Synthesis in Vitro of Rabbit Hemorrhagic Disease Virus Subgenomic RNA by Internal Initiation on (−)Sense Genomic RNA

MAPPING OF A SUBGENOMIC PROMOTER*  

Rabbit hemorrhagic disease virus (RHDV), a positive-strand RNA virus, is the type species of the Lagovirus within the Caliciviridae. In addition to the genomic RNA of 7.4 kb, a subgenomic mRNA (sgRNA) of 2.2 kb, which is identical in sequence to the 3′-one-third of the genomic RNA, is also synthesized in RHDV-infected cells. Numerous RNA viruses make sgRNA for expression of their 3′-proximal genes. A relevant mechanism for viral gene expression is the regulation of sgRNA synthesis by specific promoter elements. In this study, we have investigated in vitro the sgRNA synthesis mechanism using recombinant RHDV RNA-dependent RNA polymerase produced in baculovirus-infected insect cells and synthetic RHDV (−)RNA templates of different lengths containing regions located upstream of the subgenomic start site. We report evidences supporting that the sgRNA of RHDV is synthesized in vitro by internal initiation (subgenomic promoter) on (−)RNA templates of genomic length. The deletion mapping of the subgenomic promoter starting from minus-strand genomic length RNA showed that a sequence of 50 nucleotides upstream of the sgRNA start site (+1) is sufficient for full subgenomic promoter activity in an in vitro assay using recombinant RHDV RNA-dependent RNA polymerase. This study reports the first description of a subgenomic promoter in a member of the Caliciviridae.

Positive-strand RNA viruses are defined by the translatability of their genomic RNAs (gRNA). For some of them, such as picornaviruses and flaviviruses, the gRNA is the only viral mRNA in the infected cells. Nevertheless, there are many other viruses in which one or more subgenomic mRNAs (sgRNAs) are also synthesized. SgRNA of positive-strand viruses are 3′-terminal and identical to the gRNA for most of their length but have deletions at the 5′ ends (with respect to gRNA) to bring their 5′ ends close to the start codon of downstream (on genomic RNA) ORFs. In these cases, some of the viral genes are translated from the sgRNA species, thus creating the potential for independently regulating the levels of viral proteins in infected cells. SgRNA synthesis has been more studied in plant viruses than in animal viruses, probably because a greater percentage of all of the plant viruses make sgRNA and also because their smaller genomes and highly efficient replication make them more amenable to studies on RNA replication mechanisms, especially using cell-free extracts. Several basic mechanisms for generating subgenomic RNAs have been defined (1). The most widely recognized model is internal initiation on longer-than-subgenomic-length (−)strand templates in which the RNA-dependent RNA polymerase (RdRp) internally initiates (−)strand sgRNA synthesis. Initiation of RNA synthesis occurs at selected sites called promoters, which have been characterized in several plant viruses (2–11) and the alphavirus (12). Most of the characterized viral RNA promoters are located at the 3′ end of the RNA templates, and they often have stem-loop structures or consist of short single-stranded regions with unique primary sequences (13–15). The best characterized cases are the subgenomic promoters for brome mosaic virus and Turnip crinkle virus. The second mechanism is premature termination during (−)strand synthesis from the genomic RNA template, giving a subgenomic-length (−)strand (16). This would then serve as template for end-to-end (−)strand synthesis.

