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Processing of Rabbit Hemorrhagic Disease Virus Polyprotein

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Expression of rabbit hemorrhagic disease virus (RHDV) cDNAs in vitro with rabbit reticulocyte lysates and in *Escherichia coli* have been used to study the proteolytic processing of RHDV polyprotein encoded by ORF1. An epitope tag was used for monitoring the gene products by a specific antibody. We have identified four gene products with molecular masses of 80, 43, 73, and 60 kDa, from the amino to the carboxy terminus of the polyprotein. The amino-terminal sequences of the 43- and 73-kDa products were determined and indicated that RHDV 3C proteinase cleaved Glu-Gly peptide bonds.

Rabbit hemorrhagic disease virus (RHDV), the causative agent of a lethal disease in rabbits (25, 28), has been included as a member of the *Caliciviridae* family. Its small icosahedral capsid of 40 nm in diameter is composed of a single protein of 60 kDa (VP60) (28) and contains the 7.5-kb genome that consists of a single-stranded RNA of positive polarity. Viral particles also encapsidate an abundant subgenomic RNA of about 2.2 kb, which is coterminal with the 3′ end of the viral genome (23).

The full-length genomic RNA has been cloned and sequenced. It contains a long open reading frame (ORF) with coding capacity for a large polyprotein of 256 kDa (22). As in other members of the family, it contains the motifs of a helicase (2C-like protein), proteinase (3C-like protein), and RNA-dependent RNA polymerase (3D-like protein). These nonstructural polypeptides are located toward the amino-terminal region of the polyprotein, whereas the structural protein VP60 constitutes the carboxyl end. RHDV differs from other caliciviruses in the fact that ORF1 contains both the nonstructural and the capsid protein genes, while in other caliciviruses the capsid protein is encoded by a separate ORF. For this reason, the VP60 of RHDV can be synthesized in two ways: by translation of the subgenomic RNA (5) or by proteolytic processing of the polyprotein (6).

Proteolytic processing, carried out by viral proteinases, of the polyprotein of other positive-strand RNA viruses, such as the picornaviruses (26), is well known. In RHDV, a region located upstream of the putative RNA polymerase, which might correspond to the 3C proteinase of picornavirus by computer analysis, has been described (22). Furthermore, this proteolytic activity has been included in the same family of trypsin-related proteinases on the basis of amino acid sequence alignments and site-directed mutagenesis that identify conserved residues which have been proposed to correspond to the catalytic triad of cysteine proteinases (6). Although this proteolytic activity has been characterized, little is known about its cleavage specificity for RHDV polyprotein.

Since the virus could not be propagated in primary cultures or in established cell lines, in this work we have studied the proteolytic processing of RHDV polyprotein by using two in vitro approaches: coupled transcription-translation experiments and bacterial expression studies with an epitope tag to label the resulting products and to further determine the amino-terminal sequence of these products. To study the proteolytic processing of RHDV polyprotein in rabbit reticulocyte lysates, we cloned the full-length cDNA or selected fragments in the plasmid expression vectors pRSET (A, B, or C) (Invitrogen) or pT7-7 (31). These constructs (summarized in Table 1) allowed the expression of the foreign cDNA cloned downstream from the T7 RNA polymerase promoter. A schematic diagram showing the fragments of the RHDV cDNA used for the coupled in vitro transcription-translation experiments is presented in Fig. 1.

RHDV cDNA cloned in the pRSET or in pT7-7 vectors was used in the DNA-directed in vitro translation system as specified in the protocol of the manufacturer (Promega) with [^{35}S]methionine as a tracer. For 1 μg of circular plasmid DNA, 10 U of T7 RNA polymerase, 10 μCi of Tran[^{35}S]Label (ICN), and 10 μl of rabbit reticulocyte lysate were used. For kinetic analysis, aliquots were withdrawn at different time intervals, diluted with sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). For fluorography, gels were stained with Coomassie blue, incubated for 30 min in 1 M sodium salicylate solution, dried, and subjected to autoradiography.

When plasmids pRC49, pRC30, and pT7VP60 were transcribed and translated in vitro in a coupled reaction, major products of the expected size (Table 1) were observed and no proteolytic processing products were detected after 150 min of incubation (Fig. 2, lanes 3, 4, and 8). The minor bands observed in these lanes are probably due to some degree of internal initiation.

