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Characterization of the In Vitro Activity of the RNA-Dependent RNA Polymerase Associated with the Ribonucleoproteins of Rice Hoja Blanca Tenuivirus

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An RNA-dependent RNA polymerase (RdRp) activity associated with the ribonucleoproteins of rice hoja blanca tenuivirus (RHBV) was detected and analyzed. Conditions for in vitro RNA synthesis and for coupled RNA synthesis-translation of RHBV were established. In both cases, synthesis of the viral and viral complementary genomic and subgenomic RNA3 and RNA4 were observed, demonstrating that both transcription and replication occurred. Though coupling of RNA synthesis to translation allowed efficient translation of the newly synthesized subgenomic RNAs, studies of the effect of various inhibitors of protein synthesis revealed that RNA synthesis was independent of translation. Primer extension experiments demonstrated that in the presence of capped exogenous RNAs, a stretch of 10 to 16 nonviral nucleotides was added to the 5′ end of a population of newly synthesized viral complementary RNA4. It appears that in addition to RdRp activity, RHBV-associated protein(s) also possessed cap-snatching capacity.

Rice hoja blanca virus (RHBV) is a species of the genus *Tenuivirus*, which also includes rice stripe virus (RSV), the type species, maize stripe virus (MStV), rice grassy stunt virus, and European wheat striate mosaic virus. Tenuiviruses are characterized by fine filaments, 3 to 8 nm wide, that may adopt a circular conformation. Their genomes are composed of four or five single-stranded RNAs. The RNAs are encapsidated by the nucleocapsid (NC) protein forming ribonucleoproteins (RNPs). The 5′ and 3′ ends of the genomic RNAs (gRNAs) are complementary to each other and can base pair to form panhandle structures (reviewed in reference 25).

Only the genome of RSV has been entirely sequenced. RNA1 of RSV is of negative polarity (36) and encodes a putative RNA-dependent RNA polymerase (RdRp). RNA2 to -4 resort to the ambisense coding strategy, i.e., the 5′-proximal region of each strand, viral (v) and viral complementary (vc), contains an open reading frame (ORF) separated by an intergenic region (IR). RSV v-strand RNA2 (vRNA2) encodes a nonstructural (NS) protein of 22.7 kDa (NS2), and viral complementary-strand RNA2 (vcRNA2) codes for a 94-kDa protein (NSvc2) (31). v- and vcRNA3 code for the NS3 protein and NC, respectively (17, 38). RNA4 contains the ORF of NS4 in the v strand and of NSvc4 in the vc strand (16, 39). An RNA5 segment of negative polarity encoding a protein referred to as NS5 has been reported for MStV (14), and a similar-size RNA has been detected in a single isolate of RSV (15).

RHBV causes an important endemic disease in rice cultures in tropical America. Its tetrapartite genome has been characterized (26). The three smallest RNA segments have been sequenced; they are ambisense in genome organization (7, 8, 27). Similarly to RSV, the RHBV v- and vcRNA2 code for the NS2 (23 kDa) and NSvc2 (94 kDa) proteins, the v- and vcRNA3 code for the NS3 (23 kDa) and NC (35 kDa) proteins, and the v- and vcRNA4 code for the NS4 (20 kDa) and NSvc4 (32 kDa) proteins. The mRNAs of RHBV (28), like those of MStV (14) and RSV (29), are synthesized via a cap-snatching mechanism similar to the one first described for influenza virus (reviewed in reference 18).

Another feature of tenuiviruses is the presence of an RdRp associated with the RNPs (2, 24, 32, 33), a property that they share with negative-strand RNA viruses. The RdRps of RSV and rice grassy stunt virus have been reported to produce RNAs in vitro whose sizes correspond to gRNA2 to -4, while the RSV RdRp in addition synthesizes an RNA of the size of gRNA1 (34). However, no clear delineation between transcription activity and replication activity was demonstrated. Using a solubilized preparation of the RSV RdRp, Barbier et al. (2) showed that the enzyme was able to copy a model RNA template of 50 nucleotides comprising the 16 to 17 conserved nucleotides of both 5′ and 3′ ends of the RNAs or a 3′-terminal half molecule of this model template. However, it failed to use virion RNAs as templates.

In this study, the RdRp activity associated with RHBV RNPs was analyzed. It is shown that in vitro, the RHBV RdRp has both transcription and replication activities. These activities can be coupled to translation, but the two events appear to be independent. The RdRp is also able to synthesize a population of RNA4-related molecules containing nonviral nucleotides at their 5′ ends, which probably originated from a cap-snatching mechanism.

MATERIALS AND METHODS

Virus purification. RHBV RNPs were purified by a modification of the method of Morales and Niessen (20). In the first step, the potassium phosphate buffer was 0.1 M instead of 0.2 M and chloroform-carbon tetrachloride was omitted. The cesium sulfate (or chloride) gradient was replaced by a linear sucrose gradient (10 to 40%) prepared in 0.05 M potassium phosphate (pH 7.6), and centrifugation was for 3 h at 40,000 rpm in an SW50.1 rotor. The purified virions were centrifuged at 183,000 × *g* for 90 min. The pellet was resuspended in 0.01 M potassium phosphate (pH 7.6) and could be kept at 4°C for over 2 weeks or at −20°C in 30% glycerol for over a year without significant loss of RdRp activity. The concentration of the virus preparations was estimated as described elsewhere (20).

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TABLE 1. Features of different riboprobes obtained from clones of RNAs 3 and 4 of RHBV

RHBV RNA	Riboprobe[a] (length in nt)	Restriction enzyme and position[b]	Plasmid
RNA3	g3vc (162)	*Ssp*I$_{168}$	R3-1[c]
	3v (615)	*Bam*HI$_{MCS}$	R3-1sc
	g3v (179)	*Tth*111I$_{2174}$	RHBC-8[c]
	3vc (872)	*Bam*HI$_{MCS}$	RHBC-8sc
RNA4	g4vc (160)	*Eco*NI$_{88}$	R4-26sc[d]
	4v (502)	*Eco*NI$_{88}$	R4-26sc[d]
	g4v (159)	*Pvu*II$_{1883}$	RHB4C-341[d]
	4vc (899)	*Eco*RV$_{1942}$	RHB4C-341sc

[a] See also Fig. 2.
[b] Enzyme used for plasmid linearization; numbering is based on that of the vRNAs.
[c] Described in reference 24.
[d] Described in reference 27.

RNA purification. When total RNAs were purified from RHBV RNPs by proteinase K digestion followed by CsCl centrifugation (26), they are referred to as CsCl-purified RHBV RNAs. When they were obtained, after proteinase K treatment, by phenol extraction and ethanol precipitation of the RHBV RNPs, they are referred to as phenol-extracted RNAs.