Rabbit hemorrhagic disease virus (RHDV) is a member of the Caliciviridae (17–19). The genome of RHDV consists of a single positive-stranded RNA of 7.4 kb (20) that has a virus-encoded protein, VPg (21), attached covalently to the 5′ end (22) and is polyadenylated at the 3′ end. Viral particles also encapsidate a VPg-linked polyadenylated subgenomic RNA of approximately 2.2 kb (22), which is co-terminal with the 3′ end of the viral genome. Progress on the replication of caliciviruses has been negligible (23) compared with the advances made with the picornaviruses. Because of the lack of a cell culture system for most caliciviruses such as RHDV, recombinant DNA technology has been crucial for the production and characterization of viral proteins. The data obtained from the in vitro translation (21) and Escherichia coli expression studies (24) revealed that the viral RNA is translated into a polyprotein that is subsequently cleaved to give rise to mature structural and nonstructural proteins. The extensive sequence similarities between the RdRp of picornaviruses (3Dpol) and the RHDV polyprotein cleavage product p58 (21, 25) suggested that this polypeptide might have a similar role in RHDV genome replication. Recently, the structure of the recombinant RdRp from RHDV produced in E. coli has been determined by x-ray crystallography showing a close structural similarity to the RdRps from poliovirus and hepatitis C virus (26). Previous studies of the purified recombinant RHDV enzyme also showed RdRp activity acting on synthetic RHDV positive subgenomic RNA in the presence or absence of an oligo(U) primer (27–28). Template-
sized products were synthesized in the oligo(U)-primed reactions as described for EMCV 3D<sup>pol</sup> (29), whereas in the absence of added primer, RNA products up to twice the length of the template were made, suggesting that the newly made negative-strand RNA was covalently linked to the plus-strand RNA template (27). Despite the structural and functional similarities found between the *Picornaeviridae* and *Caliciviridae*, the synthesis of a sgRNA in the *Caliciviridae* denotes a significant discrepancy concerning their gene expression strategies.

In this work, we have intended the study of RHDV sgRNA formation using synthetic (–) strand RNA was covalently linked to the plus-strand RNA template were made, suggesting that the newly made negative-strand RNA (gRNA) or negative sense genomic RNA (gRNA) were made using primer pairs RHDV10/RHDV14 and RHDV1/ RHDV9, respectively (Table 1). The PCR used to produce positive (sgRNA) or negative sense subgenomic RNA (sgRNA) were made using RHDV/ RHDV9 and RHDV3/RHDV14 primer pairs, respectively (Table 1). The PCR used to produce a series of 5′-truncated (–)strands (Fig. 4, RNA h and RNA i) were made using primer pairs RHDV11/14 and RHDV12/14, and the series of 3′-truncated (–)gRNAs (Fig. 4, RNA a, RNA b, RNA c, RNA d, RNA e, and RNA g) were made using RHDV29/ RHDV39, RHDV49/ RHDV59, RHDV69/ RHDV79, and RHDV89/ Table 1). The RNA transcripts were made using the large scale RNA production system Ribomax (Promega). The resulting positive and negative transcripts were analyzed by electrophoresis on denaturing agarose gels, and their concentrations were measured spectrophotometrically.

**Gel Electrophoresis and Protein Concentration**—RNA was analyzed on 1% formaldehyde-agarose gels (31). SDS-PAGE was performed as described elsewhere (32). The protein concentration was measured using the Bio-Rad protein assay.

**Preparation of Oligo(U)—**Oligo(U) was made by alkali hydrolysis of poly(U) as described previously (33). The size of the resulting oligo(U) was investigated by end-labeling with [γ-<sup>32</sup>P]ATP (ICN) and electrophoresis on denaturing 6% polyacrylamide gels.

**Enzymatic Assays—**In vitro RHDV polymerase assays were performed as described previously (27) with some modifications. The reactions were carried out in a final volume of 50 μl containing appropriate concentrations of 3D<sup>pol</sup> (1–10 μl of baculovirus-infected H5 cell-free extract), 50 mM HEPES, pH 8.0, 10 μM ATP, 10 μM CTP, 10 μM GTP, 5 μM UTP, 4 mM dithiothreitol, 3 mM magnesium acetate, 6 μM zinc chloride, 50 μM of ribonucleoside inhibitor (Promega), 25 μM of [α<sup>32</sup>P]rUTP, and 14–28 μM of the appropriate synthetic RNA template or poly(A) template in the absence (−) or presence (+) of oligo(U) primer. After incubation at 30 °C for 60 min, the reaction mixtures were phenol-chloroform-extracted and ethanol-precipitated in the presence of 0.3 M sodium acetate (pH 6.0) and 20 μg of carrier tRNA. The sediments were dissolved in electrophoresis sample buffer, loaded onto 1.2% agarose gels, and analyzed at 60–70 V. The gels were then dried, and the 32P-labeled RNA was detected by autoradiography.