The coupled transcription-translation of plasmid pRC44, which has the coding capacity for a protein of 256 kDa, did not yield a polypeptide product of the expected size (Fig. 2, lanes 5, 6, and 7). After short incubation periods (30 min), three major labeled products smaller than the 150-kDa marker could be observed (lane 5). After longer reaction intervals (90 or 150 min), four major polypeptide products with approximated molecular masses of 80, 73, 60, and 43 kDa were obtained (lanes 6 and 7). Results obtained with the negative controls without added DNA (lane 1) or with 1 μg of plasmid vector pRSETA without insert DNA (lane 2) support the conclusion that the labelled products observed are virus specific. To demonstrate further the viral origin of these polypeptides, immunoprecipitation experiments with sera from RHDV-immunized rabbits were performed. Only the 60-kDa product was recognized by antibodies against RHDV (results not shown). It should be mentioned that the 60-kDa product obtained in the coupled transcription-translation of plasmid pRC44 (lanes 6 and 7)

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TABLE 1. Recombinant plasmids containing RHDV cDNA used for in vitro translation experiments and *E. coli* expression as fusion proteins with β-galactosidase

Construct[a]	Vector	ORF1 residues	DA3 label location	Size (kDa) of:	
				Expected unprocessed product	Observed labelled product
pRC49	pRSETA	9–1032		112	
pRC30	pRSETB	1170–2344		130	
pT7VP60	pT7-7	1766–2344		60	
pRC44	pRSETA	9–2344		256	
pRC59N	pUR291	9–1577	489	288	195
pRC56P	pUR291	438–1577	1031	241	43
pRC37B	pUR291	883–1796	1649	216	73
pRC40B	pUR291	883–2344	1649	276	73

[a] All the constructs were made by standard techniques (30) and were checked by restriction analysis and DNA sequencing.

seems to be slightly smaller than the VP60 protein produced by pT7VP60 (lane 8) and that found in RHDV virions (4) when analyzed by SDS-PAGE.

The lack of major proteolytic processing of the polypeptide products of clones pRC49 and pRC30 demonstrates that these large regions of the RHDV polyprotein do not code for viral proteases capable of autocatalytic cleavage. Therefore, the autocatalytic cleavage observed in clone pRC44, which has the combined coding capacity of clones pRC49 and pRC30, should be attributed to the inclusion in pRC44 of ORF1 residues 1033 to 1169 (not present in pRC30 or pRC49). Considering that this ORF1 region contained amino acid residues His-1135 and Asp-1152, involved in the catalytic triad of RHDV 3C proteinase (6), these data suggested the direct involvement of the RHDV 3C protease in the autocatalytic cleavage of the RHDV polyprotein.

To locate the polyprotein cleavage products in the RHDV genome, we expressed in *Escherichia coli* several RHDV cDNA fragments (Table 1), including the region coding for the 3C-like proteinase, as fusion proteins with the β-galactosidase. To do this, we used the pUR291 vector (29). For the detection of the specific cleavage products of RHDV polyprotein, we used a well-characterized linear epitope (residues 362 to 373) from transmissible gastroenteritis virus N protein recognized by the monoclonal antibody DA3 (20). The insertions were made (Fig. 1) between amino acid residues 1649 and 1650 (putative RNA polymerase), residues 1031 and 1032 (close to the region homologous to 3C proteinase), and residues 489 and 490 (next to the region homologous to picornavirus 2C protein) (22). No flag insertions were made at sites of possible 3C cleavage as judged from previous works (6). To introduce this epitope tag at the selected locations, we synthesized two 36-mer complementary oligonucleotides coding for the DA3 epitope with *Bgl*II-compatible ends. The phosphorylated and annealed oligonucleotides were ligated to the dephosphorylated *Bgl*II site at nucleotide 4955 of RHDV AST/89 cDNA. To insert the flag at amino acid positions 1031 to 1032 and 489 to 490, we used *Pst*I and *Nsi*I restriction sites located at nucleotides 3101 and 1475, respectively, of RHDV AST/89 cDNA. To do this, the cohesive ends of the phosphorylated and annealed oligonucleotides were filled with the Klenow fragment of DNA polymerase and then ligated to the previously blunt-ended *Pst*I and *Nsi*I restriction sites.

E. coli XL1-blue cells transformed by pUR recombinant plasmids were grown to the plateau stage by overnight incubation at 37°C in Luria-Bertani medium containing 100 μg of ampicillin per ml. The culture was then diluted in fresh medium and further incubated at 37°C until it reached an optical density at 600 nm of 0.6. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 200 μM, and the culture was further incubated for 2 h. Following this, cells were harvested by centrifugation, disrupted with sample buffer, and analyzed by SDS-PAGE. For detection of the expression products, the cell extracts analyzed by SDS-PAGE were transferred to nitrocellulose filters (7) and incubated with monoclonal antibody DA3 or a polyclonal serum against RHDV. The presence of antigen-antibody complexes was revealed with peroxidase-conjugated anti-mouse antibodies or protein A, respectively.