Preparation of cDNA subclones. cDNAs of CsCl-purified RHBV RNA3 and -4 were cloned into pBluescript II KS+ or SK− (Stratagene) as described previously (24, 27). To avoid nonspecific hybridization signals due to the IR contained in the transcripts of the RHBV cDNA clones, a subcloning step deleting the IR was carried out in three of the clones. Deletions were performed by digestion of plasmids RHB4C-341, R3-1, and RHBC-8 by pairs of restriction enzymes, i.e., *Stu*I-*Pst*I, *Stu*I-*Kpn*I, and *Bgl*II-*Pst*I, respectively. The plasmids were blunt ended by using T4 DNA polymerase (Boehringer Mannheim) and religated with T4 DNA ligase (Boehringer Mannheim) as indicated by the manufacturer. The subclones are designated by the names of their parental plasmids followed by the suffix sc (Table 1). Plasmid R4-26 was previously subcloned to yield R4-26B (27) designated here R4-26sc (Table 1).

Preparation of transcripts and/or riboprobes. Plasmids containing the ORFs of proteins encoded by v- or vcRNA3 or -4 were linearized with appropriate restriction enzymes prior to transcription using T3 or T7 RNA polymerase. Plasmids R3-1 and RHBC-8 (24) were linearized with *Kpn*I in the multiple cloning site (*Kpn*I$_{MCS}$) to yield the NS3 and NC ORFs, respectively. Plasmid R4-26 was linearized with *Xba*I$_{MCS}$ to yield the NS4 ORF, and plasmid RHB4C-341 was linearized with *Bam*HI$_{MCS}$ to yield the NSvc4 ORF. In addition, transcripts of about 245 nucleotides and devoid of any ORF (referred to as SK− RNAs) were obtained by digestion of pBluescript II SK− with *Pvu*II and transcription by T3 RNA polymerase.

The transcripts were synthesized essentially as described by the manufacturer (Stratagene). Transcription reaction mixtures (25 μl) contained 1 μg of linearized DNA, 1× Stratagene transcription buffer (40 mM Tris-HCl [pH 8], 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine), 10 mM dithiothreitol (DTT), 25 U of RNasin (Promega), 1.25 μg of bovine serum albumin (New England Biolabs), 2 mM each nucleoside triphosphate (NTP), and 10 U of T3 or 63 U of T7 RNA polymerase (Stratagene). When the transcripts were labelled to serve as riboprobes, the concentration of CTP was reduced to 0.2 mM and 370 kBq (10 μCi) of [α-^{32}P]CTP (14.8 TBq/mmol [400 Ci/mmol]; Amersham) was added. When the SK− RNA transcript was capped, the total volume was raised to 100 μl, the concentrations of ATP, UTP, and CTP were reduced to 0.5 mM each, that of GTP was reduced to 0.05 mM, and 0.5 mM $m^7G(5')ppp(5')G$ (Pharmacia) was included. After incubation for 1 h at 37°C, the transcribed RNAs were extracted as indicated previously (19).

RNA synthesis and coupled RNA synthesis-translation in vitro. For the sake of clarity, RNA synthesis and coupled RNA synthesis-translation will be referred to as incubation without and with, respectively, nuclease-treated rabbit reticulocyte lysate (designated retic. lysate). The assays were based on the protocol of Vialat and Bouloy (37). The incubation mixtures (10 μl) contained 5 μg of RHBV RNPs, 32 mM HEPES (pH 7.4), 2.5 mM DTT, 4 mM magnesium acetate, 100 mM KCl, 0.6 μg of bovine liver tRNA, 10 U of RNasin, 30 μM each amino acid, 1 mM each NTP (or without NTPs when specified), and, when indicated, 40% lysate. To obtain labelled RNA products, the final concentration of unlabelled CTP was reduced to 0.05 mM and 370 kBq (10 μCi) of [α-^{32}P]CTP was added. In some experiments, 0.1 μg of uncapped or capped SK− RNA was added to the incubation mixture. When indicated, translation inhibitors (10 μM edeine, 350 μM cycloheximide, and 200 μM puromycin) were included separately. In all cases, incubations were performed for 2 h at 32°C. After treatment of the mixtures with 1 mg of proteinase K per ml in 1% sodium dodecyl sulfate (SDS)–5 mM EDTA for 30 min at 56°C and filtration through Sephadex G-50, the RNAs were phenol-chloroform extracted and ethanol precipitated with 10 μg of glycogen (Appligene).

Gel electrophoresis. Analyses of RNAs on a 1.8% agarose gel under native or denaturing glyoxal conditions were performed as described previously (26). After electrophoresis, the gel was transferred overnight by capillarity onto a positively charged nylon membrane (Boehringer Mannheim) before hybridization and/or exposure.

Northern blots. Hybridization was performed essentially as described previously (26). It was carried out with radiolabelled in vitro-transcribed riboprobes ($\sim 5 \times 10^5$ cpm/ml) or with radiolabelled in vitro-transcribed RNAs phenol extracted from an incubation of RHBV RNPs without lysate ($\sim 5 \times 10^5$ cpm/ml) or with lysate ($\sim 10^5$ cpm/ml).

Translation and protein analysis. Two types of translation experiments were carried out: translation coupled with RNA synthesis and classical translation of added RNA templates. Translations coupled with RNA synthesis were performed in the conditions described for coupled RNA synthesis-translation except that the unlabelled methionine and cysteine were replaced by 555 kBq (15 μCi) of a [^{35}S]methionine-cysteine mixture (>37 TBq/mmol [1,000 Ci/mmol]; Amersham). Classical translation experiments were carried out under similar conditions except that the viral RNPs were replaced by RNA templates, either (i) RNAs phenol extracted and ethanol precipitated from incubations of RHBV RNPs without or with NTPs and/or retic. lysate (see above), (ii) 0.5 μg of each RNA transcribed from clones encoding the ORF of v- or vcRNA3 or -4, (iii) 0.7 μg of CsCl-purified RHBV RNAs, or (iv) the phenol-extracted RNAs from 5 μg of RHBV RNPs.

The translation products were analyzed by 0.1% SDS–12.5% polyacrylamide gel electrophoresis (PAGE) as described previously (26) except that after migration, the gel was electrotransferred (22) for 2 h at 350 mV onto a nitrocellulose membrane (0.45 μm; Schleicher & Schuell) before exposure.

