) remained seronegative for the entire period of observation (24 days). The seroconversion per se indicated that an active infection was in progress, since the minute amount of viral material administered would probably not be sufficient to stimulate the immune system. Moreover, passive immunization would not explain the seroconversion of the animals in group B. For comparison Table 3 also reports the anti-RHDV titers in two convalescent rabbits after infection with RHDV on day 0 (Rc1 and Rc2). As mentioned above, the higher antibody titers of Rc1 and Rc2 probably reflect the ability of RHDV to replicate to a wider extent than RCV.

To extend these results, the presence of viral nucleic acid and proteins was assessed in various organs from six rabbits in group A (rabbits 1 to 6), sacrificed at 3, 5, and 6 days p.i. For the same purpose, two rabbits from group B (rabbits 7 and 8) were also sacrificed 7 days p.i. for all these animals, RT-PCR yielded a specific band (340 bp) from the intestine, starting at the earliest time point tested (Fig. 6A, lanes 1 and 4). In agreement with the serological data, a smaller amount of virus was present in the two group B rabbits (lanes 6 and 7), as reflected by the visualization of a specific band only after hybridization to a radiolabeled probe (Fig. 6B). Under the same experimental conditions, no positive signal was detectable from the liver and the spleen of rabbits 1 to 8 (data not shown).

A protein of 60 kDa was detected by Western blot analysis in all but one of the intestinal extracts from the experimentally infected animals (Fig. 7). As described above, a 30-kDa protein in addition to VP60 was detectable in several samples. The capsid protein could not be detected in extracts from group B animals (Fig. 7, lanes 9 and 10).

Later, the rest of the animals (Table 3, rabbits 9 through 14) and two seronegative control rabbits were challenged by intramuscular injection of 10^3 50% lethal doses of RHDV. The animals from groups A and B survived, indicating that the immune response induced by RCV was sufficient to confer protection against virulent RHDV. In contrast, rabbits from group C and the negative controls showed the typical symptoms of RHDV infection and were sacrificed.

EBHSV and RHDV have an extremely restricted host range and fail to cross-infect and to provide cross-protection (5, 7, 19) to rabbits and hares, respectively. To determine if the host range of RCV was equally restricted, six seronegative hares and six rabbits were experimentally infected by the oronasal route. Serological tests performed at 5-day intervals demonstrated the previously observed pattern of seroconversion in rabbits. On the other hand, no seroconversion was observed in hares, indicating that no virus replication was occurring in this host. This was confirmed by the negative RT-PCR results obtained with the organs of two hares sacrificed 5 days after administration of RCV (data not shown). As expected, when the animals previously inoculated with RCV were challenged with wild-type RHDV (rabbits) or EBHSV (hares), all the rabbits were fully protected, while the hares displayed the typical symptoms and course of EBHS.

DISCUSSION

We have identified a new rabbit calicivirus by analysis of its antigenic features and through detection of the viral RNA, visualization of the major capsid protein, determination of part of the genome sequence, and experimental reproduction of the infection. Several lines of evidence indicate that the new virus is more strictly related to RHDV than to EBHSV, the other known member of the group of caliciviruses of lagomorphs. In particular, we have shown that (i) serum antibodies in response to the new virus compete with RHDV- but not EBHSV-specific antibodies for recognition of the respective antigen; (ii) the immune response to the new virus confers cross-protection to rabbits against subsequent infection by RHDV; (iii) the new virus can be experimentally transmitted to rabbits but not hares; and (iv) the primary sequence of the capsid protein is more closely related to RHDV than to EBHSV.

Since the newly identified virus does not retain several important features of RHDV, namely, its well-characterized clinical symptoms and the associated high mortality (hence the name rabbit hemorrhagic disease) and also differs with regard to the sites of accumulation in the host, we propose to name the new virus rabbit calicivirus (RCV). Rabbits infected by RCV appear healthy, and no histopathological lesions could be detected after necroscopy.