The size of the DA3-labelled product expressed by construct pRC59N was 195 kDa (Fig. 3, lane 1), which was 93 kDa smaller than expected (288 kDa). Considering the location of the epitope tag, i.e., closer to the 5′ end of the RHDV cDNA

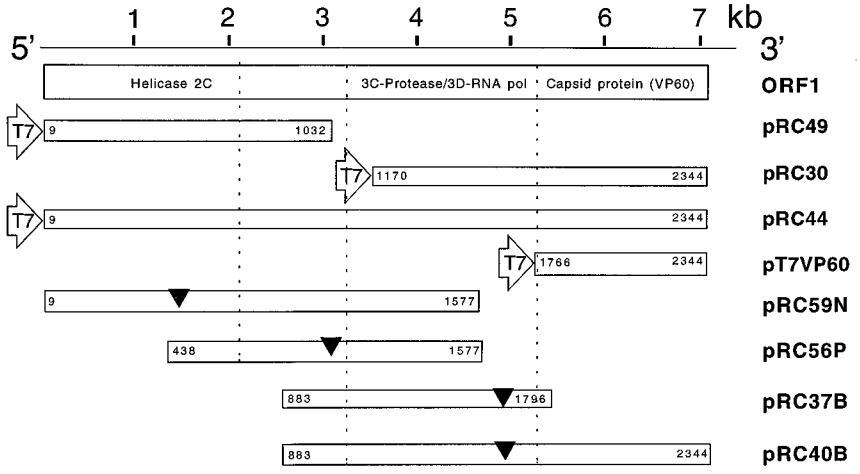

FIG. 1. Diagram of the RHDV genome showing the cDNA clones pRC49, pRC30, pRC44, and pT7VP60 used for the in vitro transcription and translation experiments and plasmids pRC59N, pRC56P, pRC37B, and pRC40B, which were expressed in *E. coli* as fusion proteins with β-galactosidase. Dashed vertical lines denote hypothetical proteolytic processing sites. Numbers inside the boxes indicate the polyprotein amino acid residues included in each construct. The solid inverted triangles represent the location of the monoclonal antibody DA3 epitope.

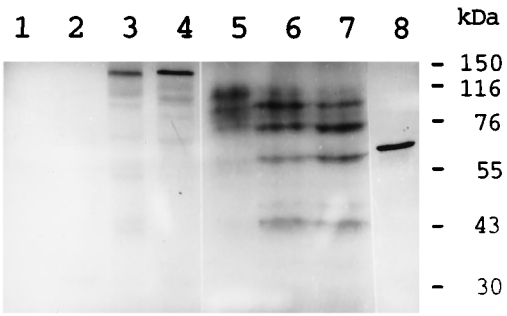

FIG. 2. SDS-PAGE analysis of the products obtained by coupled transcription and translation without DNA (lane 1), with 1 μg of pRSETA vector without insert DNA (lane 2), with 1 μg of pRC49 (lane 3), with 1 μg of pRC30 (lane 4), with 1 μg of pRC44 (lanes 5 to 7), and with 1 μg of pT7VP60 (lane 8). Incubation time was 150 min, except for lanes 5 (30 min) and 6 (90 min).

(Fig. 1), one or more unlabelled peptides equivalent to the 93 kDa must be removed from the C terminus of the 288-kDa precursor. The expression experiments with construct pRC56P, in which the epitope flag was placed 540 amino acid residues downstream with respect to its location in construct pRC59N, yielded two labelled polypeptide products of 94 and 43 kDa (lane 2). The fact that both proteins had the same amino-terminal sequence indicated that the epitope label was included within the 43-kDa product which originates from the amino region of the 94-kDa polypeptide precursor by an autoproteolytic cleavage. Two faint bands with higher molecular masses than 94 kDa were also visible in this lane and might represent incompletely processed products.

The use of constructs pRC37B and pRC40B, in which the DA3 flag was placed at ORF1 residue 1649, slightly 5′ of the VP60 amino terminus (residue 1766), gave rise to the detection of polypeptide products of 73 kDa (Fig. 3, lanes 3 and 4). The use of a polyclonal serum against RHDV to probe a blot containing constructs pRC37B and pRC40B allowed the detection of a 60-kDa polypeptide product (Fig. 4, lane 4) expressed only from construct pRC40B, whose SDS-PAGE mobility was slightly lower than that of VP60 from purified RHDV (Fig. 4, lane 5). This fact and the location of the epitope tag, i.e., very close to the 3′ end of the RHDV cDNA pRC37B, suggested that the 73-kDa product derived from a genomic region located immediately 5′ of the VP60 coding sequence which constituted the carboxy terminus of ORF1.