Primer extension. Primer extension experiments were performed essentially as described elsewhere (23, 28). The primer 5′-CCUAGAUAUCGACAUGUUU GUUC-3′) annealed to nucleotides 1941 to 1963 of vcRNA4. In a 10-μl mixture, 15 pmol of primer was 5′ end labelled with 2.2 MBq (60 μCi) of [γ-^{32}P]ATP (259 GBq/mmol [7 Ci/mmol]; ICN), using 10 U of T4 polynucleotide kinase (Bethesda Research Laboratories [BRL]) in 1× forward reaction buffer (60 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 330 μM ATP, 15 mM 2-mercaptoethanol; BRL). The radiolabelled primer was coprecipitated with unlabelled RNAs and was phenol extracted and ethanol precipitated from an RNA synthesis incubation of RHBV performed without or with NTPs and/or retic. lysate and/or uncapped or capped SK− RNAs. The pellet was resuspended in 12 μl of H_2O, boiled for 2 min, and placed on ice; 4 μl of 0.8 M NaCl was then added, and the mixture was incubated at 37°C for 2 to 5 min. After addition of avian myeloblastosis virus reverse transcriptase buffer (Promega), 0.5 mM each dNTP, 10 mM DTT, 40 U of RNasin, and 10 U of avian myeloblastosis virus reverse transcriptase (Promega), incubation (50 μl) was carried out for 1 h at 42°C. The extended products were phenol extracted, ethanol precipitated, and loaded onto a 10% polyacrylamide–7 M urea sequencing gel. After migration, the gel was exposed to an X-ray film at −80°C.

RESULTS

RNA synthesis and coupled RNA synthesis-translation. A detailed comparison (not shown) of assay conditions for in vitro RHBV RNA synthesis, coupled or not to translation, indicated that the activity of the RdRp is optimal at 4 mM magnesium acetate. As this coincided with the optimum magnesium ion concentration for in vitro translation, all further in vitro RNA synthesis assays with RHBV RdRp were performed at this concentration.

To analyze the products synthesized by the RHBV RdRp, all incubations were performed in the presence of [α-^{32}P]CTP plus, where indicated, unlabelled NTPs. After phenol extraction, the newly synthesized, radiolabelled RNAs were separated by denaturing agarose gel electrophoresis. In the absence of both unlabelled NTPs and retic. lysate, no RNA synthesis was detected (Fig. 1A, lane 1). In the presence of the unlabelled NTPs, the profiles of the RNAs synthesized by the RdRp were similar irrespective of whether the reaction was carried out without (lane 3) or with (lane 4) retic. lysate, except that the level of RNA synthesis was generally higher without retic. lysate. Six bands were observed (lanes 3 and 4), of which the two slowest-migrating bands of 3.5 and 2.7 kb were discernible only upon long exposures (compare lanes 4 and 4*; see also Fig. 6). Three bands had sizes that correspond to the gRNA 2 (3.5 kb), -3 (2.3 kb), and -4 (2 kb). The band of 2.7 kb

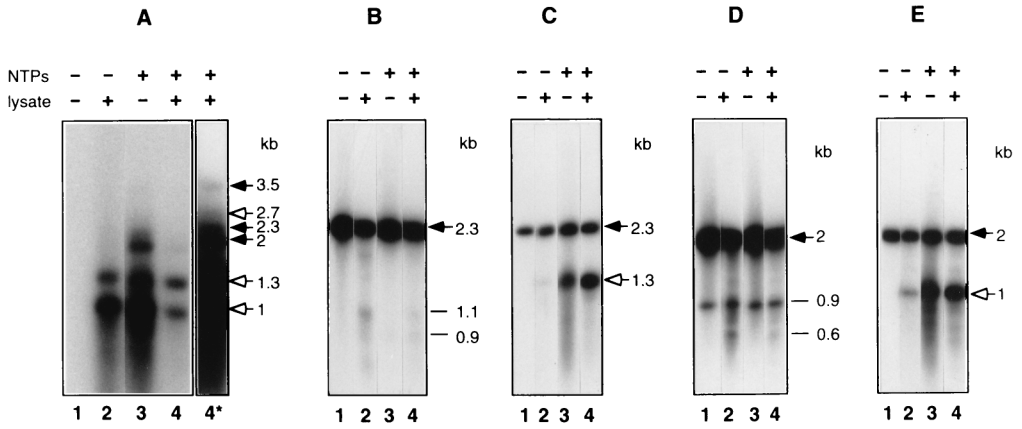

FIG. 1. Analysis of RNAs synthesized in vitro by the RHBV RdRp. After synthesis, extraction, and glyoxal treatment, the RNAs were separated on a native agarose gel, blotted onto a nylon membrane, and autoradiographed. (A) Labelled RNAs detected by autoradiography; (B to E) unlabelled RNAs visualized by hybridization with radiolabelled riboprobes (Fig. 2) specific for vRNA3 (3v; B), vcRNA3 (3vc; C), vRNA4 (4v; D), and vcRNA4 (4vc; E). Reactions were performed without (−) or with (+) unlabelled NTPs and/or retic. lysate. Lane 4*, four-times-longer exposure of lane 4. In all panels, the 0.24- to 9.5-kb RNA ladder (BRL) served to verify the positions of the RHBV RNAs (not shown). The sizes of the RNAs are indicated to the right of each panel. Closed and open arrows correspond to the putative gRNAs and sgRNAs, respectively. sgRNA bands, detectable only by Northern analyses, are indicated by horizontal lines. All lanes of panels B and C were from the same gel, and those of panels D and E were from another gel.

was tentatively assigned to subgenomic vcRNA2 (sg-vcRNA2). The 1.3- and 1-kb bands could correspond to sg-vRNA2 or subgenomic RNA3 (sgRNA3) and -4 of undetermined polarity. RNA synthesis was also observed in incubations without unlabelled NTPs but with retic. lysate (Fig. 1A, lane 2), but high-molecular-weight RNA species were virtually absent, indicating that the free NTPs present in the retic. lysate became limiting in the course of RNA synthesis.

Northern blot analysis. To identify the population of RNAs obtained, Northern blot experiments were performed. Unlabelled RNAs synthesized by the RHBV RdRp were electrophoretically separated as for Fig. 1A and transferred onto a nylon membrane before hybridization with radiolabelled riboprobes which specifically recognize the v- and vcRNA3 and -4. The probes are designated 3v, 3vc, 4v, and 4vc, respectively, based on the RNA segment and the polarity of the strand to which they hybridize (Fig. 2 and Table 1). They detect both g- and sgRNAs. Hybridization of the 3vc probe to the 1.3-kb product (Fig. 1C) and of the 4vc probe to the 1-kb product (Fig. 1E) indicated that these RNAs correspond to sg-vcRNA3 and -4, respectively. These two RNA species appeared only in the presence of retic. lysate or NTPs or both (Fig. 1C and E, lanes 2 to 4), clearly indicating that transcription had occurred. Similarly, products of 1.1 and 0.9 kb could be faintly detected with the 3v probe, indicative of low levels of sg-vcRNA3 synthesis (Fig. 1B, lanes 2 and 4). The intense 1-kb RNA species visible by direct analysis of the radiolabelled RNAs (Fig. 1A, lanes 2 to 4) could correspond to a mixture of sg-vcRNA4 and small amounts of sg-vRNA3.