**RESULTS**

**Production of Recombinant RHDV 3D<sup>pol</sup> Using Baculovirus-infected Insect Cells**—Previous functional (27, 28) and structural (26) studies on RHDV 3D<sup>pol</sup> used a recombinant protein produced in *E. coli*, which nonetheless showed enzymatic activity on homopolymeric and heteropolymeric RNA templates. In this work, we have selected insect cells as the host system to produce recombinant 3D<sup>pol</sup>. For this purpose, a suitable vector was made (see “Experimental Procedures”) after PCR amplification of the previously identified 3D<sup>pol</sup>-coding region. The resulting gene construct included an added ATG initiator codon.

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**Table I**

| Name | Position in RHDV | Primer sequence<sup>a</sup> | Polarity<sup>b</sup> |
|------|------------------|----------------------------|-------------------|
| RHDV 1 1–32 | GTGAAATTTAGCGGGCTATGTTGCGCTTAC | + |
| RHDV 2 3763–3780 | ACATCAAACTTCTTTCTGC | + |
| RHDV 3 5140–5154 | GTCGCCGCACAGGCC | + |
| RHDV 4 5196–5216 | GACGCGCTAACGAGCTTAG | + |
| RHDV 5 5246–5286 | TACTGCGAGCATGAAGAGACT | + |
| RHDV 6 5266–5282 | GATGGTAGTATGAGGGAAACCGGC | + |
| RHDV 7 5296–5310 | GACATCCCCCATGTTGCCATCG | + |
| RHDV 8 5778–5779 | GTATCCCCCATGTTGCCATCG | + |
| RHDV 9 7437–7441 | ACCCGCGGCTCAATACGACTCTATA GAGGGG(T)23ATAGCTTACTTTAAC | + |
| RHDV 10 1–21 | CCGCGGCGCTTAATACGACTCTATA GAGGAATATGCGGCTTATG | + |
| RHDV 11 5039–5061 | GCGCCGCGGCCGCG | + |
| RHDV 12 5196–5216 | GACACGAGCATGAAGAGACT | + |
| RHDV 13 5266–5282 | GACATCCCCCATGTTGCCATCG | + |
| RHDV 14 7437–7441 | (T)23ATAGCTTACTTTAAC | + |

<sup>a</sup> The sequences are written from 5′ to 3′. Nucleotide residues not related to RHDV AST/89 sequence (GenBank<sup>TM</sup> accession number Z49271) are underlined. The T7 promoter sequence included is indicated in boldface.

<sup>b</sup> Polarity refers to homology (+) or complementarity (−) to RHDV genomic RNA.

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*Internal Initiation at a Calicivirus Subgenomic Promoter*

