Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5019–5023, July 1986
Biochemistry

Detection and sequence of plus-strand leader RNA of sonchus yellow net virus, a plant rhabdovirus

(negative-strand RNA virus/viral RNA transcripts)

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Communicated by Myron K. Brakke, March 18, 1986

ABSTRACT **Tobacco infected with the plant rhabdovirus sonchus yellow net virus (SYNV) contains short, 139- to 144-nucleotide (nt) transcripts complementary to the 3′ terminus of the negative-strand genomic RNA. These transcripts are similar to the leader RNAs associated with several animal rhabdovirus infections in that they are encoded by the same region of the genome, but the SYNV transcripts are nearly 3 times longer than the animal rhabdovirus leader RNAs. The SYNV leader RNAs differ markedly in sequence from the leader RNAs associated with strains of vesicular stomatitis virus and rabies virus, although the first 30 nt of all three transcripts are rich in adenylate residues. The nucleotide sequence determined directly from SYNV RNA and from recombinant DNA clones derived from SYNV RNA reveals a possible initiation site for transcription of the N-protein mRNA that is located 147 nt from the 3′ end of genomic RNA. The sequence (UUGU) at this site is complementary to the first 4 nt of the N-protein mRNAs of animal rhabdoviruses. In SYNV, the first AUG codon in the putative N-protein mRNA is located 57 nt downstream (at positions 203–205 in the viral genome) and is followed by an open reading frame for the remainder of the 1020 nt determined in these experiments.**

Sonchus yellow net virus (SYNV) is a negative-stranded plant rhabdovirus that has several properties in common with animal rhabdoviruses. For example, purified virions are enveloped, have a bacilliform structure, and contain a nonsegmented RNA genome with an estimated length of 13,000 nucleotides (nt) (1). As in other rhabdoviruses, polyadenylylated RNA species complementary to >95% of the genomic RNA are synthesized during infection (2). In addition, the pattern of polypeptides resolved by polyacrylamide gel electrophoresis of dissociated SYNV particles and the locations of the polypeptides in the virion (3) fit the nomenclature proposed for other rhabdoviruses (4). The four most abundant SYNV proteins are a surface glycoprotein, G (M_r 77,000); a nucleocapsid protein, N (M_r 64,000); and two proteins designated M-1 (M_r 45,000) and M-2 (M_r 39,000). Purified preparations of SYNV always contain at least one minor protein of high molecular weight (M_r 200,000), and frequently, two or three high molecular weight polypeptides are present in low abundance (3). These high molecular weight polypeptides become highly labeled when undissociated SYNV particles are iodinated with ^{125}I, suggesting that they are located on the surface of the virion. In contrast, the high molecular weight proteins (L proteins) of vesicular stomatitis virus (VSV) and other rhabdoviruses, essential for *in vitro* RNA-directed RNA polymerase activity, are located within the viral envelope (5). Thus, the L proteins detected in SYNV preparations do not appear to be similar to those in VSV and other rhabdoviruses. Despite the lack of direct evidence for an internal L protein or for endogenous polymerase activity associated with purified virus preparations, we presume that an L protein is encoded by the SYNV genome, because SYNV-complementary RNAs found in infected tobacco consist of five discrete poly(A)$^+$ RNA species (6). The largest of these complementary RNAs is of sufficient size (6600 nt) to encode an L protein. The four smaller RNAs could serve as mRNAs for the G, N, M-1, and M-2 proteins. SYNV, therefore, appears to be similar to the animal rhabdoviruses in its structure and mode of replication.

Several animal rhabdoviruses are known to initiate transcription by synthesis of short positive-strand leader RNAs that are coterminal with the 3′ ends of their respective genomes (7–11). Because the leader RNAs contain signals for binding N protein (12) and a host transcription factor (8, 13), they are thought to play an important regulatory role during the rhabdovirus infection cycle. The leader RNA of VSV is also thought to regulate host cellular RNA synthesis (14), because experiments have shown that the leader RNA inhibits host transcription by RNA polymerases II and III *in vitro*. In addition, the 3′ end of the VSV genome, encoding the leader RNA, contains a site for viral polymerase entry and initiation (15, 16). Since this region of the genome appears to function in the control of several aspects of viral transcription and replication, structural comparisons of the RNAs of plant and animal rhabdoviruses may provide important information about replication of viral functions. An understanding of how these molecules interact within the cell to influence the metabolism of their respective hosts may also help identify regulatory functions in plant and animal cells.