The 4v probe hybridized to a product of 0.9 kb under the four different assay conditions tested (Fig. 1D). The fact that hybridization was detected in conditions where no RNA synthesis occurs (Fig. 1A, lane 1) demonstrates that sg-vRNA4 was present in the virions, in accordance with previous results (26). It is possible that some 0.9-kb product was synthesized but that its amount was too low to be detected above the level of endogenous RNA. In addition, in the reactions with NTPs or retic. lysate or both, the 4v probe also faintly hybridized to a product of 0.6 kb, suggesting partial synthesis of sg-vRNA4 (Fig. 1D, lanes 2 to 4).

In all conditions, probes 3v and 3vc also hybridized to a 2.3-kb fragment (Fig. 1B and C), indicating that this product corresponds to gRNA3; similarly, the 4v and 4vc probes hybridized to a 2-kb product which corresponds to gRNA4 (Fig. 1D and E). In all cases, the high level of endogenous gRNA3 and -4 of both polarities existing in the RNPs (Fig. 1B to E, lanes 1) largely hindered detection of de novo synthesis of gRNAs.

Due to this interference, it was not possible to determine the possible occurrence of replication. Consequently, a system

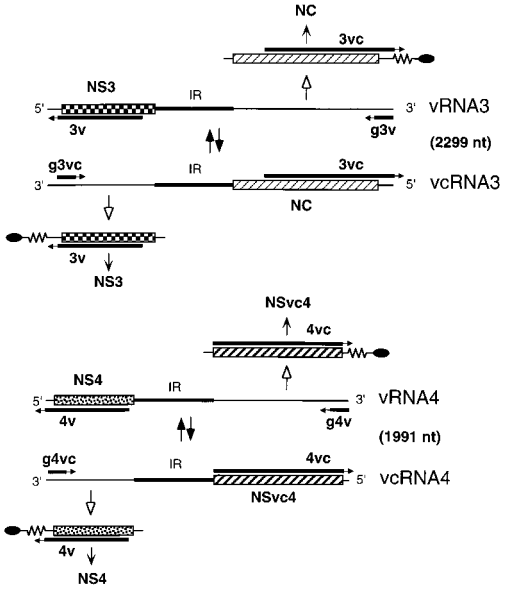

FIG. 2. Schematic scale-size representation of v- and vcRNA3 and -4 of RHBV and of the riboprobes used in the Northern blot experiments. Vertical arrows indicate replication (⇅), transcription (↓), and translation (↓). Horizontal arrows represent the riboprobes and indicate the 5′-3′ polarity. Riboprobes are designated 3 or 4 and v or vc, based on their hybridization target, and are preceded by g when they hybridize only to gRNAs. Boxes represent ORFs; dotted and hatched boxes correspond to ORFs of v- and vcRNAs, respectively. ●, cap structure; -〰, nonviral nucleotides (nt).

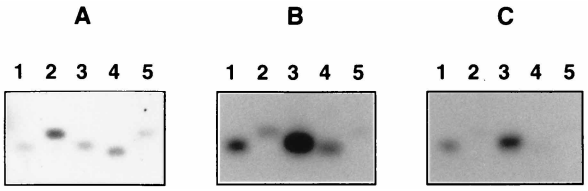

FIG. 3. Analysis of the replication activity of the RHBV RdRp. Unlabelled riboprobes specific to full-length RHBV RNA3 or -4 (Fig. 2) were run on a native agarose gel and blotted onto a membrane; this was followed by autoradiography. (A) Staining with methylene blue; (B and C) hybridization with radiolabelled RNAs obtained from incubations of RHBV RNPs without (B) or with (C) retic. lysate. Lanes: 1, g3v; 2, g3vc; 3, g4v; 4, g4vc; 5, SK− RNA as a negative hybridization control.

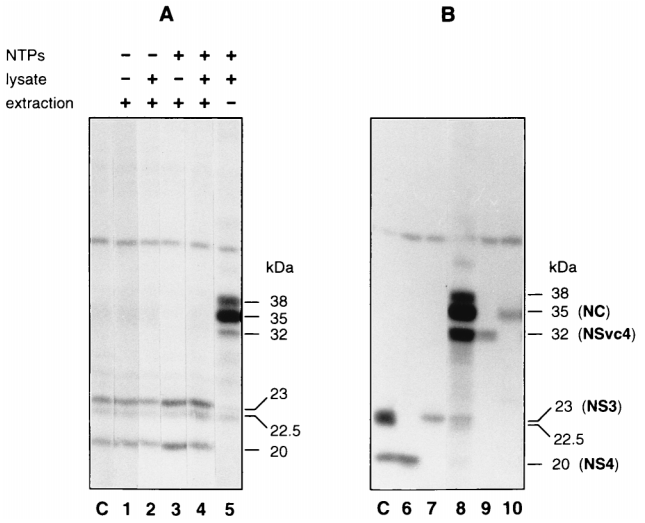

FIG. 4. Autoradiogram of the ^{35}S-labelled translation products of RHBV RNAs produced in transcription-replication reactions and separated by SDS-PAGE. (A) Lanes 1 to 4, the RNAs synthesized without (−) or with (+) NTPs and/or retic. lysate (designated lysate) were first phenol extracted (designated extraction) prior to translation, as indicated above the lanes. Lane 5, translation was coupled with RNA synthesis (i.e., without extraction). Lane C, translation was performed with RNAs phenol extracted from 5 μg of RHBV RNPs as a control. (B) Identification of the translation products by comigration with proteins translated from transcripts of clones containing the various ORFs of RNA3 and -4. Lanes 6, 7, 9, and 10 contain the products corresponding to NS4, NS3, NSvc4, and NC, respectively. Lane C, translation products of 0.7 μg of CsCl-purified RHBV RNAs as a control. Lane 8, coupled RNA synthesis-translation. The sizes of proteins are shown to the right of each panel.

which allowed the detection of newly synthesized gRNAs was established. To this end, small riboprobes 159 to 179 nucleotides in length that could hybridize only to the 3′ end of v- or vcRNA3 or -4, and therefore could detect only gRNAs, were produced. They were designated g3v, g3vc, g4v, and g4vc, respectively, based on their hybridization targets (Fig. 2 and Table 1). Three parallel sets of these unlabelled probes (Fig. 3, lanes 1 to 4) and of uncapped SK− RNA (Fig. 3, lanes 5) which served as a negative control (see Materials and Methods), were run on a native agarose gel and blotted onto a nylon membrane. One part of the membrane was stained with methylene blue to verify transfer efficiency of the RNAs (Fig. 3A), whereas the other parts were hybridized with radiolabelled in vitro-synthesized RNAs phenol extracted from RHBV RNPs incubated without (Fig. 3B) or with (Fig. 3C) retic. lysate. A very low level of hybridization was observed with SK− RNA (Fig. 3B, lane 5) compared to the intense hybridization of the vRNA bands (Fig. 3B, lanes 1 and 3). Previous experiments (not shown) demonstrated that the IR is responsible for this nonspecific signal. Therefore, the cross-hybridization observed here is probably due to nonspecific hybridization of the probe with the IR of the radiolabelled RHBV RNAs. In the presence, and in particular in the absence, of retic. lysate, v- and vcRNA3 (Fig. 1B and C, lanes 1 and 2) and -4 (Fig. 1B and C, lanes 3 and 4) were detected, the vRNAs forming the most prominent bands. The predominance of the vRNA bands over the vcRNA bands was not the result of a larger amount of the corresponding unlabelled probes blotted onto the membrane, as reflected by the stained bands in Fig. 3A. Thus, under these conditions, replication occurred and synthesis of the vRNA strands predominated.