Replication of RHDV most probably occurs in the liver (10), and massive virus accumulation is observed in the liver and the spleen. In this respect, RCV is clearly different from RHDV, because the largest amounts of RCV are invariably found in the intestine. We do not know the biological relevance of detecting very small amounts of RCV in the liver of one rabbit and the spleen of another two. This event is relatively rare, since it occurred in only 3 of 13 animals (5 naturally infected, 6 experimentally infected, and 2 that had acquired the infection upon contact) examined in detail. It should be pointed out that even in the case of RHDV, the initial steps of the infection and the details of viral tropism are ill-defined. A recent study (12) demonstrated the presence of RHDV in various organs at early times (hours) following experimental infection. RHDV was detected (by RT-PCR) in the lung, liver, and spleen between 2 and 18 h p.i.; the intestine was not examined in this study. Unfortunately, very high viremia in the following hours obscured the meaning of PCR-positive results from other organs such as the kidney, thymus, tonsil, and lymph nodes.

The RCV capsid protein was detected by Western blotting from intestine extracts that also yielded the stronger bands

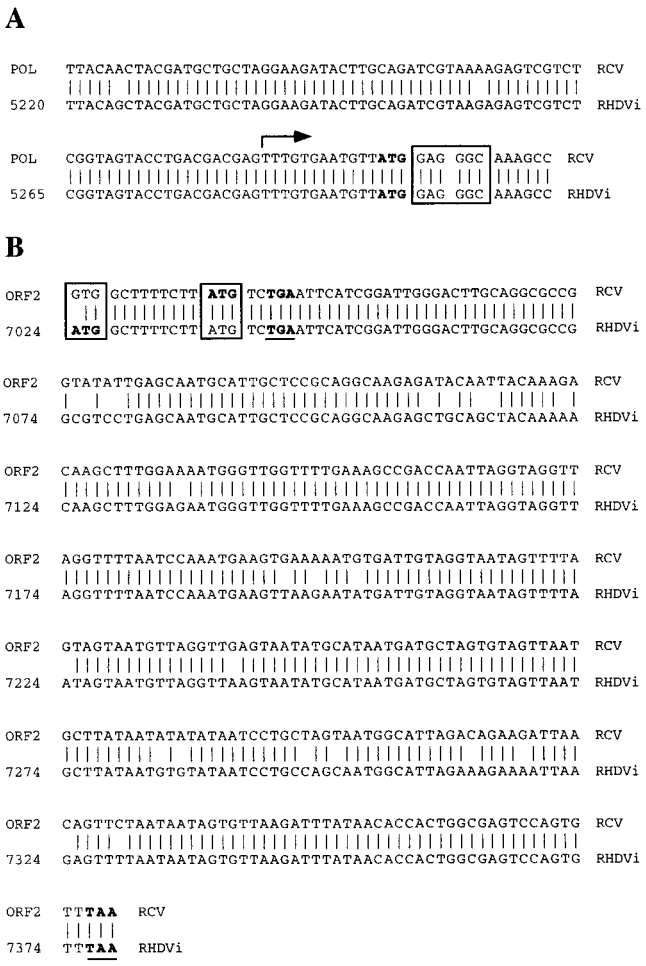

FIG. 5. 3′ genome organization. RCV sequences were aligned to the homologous sequences from the Italian RHDV isolate. (A) Boundary between the RNA polymerase (POL) gene and VP60. The positions of the subgenomic RNA 5′ end (arrow) (19) and of the VP60 translation start codon (in boldface type) (26) of RHDV are highlighted. Boxed codons: P1 and P1′ amino acids in the target site of the RHDV 3C-protease. (B) Sequence and alignment of the ORF2 nucleotide sequence. Boxes indicate the putative ORF2 initiation codons of RHDV and RCV. The boldface underlined sequence indicates the termination codons of VP60 and ORF2. Base numbering is according to the Italian RHDV isolate complete genomic sequence.