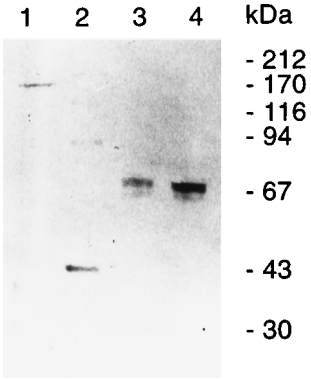

FIG. 3. Western blot analysis of extracts from IPTG-induced recombinant bacteria with monoclonal antibody DA3. Lanes: 1, pRC59N; 2, pRC56P; 3, pRC37B; 4, pRC40B.

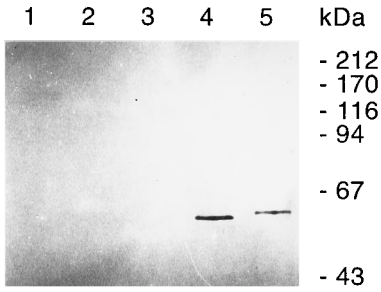

FIG. 4. Western blot analysis of extracts from IPTG-induced recombinant bacteria with a polyclonal serum against RHDV. Lanes: 1, pRC59N; 2, pRC56P; 3, pRC37B; 4, pRC40B; 5, purified RHDV virions.

To determine the exact localization of the proteolytic processing sites and also the specificity of 3C-like proteinase of RHDV, extracts of the strains harboring plasmids pRC56P, pRC37B, and pRC40B were separated by electrophoresis and transferred to Immobilon filters in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer (pH 11.0) plus 10% methanol for 30 min at 50 V and room temperature (21). To establish the amino terminus of the processed products, the relevant bands were excised from the filters and subjected to N-terminal sequence analysis by sequential degradation on an Applied Biosystems 477A sequenator. The phenylthiohydantoin derivatives were identified and quantified with an on-line analyzer (model 120A). The 43-kDa polypeptide, and its 94-kDa precursor, originated by the processing of construct pRC56P, yielded the sequence Gly-Ala-Asn-Arg-Phe-Asn, which corresponded to residues 719 to 724 of RHDV polyprotein. The amino-terminal sequence of the 73-kDa polypeptides from clones pRC37B and pRC40B was Gly-Leu-Pro-Gly-Phe-Met, which corresponded to residues 1109 to 1114 of RHDV polyprotein. The amino-terminal sequence of the 60-kDa polypeptide detected by the anti-RHDV serum could not be experimentally determined. Nevertheless, as the amino acid residues located at positions 718 and 1108, preceding the RHDV polyprotein cleavage products p43 and p73, corresponded to glutamic acid, it can be postulated that the cleavage by 3C-like proteinase of RHDV could also take place at the VP60 Glu-Gly peptide bond (residues 1767 and 1768). This hypothesis was confirmed by site-directed mutagenesis (Glu/Gly substitution of ORF1 residue 1767) of cDNA clone pRC40B (data not shown). The resulting mutant clone gave rise to a product of 133 kDa, which could be detected by both monoclonal antibody DA3 and the anti-RHDV serum.

In this report, we have shown that there is a 3C-like proteolytic activity codified by the RHDV genome, which is able to cut polyprotein substrates obtained by in vitro translation or by expression in *E. coli*. A similar activity has been very well characterized by sequence comparison analysis (2, 14), site-directed mutagenesis (6, 11), or X-ray crystal structure determination (1) in different animal viruses (picornavirus or calicivirus) or picornavirus-like plant viruses (potyvirus, comovirus, or nepovirus) (18).

Despite the very well characterized catalytic triad of the 3C proteinase, very little is known about the processing of the calicivirus polyprotein. The presence of a Glu or Gln residue at position P1 of the cleavage site because of the existence of a His in the substrate-binding pocket of the proteinase 3C of picornaviruses has been proposed (14). 3C-like proteinases of nepovirus (3, 19) and 2A proteinases (32), which do not contain a His in their binding pocket, do not have Glu or Gln at position P1. For feline calicivirus (FCV), a Glu-Ala cleavage

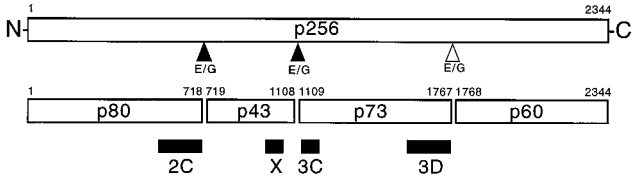

FIG. 5. Proposed polyprotein processing by RHDV 3C-like proteinase. Solid triangles indicate the cleavages determined by amino acid sequencing. The open triangle indicate the processing site found by site-directed mutagenesis. Solid rectangles indicate regions homologous to picornavirus proteins 2C, 3C, and 3D as described by Meyers et al. (22). X represents a domain conserved among several caliciviruses and astroviruses. Numbers indicate polyprotein amino acid residues.

has been reported (9) to be responsible for the generation of the mature capsid protein, although in this virus the capsid protein is synthesized from an independent ORF.