In this article, we report the presence of small, plus-strand leader RNAs in plants infected with SYNV. Like the leader RNAs associated with animal rhabdovirus infections (7–11), the 5′ ends of the SYNV-associated leader RNAs are encoded at the 3′ end of viral genomic RNA. However, the SYNV leader RNAs are about 3 times longer than, and contain few sequence similarities with, the animal rhabdovirus leader RNAs.

MATERIALS AND METHODS

Virus Growth and RNA Purification. SYNV (American Type Culture Collection PV 263) was propagated (17) and purified as described (1). SYNV RNA was separated from $NaDodSO_4$-disrupted virions by sucrose density gradient centrifugation (1) and the 44S fraction, representing full-length RNA, was recovered by ethanol precipitation. RNA from healthy or SYNV-infected tobacco was isolated and fractionated by oligo(dT)-cellulose chromatography into poly(A)$^+$ and poly(A)$^-$ RNA fractions (2).

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Abbreviations: SYNV, sonchus yellow net virus; VSV, vesicular stomatitis virus; nt, nucleotide(s); u, unit(s).

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RNA Sequencing. Full-length SYNV genomic RNA was labeled at the 3′ end with cytidine 3′,5′-[5′-^{32}P]bisphosphate (pCp), using bacteriophage T4 RNA ligase as described (18). Chemical sequencing of end-labeled RNA was conducted as described by Peattie (19), and enzymatic sequencing (20) was performed with the following specific ribonucleases: T_1 (G-specific), U2 (A-specific), Phy M (A- and U-specific), *Bacillus cereus* (U- and C-specific), and CL3 (C-specific). RNase T_2 (nonspecific) was used to determine the 3′-terminal nucleotide. Dideoxynucleotide sequencing was performed using the synthetic oligonucleotide primer described below and reverse transcriptase (21). All enzymes used in these experiments and those listed below were obtained from Bethesda Research Laboratories unless stated otherwise.

Identification of Leader RNA. Tests to identify leader RNA were conducted as described for VSV (7) by hybridization of 3′-end-labeled SYNV viral RNA with various RNA fractions from SYNV-infected or healthy tobacco. End-labeled viral RNA containing $\approx 10^5$ cpm (0.1 pmol) was added to 15 μg of tobacco RNA in a total hybridization volume of 35 μl and incubated 5 hr at 65°C in 20 mM Tris, pH 7.6/400 mM NaCl. Subsequently, ribonucleases T_1 [50 units (u)/ml], T_2 (500 u/ml), and A (10 μg/ml) were added and the mixture was incubated at 37°C for 45 min. After proteinase K treatment (400 μg/ml for 20 min at 37°C), phenol extraction, and ethanol precipitation, the RNase-resistant duplexes were heat-denatured (3 min at 95°C) in 100 mM Tris borate, pH 8.2/50% (vol/vol) formamide/5 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol. The samples were fractionated by electrophoresis in sequencing gels of various acrylamide concentrations, and end-labeled RNA was detected by autoradiography.