Translation products of the in vitro-synthesized RNA species. In parallel to the analysis of the RNAs synthesized by the RdRp, the nature of the proteins produced by these RNAs was also investigated by SDS-PAGE. As controls, RNAs phenol extracted from 5 μg of RNPs (Fig. 4A, lane C) or 0.7 μg of CsCl-purified RHBV RNAs (Fig. 4B, lane C) were translated in vitro, using a [^{35}S]methionine-cysteine mixture, and the products analyzed (proteins of 23, 22.5, and 20 kDa) were detected. In Fig. 4B, the 23- and 20-kDa proteins were identified as NS3 (lane 7) and NS4 (lane 6), respectively, by comigration with the proteins obtained from transcripts containing the ORFs of vRNA3 and -4, respectively. The 22.5-kDa product could correspond to the NS2 protein encoded by vRNA2 (8, 26).

In all incubation conditions tested (Fig. 4A, lanes 1 to 4), the in vitro-synthesized RNAs first phenol extracted and subsequently translated produced the same translation products as RNAs directly phenol extracted from RHBV RNPs (Fig. 4A, lane C). Since in these conditions RNA synthesis has occurred (Fig. 1A) yet the level of the proteins synthesized has not increased (Fig. 4A), it appears that the level of RNAs newly synthesized during transcription-replication is too low to induce detectable increase of protein synthesis compared to the level of protein synthesis triggered by the endogenous preexisting RNAs (Fig. 4A, lane C).

Analysis of the translation products synthesized during coupled RNA synthesis-translation revealed high levels of 38-, 35-, and 32-kDa proteins and barely discernible levels of the NS3, 22.5-kDa, and NS4 proteins (Fig. 4A, lane 5; Fig. 4B, lane 8). The 35- and 32-kDa products were identified as NC (Fig. 4B, lane 10) and NSvc4 (Fig. 4B, lane 9), respectively, by comigration with proteins obtained from transcripts bearing the corresponding ORFs. The NC and NS4 proteins were also identified by immunoprecipitation with antisera raised against these proteins (not shown). In RSV, the calculated size of NSvc2 based on the nucleotide sequence is 94 kDa (31), but when RNA containing the NSvc2 ORF was translated in vitro, only products of 37, 33, and 22 kDa were detected (12). By analogy, the 38-kDa protein of RHBV could correspond to the 37-kDa protein of RSV. RHBV proteins that could correspond to the two other smaller proteins produced from RSV vcRNA2 are not visible; if they are synthesized, they could be masked by the other proteins encoded by vcRNA3 or -4, or they could be unstable. On the other hand, analysis of the transcription-replication products of RHBV produced an RNA fragment of 2.7 kb which could correspond to sg-vcRNA2 (Fig. 1A). Since two of the three most intense proteins (NC and NSvc4) produced in these conditions derived from expression of vc strands of RHBV, the presence of a putative NSvc2 is compatible with the results of RNA synthesis.

Analysis of the proteins obtained directly from coupled RNA synthesis-translation (Fig. 4A, lane 5) or from a similar

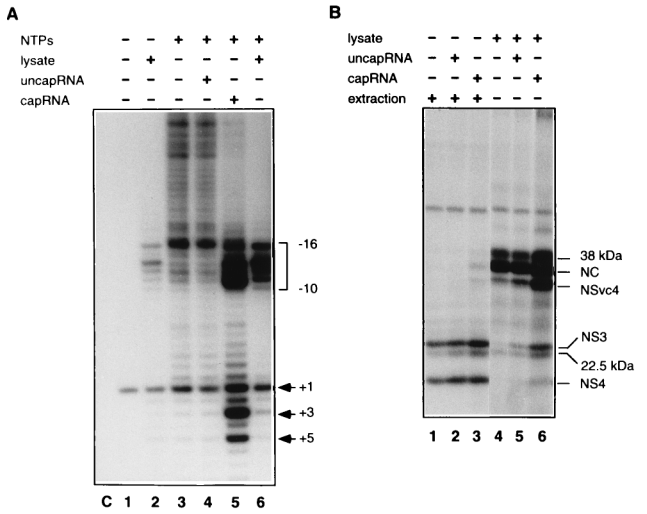

FIG. 5. Analysis by primer extension (A) and by in vitro translation (B) of the RHBV RNAs synthesized in the absence or presence of retic. lysate or SK− RNA. The autoradiograms are presented. (A) Sequencing gel. After extraction, the 5′ ends of the RNAs newly synthesized in incubations without (−) or with (+) unlabelled NTPs and/or retic. lysate (designated lysate) and/or exogenous uncapped (uncapRNA) or capped (capRNA) SK− RNAs as indicated above the lanes were analyzed by primer extension for vcRNA4. Lane C, capped SK− RNA as a control. The sequence of a clone containing the 5′ end of RHBV vcRNA4 served as a ladder (not shown). Primer extensions to positions +5, +3, +1, −10, and −16 are indicated. (B) SDS-polyacrylamide gel. Translations in the presence of a [^{35}S]methionine-cysteine mixture were performed on RNAs phenol extracted (designated extraction; lanes 1 to 3) from incubations without retic. lysate (lane 1) or without retic. lysate and in the presence of uncapped (lane 2) or capped (lane 3) SK− RNA. Translations coupled to RNA synthesis (i.e., without extraction; lanes 4 to 6) were carried out in the absence (lane 4) or presence of uncapped (lane 5) or capped (lane 6) SK− RNA. Other details are as for Fig. 4.