TABLE 3. Antibody titers after experimental infection of rabbits with RCV

Rabbit	Titer on day p.i.[a]:						
	0	3	5	6	11	24[b]	33
1A	<1/5	<1/5†					
2A	<1/5	<1/5†					
3A	<1/5	<1/5	1/20†				
4A	<1/5	<1/5	1/20†				
5A	<1/5	<1/5	1/80	1/160†			
6A	<1/5	<1/5	1/20	1/80†			
7B	<1/5	<1/5	<1/5	1/20†			
8B	<1/5	<1/5	<1/5	1/10†			
9A	<1/5	<1/5	1/80	1/160	1/320	1/320	1/320
10A	<1/5	<1/5	1/20	1/80	1/80	1/160	1/320
11B	<1/5	<1/5	<1/5	1/10	1/40	1/160	1/160
12B	<1/5	<1/5	<1/5	1/20	1/40	1/80	1/320
13C	<1/5	<1/5	<1/5	<1/5	<1/5	<1/5	
14C	<1/5	<1/5	<1/5	<1/5	<1/5	<1/5	
Rc1[c]	<1/5	1/40	1/320	1/320	1/5,120	ND[d]	ND
Rc2[c]	<1/5	1/10	1/640	1/1,280	1/10,240	ND	ND

[a] Rabbits 1 to 6 were sacrificed (†) the same day of the last serological sampling. Rabbits 7 and 8 were sacrificed the next day.
[b] On day 24 p.i., rabbits 9 to 14 were subjected to RHDV challenge; rabbits 13 and 14 died between 36 and 48 h post challenge.
[c] Convalescent rabbits.
[d] ND, not done.

after PCR. The specific recognition of a 30-kDa protein in addition to the full-size capsid protein is paralleled by similar results with a subset of the RHDV samples. The presence of a polypeptide of about 30 kDa is also reminiscent of the findings in a calicivirus causing gastroenteritis in humans (Norwalk virus). It must be noted, though, that the smaller product of Norwalk virus corresponds to the C-terminal half of the capsid protein, while the N-terminal half is not detected (13, 17). The opposite is true for RHDV and RCV.

Because of the apparent inability of the new virus to accumulate to high titers in the organs examined, its purification and detailed biochemical analysis are rather difficult: in particular, we were not able to observe the viral particles by electron microscopy in spite of several attempts. Preliminary experiments to achieve RCV replication in vitro with an immortalized rabbit cell line (RK13) or primary cultures from the intestine and kidney of newborn rabbits also failed (data not shown), by analogy to the negative results with other caliciviruses including RHDV, EBHSV, and Norwalk virus. Further work will be required to purify sufficient amounts of the virus to reexamine these issues. Studies of RCV will also take advantage of the expression of the capsid protein gene by recombinant means and possibly by the cloning and characterization of the entire genome.

Wirblich et al. (40) have proposed to divide the caliciviruses into three groups according to their genome organization and to the strategies used to express the capsid protein. We have demonstrated that RCV belongs to the group comprising EBHSV and RHDV, on the basis of the sequence of the 3′-terminal one-third of its genome. This region includes part of the RNA polymerase gene, the capsid protein gene, and the complete ORF2 sequence. The first available initiation codon of the RCV ORF2 corresponds to the next in-frame AUG of RHDV. Neither of the AUGs is in an optimal context for translation initiation. Interestingly, in RCV the AUG is closer to the VP60 termination codon and thus would be at an advantage if translation of ORF2 occurred by a termination-reinitiation mechanism, as recently hypothesized by Herbert et al. for feline calicivirus (15).

The alignment of the capsid protein amino acid sequence of RCV, RHDV, and EBHSV highlights the relatively few differences between RCV and RHDV. Nevertheless, the extremely high degree of conservation among different RHDV isolates would argue that such differences are significant, even from a functional standpoint. About 75% of the amino acid substitutions and deletions are clustered within regions c, e, and f (according to the nomenclature of Neill [25]), where the majority of the changes among different serotypes of San Miguel sea lion virus and feline calicivirus are also clustered. This is consistent with the proposal, based on structural (31, 32) and antigenic (3, 23, 38) data, that the C-terminal half of the capsid protein of the caliciviruses constitutes the external surface of the virion. It remains to be seen if the differences at the capsid protein level between RCV and RHDV are directly responsible for their different tropism and for the lack of pathogenicity of the former. In this respect, there are several examples of a