**In Vitro Synthesis of RHDV RNAs**—Positive and negative sense gRNA, sgRNA, and a series of 3′-truncated (−)gRNAs or 5′-truncated (+)gRNAs were obtained (Fig. 4) by transcription in *vivito* of purified PCR fragments to which a modified T7 promoter was added using the appropriate primers (Table I). The PCR fragments to produce full-length positive (gRNA) or negative sense genomic RNA (gRNA) were made using primer pairs RHDV10/RHDV14 and RHDV1/RHDV9, respectively (Table 1). The PCR used to produce positive (sgRNA) or negative sense subgenomic RNA (sgRNA) were made using RHDV7/ RHDV9 and RHDV3/RHDV14 primer pairs, respectively (Table 1). The PCR used to produce a series of 5′-truncated (−)gRNAs (Fig. 4, RNA h and RNA i) were made using primer pairs RHDV11/14 and RHDV12/14, and the series of 3′-truncated (−)gRNAs (Fig. 4, RNA a, RNA b, RNA c, RNA d, RNA e, and RNA g) were made using RHDV29/ RHDV39, RHDV49/ RHDV59, RHDV69/ RHDV79, and RHDV89/ Table 1). The RNA transcripts were made using the large scale RNA production system Ribomax (Promega). The resulting positive and negative transcripts were analyzed by electrophoresis on denaturing agarose gels, and their concentrations were measured spectrophotometrically.
be mentioned that activity levels were not proportional to the presence of an added oligo(U) primer, an omission of which resulted in an almost complete loss of activity (Fig. 2). It should be noted that poly(U) polymerase activity was detectable in infected cells 1 day post-infection and increased with time up to day 3 post-infection (Fig. 2). This observation was consistent with an inhibitory component in the cell extract (data not shown). For this reason, in subsequent analyses using hetero-polymeric RNA templates, 1–10 μl of H5 extracts will be used.

RdRp Activity on Heteropolymeric RNA Templates—To investigate whether the recombinant enzyme produced in insect cells exhibited RdRp activity on heteropolymeric RNA templates, synthetic RHDV genomic and subgenomic RNAs of positive and negative polarity were made by in vitro transcription of purified PCR fragments to which a modified T7 minimal promoter sequence was added (see “Experimental Procedures”). The synthetic RHDV genomic and subgenomic RNAs differed only in that the synthetic transcripts lacked a covalently linked VPg protein at their 5′ ends and in that the 3′ terminus had a shorter poly(A) tail of only 23 residues. The synthetic RHDV genomic and subgenomic RNAs of negative polarity were complementary to the authentic viral gRNA and sgRNA and differed only in that the synthetic transcripts contained the sequence GGGGCGG included in the oligo-(U) primer used to make the amplicons (Table 1) in order to allow efficient T7 transcription initiation. The size and quantity of the RNA templates used and the labeled RNA products synthesized in the RdRp assays were analyzed by formaldehyde-agarose gel electrophoresis.

The RHDV RdRp produced in baculovirus-infected H5 cells was able to use (+)- and (−)-single-stranded RNA templates in the absence of added primers. As has been described previously using the bacterially produced RHDV RdRp (27), the major labeled products in the presence of either gRNA or sgRNA of (+) polarity were of twice the length (~15 and 4.4 kb, respectively) of the input template (Fig. 3A, lanes 1–2). When (−)gRNA was used as template (Fig. 3B, lane 1), the major synthesized product was of the size of sgRNA (2.2 kb). In the presence of (−)gRNA as the template, the products, if any, were poorly labeled indicating low polymerization efficiency, being the major observed band of twice the length (4.4 kb) of the input template (Fig. 3B, lane 2). These data suggested that the subgenomic RNA of RHDV was synthesized in vitro by internal initiation of replication on a (−)RNA template of genomic length.

Deletion Mapping of a Putative Subgenomic Promoter—To further investigate initiation of sgRNA synthesis on (−)gRNA...
templates and aiming to map the minimum sequence required for full function of the putative internal promoter, a series of deletion mutants were constructed in which the 3’-terminal sequence of (−)sgRNA was progressively deleted (Fig. 4, transcript j), or including 100 (transcripts a–c), reaching (transcript f), or surpassing (transcripts g) the transcriptional start residue (+1) for sgRNA. A series of (+)transcripts identical in nucleotide sequence to the RHDV sgRNA (Fig. 4, transcript j) or including 100 (transcript i) or 257 (transcript h) 5’-additional genomic residues were also made. The truncated RNA templates of positive and negative polarity made by in vitro transcription of appropriate ampiclons were used as templates in the absence of added primers in in vitro RHDV RdRp assays.