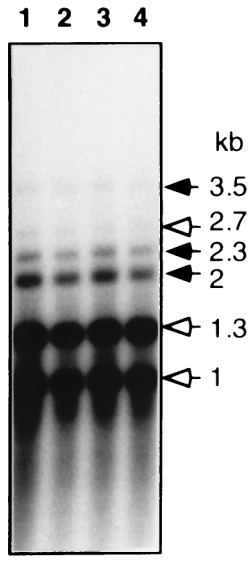

FIG. 6. Autoradiogram of an agarose gel of radiolabelled and glyoxal-treated RNAs synthesized by the RHBV RdRp in incubations containing retic. lysate, in the absence or presence of different protein synthesis inhibitors. Lanes: 1, no inhibitor; 2, with cycloheximide; 3, with puromycin; 4, with edeine. Closed and open arrows represent the putative gRNAs and sgRNAs, respectively. Other details are as for Fig. 1.

incubation but in which the RNAs were extracted prior to translation (Fig. 4A, lane 4) showed opposite results. Thus, it appeared that in the coupled reaction, selective translation of vcRNAs had occurred.

Primer extension analysis of the in vitro-produced RNA species. Previous studies have shown that a cap-snatching mechanism is used to synthesize RHBV mRNAs (28). To determine whether the in vitro conditions of RNA synthesis were adequate for cap snatching, the RNA content of incubations without or with retic. lysate were phenol extracted and analyzed for vcRNA4 by primer extension. Capped SK− RNA composed of a nonviral sequence and devoid of ORFs served as the exogenous source of capped RNAs. No extension was observed with capped SK− RNA alone (Fig. 5A, lane C). A unique extended band was observed at nucleotide +1 when incubation was performed with neither NTPs nor retic. lysate (Fig. 5A, lane 1). By convention, +1 is the position of the 5′ end of the viral RNA, +2, +3, etc., are downstream, and −1, −2, etc., are upstream. Extension to −10 to −16 by nonviral nucleotides was observed when incubation was performed in the presence of retic. lysate (Fig. 5A, lanes 2 and 6). When capped SK− RNA was included in the incubation without (Fig. 5A, lane 5) or with (not shown) retic. lysate, the extended bands were more intense. In the former case, additional intense bands were visible at positions +3 and +5; the possible origin of these bands is presented in Discussion. The −10 to −16 extensions observed are similar to the ones reported for RHBV mRNAs, indicating that the RNAs produced here could be capped. Incubations performed without retic. lysate (Fig. 5A, lane 3) or without retic. lysate but with uncapped SK− RNAs (Fig. 5A, lane 4), conditions in which no extended bands were expected, reproducibly yielded a background of bands in addition to the band at +1. This point will be developed in Discussion.

To further test possible capping of the RNAs synthesized in incubations in the presence or absence of retic. lysate, translation experiments were performed (Fig. 5B) parallel to the primer extension experiments presented in Fig. 5A. In the incubations of RHBV without retic. lysate, RNAs were phenol extracted before being translated (Fig. 5B, lanes 1 to 3). In the incubations with retic. lysate, RNA synthesis was coupled to translation (Fig. 5B, lanes 4 to 6). The profile of proteins synthesized was unchanged whether or not uncapped SK− RNA had been added to the incubations without (Fig. 5B, lanes 1 and 2) or with (Fig. 5B, lanes 4 and 5) retic. lysate. On the contrary, when capped SK− RNA was added to the incubations with retic. lysate, the translation level increased (Fig. 5B; compare lanes 4 and 6). Similarly, in the absence of retic. lysate but with capped SK− RNA, the proteins of the v strands were slightly more abundant and the proteins of the vc strands became visible (Fig. 5B, lanes 1 and 3). Therefore, in the presence of capped exogenous RNA, the newly synthesized RNAs could be capped and thus more efficiently translated.

Effects of translation inhibitors on RNA synthesis. To determine whether the RNA synthesis activity of RHBV RdRp depends on protein synthesis, inhibitors of translation were included during RdRp-dependent RNA synthesis without or with retic. lysate, in the presence of [α-^{32}P]CTP and the unlabelled NTPs. Neither cycloheximide, puromycin, nor edeine affected RNA synthesis irrespective of whether retic. lysate was added (Fig. 6) or not (data not shown). It was verified that each of these inhibitors blocked protein synthesis by more than 95%, using RHBV RNA as the template (not shown). Consequently, in vitro RHBV RNA synthesis is independent of translation.

DISCUSSION

The in vitro study of the RdRp activity associated with RHBV RNPs shows that this enzyme synthesized RNAs as large as 3.5 kb. Two types of activities have been demonstrated: transcription and replication. The transcription activity of the RdRp shows de novo synthesis of sg-vcRNA3 and -4 and possibly of sg-vcRNA2. Synthesis of sg-vRNA3 and -4 also occurs but at very low levels. The difference observed between the levels of synthesis of sg-vcRNAs and sg-vRNAs is probably related to the quantity of RNA templates available: g-vRNAs are 100-fold more abundant than g-vcRNAs (26).

The RdRp replication activity was demonstrated for RNA3 and -4. In spite of the abundance of g-vRNAs in the virions, the RdRp seems to favor the g-vcRNAs as templates to synthesize the g-vRNAs (Fig. 3B and C). This probably reflects the situation in vivo, where the g-vRNAs are more actively synthesized and are the more abundant strands in RNPs.

The RdRp appears to have different affinities for the templates depending on whether it is to produce gRNAs or sgRNAs. The more abundantly synthesized sgRNAs are the sg-vcRNAs, whereas the more abundantly synthesized gRNAs are the g-vRNAs. Some undefined factor(s), different promoters on the v- or vcRNAs, the presence of exogenous capped RNAs that may direct the RdRp toward transcription of mRNAs, and/or secondary structures probably influence differential synthesis of sg- or gRNAs by the RdRp.

No synthesis of an RNA whose size could correspond to RHBV RNA1 (9.8 kb) was observed. Toriyama and Watanabe (34) have shown that the RSV RdRp synthesizes molecules that can hybridize to RSV RNA1. Since this segment is the least abundant in RNPs, it is possible that the level of RHBV RNA1 synthesis is too low for detection.

The total level of RNA synthesized is somewhat lower in the presence of retic. lysate than in its absence, but the profile of RNAs obtained is similar. The difference observed could result from a less active RdRp due to interference of components of the retic. lysate with the RdRp or from loss of RNAs during phenol extraction in spite of all of the precautions taken.

When RNA synthesis was coupled to translation, the results were completely different from those obtained with RNAs synthesized in the presence of retic. lysate but were first phenol extracted and then translated. The possibility cannot be excluded that phenol extraction, which removes the nucleocapsid and the RdRp, has an effect on in vitro translation under these experimental conditions. It is also conceivable that in the coupled reaction, the genomic vRNAs, even if they are in much higher amounts, are encapsidated and consequently inaccessible for translation. The newly synthesized vcRNAs would probably not be encapsidated and would virtually be the only available RNAs for translation. On the other hand, after phenol extraction, the RNAs would be "naked." In this case, the most abundant RNAs, i.e., the vRNAs, would be translated.