Thus, we have determined, by direct sequencing of the processed polyprotein products p43 and p73, the presence of Glu at position P1 and Gly at position P1′. A similar approach has been used to determine the specificity of the 3C proteinase of hepatitis A virus (13). As has been hypothesized previously (6), we have experimentally determined the cleavage site at the amino terminus of the 3CD protein to be the Glu-Gly bond located at position 1108 to 1109 of the RHDV polyprotein. Taking into account the results obtained after site-directed mutagenesis at residue 1767, another processing site could be mapped between residues 1767 and 1768 of the polyprotein, inside of the capsid protein-coding region. This would partially explain the slight differences in SDS-PAGE mobility of the p60 product resulting from the polyprotein cleavage, with respect to native VP60, which has two extra amino acid residues at the amino terminus which could also be acetylated (27).

The data obtained from the in vitro translation and *E. coli* expression studies revealed that there are at least three primary cleavages of RHDV polyprotein which yield four mature products (Fig. 5). One of these products, p80, which represents the polyprotein amino terminus, contained domains homologous to those in the picornavirus 2C-like helicase protein, whereas p73 showed significant similarities to the picornavirus 3C and 3D motifs (22). Our expression studies, with either cell-free translation systems or *E. coli*, showed that there is no proteolytic processing between 3C proteinase and 3D RNA polymerase and that this cleavage is very slow or is not due to 3C proteinase. In our study, cleavage of RHDV 3CD product was not required for the activity of the proteinase. However, it has been reported that in poliovirus, the cleavage of 3CD to 3Cpro and 3Dpol, although very slow, is a prerequisite for RNA replication (15).

From our data, it can be concluded that the product immediately 5′ of the p73 (3CD) protein corresponded to p43. This protein has a low degree of homology with other caliciviruses, i.e., 28% identity with FCV and 22% identity with Norwalk virus by using the Bestfit program of the University of Wisconsin package (12). Despite this low degree of sequence homology, there are well-conserved motifs between RHDV, FCV (8), Norwalk virus (16), and human astrovirus ORF1a (33). Alignment analysis carried out by Neill (24) between the picornavirus 3A and 3B regions and the FCV 40-kDa region which lies between 2C and 3C revealed no significant homology. Whether this region of the calicivirus polyprotein harbors the VPg function is not clear, considering that it is larger than the 15-kDa polypeptide described for RHDV VPg (23). Nevertheless, it cannot be ruled out that the in vitro approach used does not provide the adequate conditions for a further cleavage of this polypeptide.

Two ways have been proposed to produce the capsid protein of the RHDV. A subgenomic origin for the virion protein has been suggested (27), considering the fact that the sequence reported starts at the Glu residue located at position 1767 of RHDV polyprotein. As the amino acid sequence was obtained from a peptide derived from a CNBr treatment of the VP60 capsid protein, the true amino terminus of the protein should correspond to the Met residue located at position 1766 of RHDV polyprotein. On the other hand, the cleavage in the carboxy terminus of p73 yields a p60 polypeptide detected by antibodies against the capsid protein, and this provides an alternative source of VP60 by means of proteolytic processing of the genome-encoded polyprotein (22). Taking into account the experimental amino acid sequence obtained (27), this p60 protein either is not present in the virion or is present at very low levels. The significance and the function of this VP60-like polypeptide remain to be established.

The data reported in this paper concerning the processing of RHDV polyprotein are consistent with those obtained by Carter et al. (10) for FCV. Using serum from immunized cats, they immunoprecipitated proteins of 80, 78, and 40 kDa, as well as the capsid protein, from the FCV-infected cells. In our hands, the hyperimmune sera against RHDV and sera from "contact" rabbits, which survived after an RHDV infection, contained only antibodies able to precipitate VP60. The observed extremely low immunogenicity of the viral nonstructural proteins and the lack of a cell system able to replicate RHDV were factors in the design of epitope tags to identify RHDV gene products.

To our knowledge, this is the first report elucidating the specificity of a calicivirus 3C-like proteinase as well as the proteolytic processing of a calicivirus polyprotein.

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