The major labeled products obtained using (+)RNA templates h, i, and j (Fig. 4) were of twice the length of the input RNA (Fig. 5B, lanes h–j), suggesting that de novo initiation, premature termination, or template terminal-labeling have not occurred in these in vitro reactions. Moreover, the presence of double-sized products suggested a template-primed synthesis mechanism (15) as described earlier for the RHDV enzyme produced in bacteria (27).

In contrast with the results found using (+)RNA templates, the use of several (−)RNA templates (Fig. 4, RNA a–d) in the RdRp reaction mixtures yielded a common major product of 2.2 kb, which was smaller than the input templates (Fig. 5A, lanes a–d). In these reactions, a second slow-moving band of up to twice the size of the RNA template was also present (Fig. 5A, lanes a–d). These results suggested that under the experimental conditions used RHDV RdRp could concomitantly perform a template-primed synthesis, giving rise to the minor double-sized products, and a de novo synthesis initiation at an internal promoter region located at the same distance to the 5′-end in all four RNA templates (a, b, c, and d), thus producing a common product smaller in size than the input templates (Fig. 5A, lanes a–d). The use of the shorter transcripts e, f, and g, which lacked 20, 50, and 512 residues from the 3′-terminal of RNA d, resulted in very low RdRp activity, and no 2.2-kb products could be observed. These data suggested that for efficient production of the 2.2-kb product (sgRNA) from a (−)RNA template a minimum of 50 nucleotide residues preceding the +1 sgRNA (nucleotide 5296 of the RHDV genome sequence) should be present as deduced from the results obtained using RNA d (Fig. 5A). Shorter 3′ regions yielded RNA templates, which were very inefficient templates in RdRp in vitro reactions. This conclusion was also supported by a Northern blot analysis, indicating that the observed 2.2-kb products made from (−)RNA templates were of positive polarity (data not shown).

**DISCUSSION**

For replication of (+)RNA viruses, the input genome should act both as mRNA for producing the necessary viral proteins and enzymes and as template for the synthesis of negative strand RNAs, which in turn will be copied to produce large amounts of newly made viral genomes to be packaged into virions. From this general scheme, it becomes evident that specific promoter sequences for the viral coded RdRp should be present at the 3′-terminal regions of both full-length plus- and minus-strand RNA templates to produce full-length (−) and (+)gRNAs. Some of these viruses also produce subgenomic RNAs as a prerequisite to allow translation of downstream ORFs. Two general mechanisms and some variations on these basic schemes could be proposed for generating less than full-length RNAs from viral genomes (for review see Ref. 1): (i) internal initiation on a (−)strand copy of the genomic RNA (2) and (ii) premature termination of the (−)strand synthesis from genomic RNA producing a subgenomic (−)RNA, which would then serve as template for (+)gRNA production (16). In further comments, we will refer to the sequences recognized by the RdRps to initiate the synthesis of sgRNAs as subgenomic promoters. Initial studies on subgenomic promoters allowed the mapping of specific sequences in some plant (36) and animal viruses (12). Since then, many subgenomic promoters have been characterized, mainly in plant viruses. The initial
mapping analyses made in brome mosaic virus (36, 37) and the alphavirus, Sindbis virus (12), revealed promoter regions of \(<100\) residues, mostly upstream (in the (+)sense) of the transcription initiation site. Other subgenomic promoters are more diverse and often more complex. They have been mapped in a variety of plant viruses (38–43). At least a portion of each promoter is located immediately upstream of the 5’ end of the resulting sgRNA. However, essential components of some sgRNA promoters were also located downstream of the sgRNA 5’ end, very distantly upstream, or even on a separate RNA molecule.