Another activity of the RdRp is associated with cap snatching. When capped SK− RNA was added during coupled RNA synthesis-translation, the level of translation was noticeably higher (Fig. 5B), although only a slight increase in the total level of RNAs synthesized was detectable (not shown). In parallel, when RNA synthesis was performed in the presence of capped SK− RNA and the RNAs were extracted prior to translation, the vcRNA-encoded proteins could be faintly detected. This result demonstrates that a modification such as capping could have occurred at the level of RNA synthesis but was perceptible only at the translation level. Moreover, the RNAs obtained in the presence of retic. lysate and/or capped SK− RNA possessed 10 to 16 additional nucleotides at their 5′ ends, as did mRNAs produced in RHBV-infected plants (28). Consequently, in the presence of capped SK− RNA and/or retic. lysate, cap snatching possibly occurs and the sgRNAs obtained are probably capped mRNAs.

The primer extension experiments also indicate that in the presence of capped SK− RNA, initiation of RNA4 synthesis is stimulated (Fig. 5A) and the RdRp starts polymerizing preferably at position +3. Positions +1 and +5 are second choices. In other conditions, position +1 is the preferred choice. Analysis of the 5′ ends of capped sg-vRNA4 of MStV (14) and RSV (29) showed that a part of the mRNAs lack the virus-encoded A residue at position +1. Similar results were also observed for the mRNAs of snowshoe hare (5), Germiston (6), and Uukuniemi (30) viruses. The mRNAs of Hantaan virus in some cases lack three nucleotides (11). The unexpected initiation at position +3 or +5 might reflect a natural but maybe less favored position for initiation of RNA4 synthesis. It is also possible that the presence of different primers, provided as capped SK− RNA or from the retic. lysate, induces a different behavior of the RHBV RdRp, as has been reported for the RdRp of influenza virus (10). In this latter case, initiation depends on the primer provided. Another possibility is that preferential initiation at positions +3 and +5 is due to the in vitro conditions used, as such initiation was not observed after analysis of the 5′ termini of the mRNAs of RHBV, MStV, and RSV extracted from infected plants (14, 24, 28, 29).

Parallel to these results, the primer extension performed on RNAs extracted from an incubation without retic. lysate, or without retic. lysate but with uncapped SK− RNA, showed numerous aspecific extended bands. The salt concentration (0.2 M NaCl) of the annealing step of the primer extension reaction might play an important role in the possible folding of the RNAs. Toriyama et al. (35) have observed the progressive appearance of RSV particles in the forms of stiff rods or closed circular particles with increasing salt concentrations from 0.1 to 1 M NaCl. In these conditions, it is possible that a population of g-vcRNA4 adopts a panhandle structure, which would explain the numerous extended bands obtained.

Addition of capped SK− RNA to the coupled RNA synthesis-translation reaction increases the translation level twofold. On the other hand, the presence of additional capped SK− RNA seems unnecessary for gRNA synthesis, which appears to be primer independent. Perhaps one of the consequences of capping the RNAs is to allow better translation of the RNAs concerned, primarily the vcRNAs, early in infection. This would then reflect a regulation mechanism of expression. Other possible functions of capping, such as differential recognition of the template depending on the primers available, as suggested for influenza virus (10), remain to be examined.

Coupling of RNA synthesis to translation allows efficient translation of the nascent RNAs, but the two steps are independent, as demonstrated by the use of translation inhibitors. Clearly, the translation machinery is not required for RNA synthesis in this in vitro system. Moreover, the RHBV RdRp appears to synthesize mRNAs efficiently in the presence of capped SK− RNA. These results differ from those for closely related bunyaviruses such as Akabane (21), Bunyamwera (1), La Crosse (3, 4), and Germiston (37) viruses, which appear to require elements of the retic. lysate to synthesize functional mRNAs as also full-length RNAs.

The sizes of sg-vcRNA3 and -4, whether capped or not, are approximately the same and correspond to transcription termination within the IR. Analysis of the newly synthesized sg-vRNA3 and -4 revealed a few discrete bands, while for sg-vcRNA3 and -4, only one homogeneous band was observed (Fig. 1), suggesting that synthesis of the sg-vcRNAs terminates

at a strong termination signal, as opposed to what is observed for the sg-vRNAs. It is conceivable that the structure and role of the IR are modulated depending on the polarity of the strand. The IRs of ambisense viruses have been suggested to adopt a hairpin structure which would play an important role in transcription termination (9). vRNA4 can adopt such a structure (27), but vRNA3 cannot (7). It is possible that another secondary structure and/or signal participates in transcription termination of vRNA3. The IR might also play a role in the switching from transcription to replication or vice versa. Whatever the case, the IR appears to be determinant for termination of transcription.