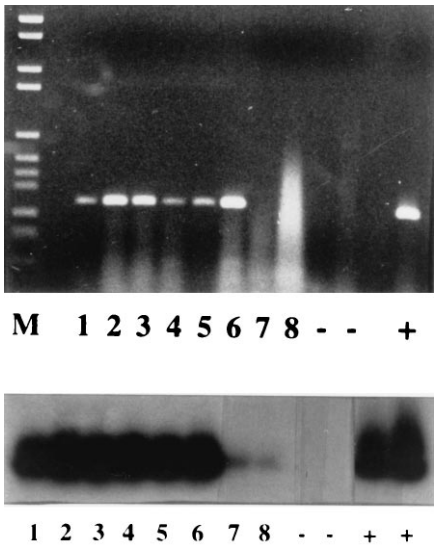

FIG. 6. Detection of viral RNA in the intestine of experimentally infected animals. (Upper) Results of RT-PCR on RNA extracted from the intestine of group A rabbits at 3 days (lanes 1 and 4), 5 days (lanes 2 and 5), and 6 days (lanes 3 and 6) p.i., and from the intestine of two group B rabbits (lanes 7 and 8) at 7 days p.i. The − and + signs indicate RT-PCR controls performed on RNA from the intestine of two seronegative rabbits and from the liver of one RHDV-infected rabbit, respectively. M, molecular weight marker VI (Boehringer). (Lower) Southern blot analysis of aliquots of the samples shown in panel A. +, 1:20 and 1:5 dilution of the positive control shown above.

single or a few mutations of a structural protein profoundly altering viral pathogenicity (see, e.g., references 9, 11, 20, 28, and 35). Maybe the most interesting paradigm is constituted by the sudden emergence and worldwide spread, in 1978, of canine parvovirus (CPV), soon followed by CPV-2a and CPV-2b. Studies of the structure, sequence, and antigenicity of CPV and comparison with the related feline panleukemia virus indicated that essentially all of the specific properties of CPV were determined by only two amino acid differences in the capsid protein VP2 (30). Thus, it has been hypothesized that CPV evolved from feline panleukemia virus-like virus via very few sequence changes (30). Clearly, the sweeping epidemics of RHD during the years 1985 to 1988 were due to the sudden emergence of RHDV (24, 26). On the other hand, retrospective serological studies show anti-RHDV antibodies in sera collected years before the initial reports of RHD (34). It follows that RHDV may have evolved from a virus already present in rabbits before 1984. Whether RCV represents such a progenitor is a matter of speculation. At present, any direct evolutionary relationships among the caliciviruses of lagomorphs remain to be demonstrated. The discovery of RCV should be taken into account in future epidemiological studies of RHD. It is not known how widespread RCV is in nature. Certainly, infection by RCV, even at very low titers like in group B animals in this work, will confer a selective advantage to rabbits exposed to RHDV. Our findings may be relevant for future vaccination campaigns, for the plans to control the rabbit population in Australia through the release of infectious RHDV, and, finally, for interpretation of the evolution of the RHDV epidemics in Australia after an accidental escape of the virus to the mainland occurred at the end of 1995 (16, 18).

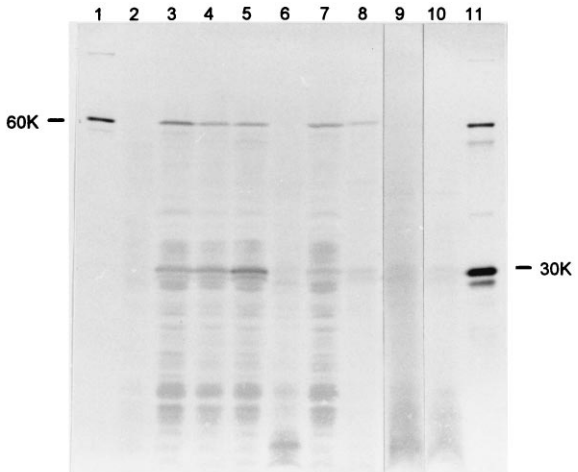

FIG. 7. Detection of the capsid protein in the intestine of experimentally infected animals. Lanes: 1, positive control, liver extract from an RHDV-infected rabbit (1:100 dilution); 2, negative control, intestine extract from a seronegative rabbit; 3 to 8, extracts from the intestine of experimentally infected rabbits (group A); 9 and 10, intestine extracts from two group B animals; 11, liver extract containing smooth RHDV particles. The positions of the capsid protein and of the 30-kDa polypeptide are indicated.

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