Rabbit hemorrhagic disease virus is a member of the Calicivirusidae, a family of (+)RNA animal viruses, which also required the synthesis of an abundant sgRNA for production of its major capsid component VP60 (44). This sgRNA has some properties, which closely resemble those of the viral genome. It is specifically packaged into virus particles and is covalently linked to VPg by its 5’ terminus whose nucleotide sequence context is very similar to that found at the genome 5’ end (22). It should be also mentioned that the synthesis of a sgRNA is consistent with the genomic organization of some calicivirus genera (Vesivirus and Norovirus) in which the VP60-coding region constituted an independent ORF2 located immediately downstream of ORF1 (45). Nevertheless, two other genera (Lagovirus and Sapovirus) lack an independent ORF for the major capsid protein whose coding sequences are fused in-frame at the 3’ region of ORF1, giving rise to a polyprotein larger than the one encoded by Vesivirus and Norovirus (45). It has been previously described that a VP60-like polypeptide can be produced by proteolysis of RHDV polyprotein (21), although this product lacked the two most N-terminal residues found in virion-derived VP60 (44). The role of this VP60-like processing product has not been investigated so far. The fact that a sgRNA is made in caliciviruses, irrespective of the translational requirements imposed by their genome organization, pointed out to the need of a separate control of gRNA and sgRNA synthesis to achieve a convenient imbalance between the levels of ORF1-derived cleavage products and the major capsid protein VP60, irrespective of the fact that the VP60-coding sequence was or not included in ORF1.

The presence of the 2.2-kb sgRNA into RHDV capsids and the above mentioned genome-like features of this sgRNA (VPg-linked, similar to 5’ sequence and 3’-poly(A) tail), which could resemble the structure of a defective genome, suggested the possibility of an independent replication mechanism for this RNA species.

The lack of a permissive cell culture for RHDV has impeded most of the molecular studies concerning the mechanisms of viral replication, although significant advances have been made in recent years using recombinant purified viral products (26, 27, 46, 47).

In agreement to these previous approaches, we have used in this paper a recombinant 3D polymerase produced using baculovirus-infected insect cells and several synthetic RHDV genomic and less than genomic-length RNAs of plus and minus polarity to investigate the mechanism of RHDV sgRNA synthesis using in vitro assays in the absence of other viral or host cell protein factors.

The possibility that the RHDV (+)sgRNA could “replicate” by a genome-independent mechanism required the existence of (−)sgRNAs in the infected cell, which could act as templates for end-to-end synthesis of the (−)gRNA. Our in vitro results indicated that a premature termination mechanism of the (−)strand synthesis was not involved in the production of less than template-length transcripts considering that the major labeled products observed were larger in size than the input (+)RNA (Fig. 5B). Nevertheless, this observation cannot rule out that such mechanism might occur in a virus-infected cell. As an additional approach to investigate the existence of independent gRNA and sgRNA replication mechanisms, we have studied the nucleotide sequence similarity or divergence between both types of RHDV RNAs by direct sequencing after their purification using agarose gel electrophoresis. It should be expected that if an independent replication mechanism exists, a sequence divergence should occur between gRNA and sgRNA as a consequence of the lack of proofreading mechanisms in RNA synthesis. Our data on direct RNA nucleotide sequence indicate 100% conservation between both RNA species (not shown), thus supporting the idea that both were derived from a common RNA template of negative polarity.

The lack of premature termination events from (+)RNAs templates in the in vitro experiments performed in this study indirectly supported the internal initiation mechanism as the putative strategy used by RHDV to produce its sgRNA. More-
Internal Initiation at a Calicivirus Subgenomic Promoter

over, our experimental data using a nested set of (−)RNA templates with common 5′ regions (derived from the genome 3′ end) and extending differing lengths toward the +1 sgRNA start (Fig. 4) also supported that the sgRNA was the result of an internal initiation mechanism on a (−)RNA template. In our studies, all of the synthetic (−)RNA templates extending at least 50 residues beyond the +1 sgRNA start (Fig. 5, lanes a–d) yielded a common major labeled product of the expected size for sgRNA. This minimal internal promoter region could not be reduced by an additional 30-nucleotide deletion (Fig. 5, lane e), which completely abolished sgRNA production. To our knowledge, this study is the first report on the mapping of a subgenomic promoter from a member of the Caliciviridae.

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