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REFERENCES

1. **Abraham, G., and A. K. Pattnaik.** 1983. Early RNA synthesis in Bunyamwera virus-infected cells. J. Gen. Virol. **64:**1277–1290.
2. **Barbier, P., M. Takahashi, I. Nakamura, S. Toriyama, and A. Ishihama.** 1992. Solubilization and promoter analysis of RNA polymerase from rice stripe virus. J. Virol. **66:**6171–6174.
3. **Bellocq, C., and D. Kolakofsky.** 1987. Translational requirement for La Crosse virus S-mRNA synthesis: a possible mechanism. J. Virol. **61:**3960–3967.
4. **Bellocq, C., R. Raju, J. Patterson, and D. Kolakofsky.** 1987. Translational requirement of La Crosse virus S-mRNA synthesis: in vitro studies. J. Virol. **61:**87–95.
5. **Bishop, D. H. L., M. E. Gay, and Y. Matsuoko.** 1983. Nonviral heterogeneous sequences are present at the 5′ ends of one species of snowshoe hare bunyavirus S complementary RNA. Nucleic Acids Res. **11:**6409–6418.
6. **Bouloy, M., N. Pardigon, P. Vialat, S. Gerbaud, and M. Girard.** 1990. Characterization of the 5′ and 3′ ends of viral messenger RNAs isolated from BHK21 cells infected with Germiston virus (Bunyavirus). Virology **175:**50–58.
7. **de Miranda, J., M. Hernandez, R. Hull, and A. M. Espinoza.** 1994. Sequence analysis of rice hoja blanca virus RNA 3. J. Gen. Virol. **75:**2127–2132.
8. **de Miranda, J. R., M. Muñoz, R. Wu, R. Hull, and A. M. Espinoza.** 1996. Sequence of rice hoja blanca tenuivirus RNA-2. Virus Genes **12:**231–237.
9. **Emery, V. C., and D. H. L. Bishop.** 1987. Characterization of Punta Toro S mRNA species and identification of an inverted complementary sequence in the intergenic region of Punta Toro phlebovirus ambisense S RNA that is involved in mRNA transcription termination. Virology **156:**1–11.
10. **Galarza, J. M., Q. Peng, L. Shi, and D. F. Summers.** 1996. Influenza A virus RNA-dependent RNA polymerase: analysis of RNA synthesis in vitro. J. Virol. **70:**2360–2368.
11. **Garcin, D., M. Lezzi, M. Dobbs, R. M. Elliott, C. Schmaljohn, C. Y. Kang, and D. Kolakofsky.** 1995. The 5′ ends of Hantaan virus (*Bunyaviridae*) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. J. Virol. **69:**5754–5762.
12. **Hamamatsu, C., S. Toriyama, T. Toyoda, and A. Ishihama.** 1993. Ambisense coding strategy of the rice stripe virus genome: *in vitro* translation studies. J. Gen. Virol. **74:**1125–1131.
13. **Huiet, L., J. H. Tsai, and B. W. Falk.** 1993. Maize stripe virus RNA5 is of negative polarity and encodes a highly basic protein. J. Gen. Virol. **74:**549–554.
14. **Huiet, L., P. A. Feldstein, J. H. Tsai, and B. W. Falk.** 1993. The maize stripe virus major noncapsid protein messenger RNA transcripts contain heterogeneous leader sequences at their 5′ termini. Virology **197:**808–812.
15. **Ishikawa, K., T. Omura, and H. Hibino.** 1989. Morphological characteristics of rice stripe virus. J. Gen. Virol. **70:**3465–3468.
16. **Kakutani, T., Y. Hayano, T. Hayashi, and Y. Minobe.** 1990. Ambisense segment 4 of rice stripe virus: possible evolutionary relationship with phleboviruses and uukuviruses (Bunyaviridae). J. Gen. Virol. **71:**1427–1432.
17. **Kakutani, T., Y. Hayano, T. Hayashi, and Y. Minobe.** 1991. Ambisense segment 3 of rice stripe virus: the first instance of a virus containing two ambisense segments. J. Gen. Virol. **72:**465–468.
18. **Krug, R. M., F. V. Alonso-Caplen, I. Julkunen, and M. G. Katze.** 1989. Expression and replication of the influenza virus genome, p. 89–142. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York, N.Y.
19. **Kujawa, A. B., G. Drugeon, D. Hulanicka, and A.-L. Haenni.** 1993. Structural requirements for efficient translational frameshifting in the synthesis of the putative viral RNA-dependent RNA polymerase of potato leafroll virus. Nucleic Acids Res. **21:**2165–2171.
20. **Morales, F. J., and A. I. Niessen.** 1983. Association of spiral filamentous viruslike particles with rice hoja blanca. Phytopathology **73:**971–974.
21. **Pattnaik, A. K., and G. Abraham.** 1983. Identification of four complementary RNA species in Akabane virus-infected cells. J. Virol. **47:**452–462.
22. **Quéméneur, E., and F. Simonnet.** 1995. Increased sensitivity of autoradiography and fluorography by membrane blotting. BioTechniques **18:**100–103.
23. **Raju, R., L. Raju, D. Hacker, D. Garcin, R. Compans, and D. Kolakofsky.** 1990. Nontemplated bases at the 5′ ends of Tacaribe virus mRNAs. Virology **174:**53–59.
24. **Ramirez, B.-C.** 1993. Exploration de la biologie moléculaire du 'rice hoja blanca virus'. Ph.D. thesis. University of Paris 7, Paris, France.
25. **Ramirez, B.-C., and A.-L. Haenni.** 1994. Molecular biology of tenuiviruses, a remarkable group of plant viruses. J. Gen. Virol. **75:**467–475.
26. **Ramirez, B.-C., G. Macaya, L. A. Calvert, and A.-L. Haenni.** 1992. Rice hoja blanca virus genome characterization and expression *in vitro*. J. Gen. Virol. **73:**1457–1464.
27. **Ramirez, B.-C., I. Lozano, L.-M. Constantino, A.-L. Haenni, and L. A. Calvert.** 1993. Complete nucleotide sequence and coding strategy of rice hoja blanca virus RNA4. J. Gen. Virol. **74:**2463–2468.
28. **Ramirez, B.-C., D. Garcin, L. A. Calvert, D. Kolakofsky, and A.-L. Haenni.** 1995. Capped nonviral sequences at the 5′ end of the mRNAs of rice hoja blanca virus RNA4. J. Virol. **69:**1951–1954.
29. **Shimizu, T., S. Toriyama, M. Takahashi, K. Akutsu, and K. Yoneyama.** 1996. Non-viral sequences at the 5′ termini of mRNAs derived from virus-sense and virus-complementary sequences of the ambisense RNA segments of rice stripe tenuivirus. J. Gen. Virol. **77:**541–546.
30. **Simons, J. F., and R. F. Pettersson.** 1991. Host-derived 5′ ends and overlapping complementary 3′ ends of the two mRNAs transcribed from the ambisense S segment of uukuniemi virus. J. Virol. **65:**4741–4748.
31. **Takahashi, M., S. Toriyama, C. Hamamatsu, and A. Ishihama.** 1993. Nucleotide sequence and possible ambisense coding strategy of rice stripe virus RNA segment 2. J. Gen. Virol. **74:**769–773.
32. **Toriyama, S.** 1986. An RNA-dependent RNA polymerase associated with the filamentous nucleoproteins of rice stripe virus. J. Gen. Virol. **67:**1247–1255.
33. **Toriyama, S.** 1987. Ribonucleic acid polymerase activity in filamentous nucleoproteins of rice grassy stunt virus. J. Gen. Virol. **68:**925–929.
34. **Toriyama, S., and Y. Watanabe.** 1989. Characterization of single- and double-stranded RNAs in particles of rice stripe virus. J. Gen. Virol. **70:**505–511.
35. **Toriyama, S., Y. Suzuki, Y. Goto, and M. Kojima.** 1994. Effects of salts on RNA polymerase activity and conformation of filamentous nucleoproteins of rice stripe virus. Ann. Phytopathol. Soc. Jpn. **60:**555–562.
36. **Toriyama, S., M. Takahashi, Y. Sano, T. Shimizu, and A. Ishihama.** 1994. Nucleotide sequence of RNA 1, the largest genomic segment of rice stripe virus, the prototype of the tenuiviruses. J. Gen. Virol. **75:**3569–3579.
37. **Vialat, P., and M. Bouloy.** 1992. Germiston virus transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. J. Virol. **66:**685–693.
38. **Zhu, Y., T. Hayakawa, S. Toriyama, and M. Takahashi.** 1991. Complete nucleotide sequence of RNA 3 of rice stripe virus: an ambisense coding strategy. J. Gen. Virol. **72:**763–767.
39. **Zhu, Y., T. Hayakawa, and S. Toriyama.** 1992. Complete nucleotide sequence of RNA 4 of rice stripe virus isolate T, and comparison with another isolate and with maize stripe virus. J. Gen. Virol. **73:**1309–1312.