Oligonucleotide Synthesis. An oligodeoxynucleotide complementary to the first 16 nt of the 3′ end of SYNV RNA was synthesized manually with a kit (supplied by Applied Biosystems, Foster City, CA) that used a solid-phase support (22) and dimethoxytritylnucleoside phosphoramidites (23). After synthesis, the 16-mer was fractionated on a 20% polyacrylamide/7 M urea gel and detected with ultraviolet light. The region of the gel containing the 16-mer was excised, and the oligonucleotide was eluted in 0.5 M CH_3COONH_4/1 mM EDTA.

cDNA Cloning. The synthetic oligonucleotide was used to prime reverse transcription of SYNV genomic RNA (24), and the resulting single-stranded cDNA was modified by addition of $\approx$15 deoxycytidylate residues (25). Tailed vector was prepared by digesting the plasmid pUC13 (26) with *Pst* I and adding $\approx$20 deoxyguanidylate residues (27). The tailed vector was digested with *Sma* I and used to prime synthesis of the second strand, essentially as described by Okayama and Berg (28). The cDNA–vector hybrid was circularized by blunt-end ligation and subsequently used to transform the JM83 strain of *Escherichia coli*. Colonies containing plasmids with SYNV-derived inserts were screened by colony hybridizations using the synthetic oligonucleotide and/or SYNV viral RNA as ^{32}P-labeled probes (6).

M13 clones containing SYNV-specific inserts were constructed using *Hin*dIII–*Eco*RI insert fragments of SYNV-recombinant plasmids. The inserts were purified by electrophoresis in 0.7% agarose gels and recovered by electrophoresis onto NA-45 DEAE membranes (Schleicher & Schuell). The ends of the purified fragments were made blunt-ended with the large (Klenow) fragment of DNA polymerase I and ligated into the *Sma* I site of bacteriophage M13, strain mp18 (33). The polarity of each insert was determined by plaque hybridization with SYNV-specific cDNA or ^{32}P-labeled SYNV RNA fragments (6). Dideoxynucleotide sequencing of M13 clones was carried out by a minor modification of described protocols (29).

RESULTS

Determination of the 3′-Terminal Nucleotide Sequence of the SYNV Genome. The sequence at the 3′ end of the genomic RNA of SYNV was determined by using a combination of techniques. Intact SYNV RNA was labeled at its 3′ end with pCp and T4 RNA ligase. Exhaustive digestion with RNase T_2 followed by thin-layer chromatography revealed that the first nucleotide at the 3′ end of the RNA is a uridylate residue (data not shown). The next 30–40 nt were determined by both enzymatic (Fig. 1*a*) and chemical sequencing methods (Fig. 1*b*). The resulting sequence data were used to synthesize an oligodeoxynucleotide (5′ AGAGACAGAAACTCAG 3′) complementary to the 3′-terminal 16 nt of the viral RNA. This synthetic oligonucleotide was used to prime RNA sequencing reactions containing dideoxynucleotides and reverse transcriptase (data not shown).

In order to verify and extend the RNA sequence data, M13 clones containing SYNV-specific inserts were constructed as described in *Materials and Methods*. One of these clones, mGS2, contained an insert of 1020 nt that hybridized to the synthetic primer, indicating that it was derived from the 3′ terminus of the genome. The nucleotide sequence obtained from this clone (data not shown) overlapped with the sequence determined directly from SYNV RNA, enabling us to extend the sequence of the SYNV genome to 1020 nt. The nick-translated probe from this cloned insert hybridized (data not shown) to a putative mRNA thought to encode the SYNV N protein. Previous studies (6) have shown that this mRNA (scRNA 3) is present in the poly(A)$^+$ RNA fraction of SYNV-infected tobacco.

Detection of Leader RNA in SYNV-Infected Tobacco. Solution hybridization techniques similar to those used to detect leader RNAs in cells infected with animal rhabdoviruses (7) were used to identify SYNV-specific leader RNA transcripts among infected tobacco RNAs. Intact SYNV genomic RNA was labeled at the 3′ end and used to probe various RNA fractions of healthy or SYNV-infected tobacco. After completion of hybridization, the RNAs in the mixture were treated with the single-stranded-specific RNases T_1, T_2, and A, and the resulting RNase-resistant duplexes were separated in denaturing polyacrylamide gels. Labeled fragments complementary to leader RNA transcripts were identified by autoradiography (Fig. 2).

Nuclease-resistant fragments resulting from hybridization of labeled genomic RNA with the poly(A)$^+$ RNA from infected tobacco leaves are shown in Fig. 2 (lane A$^+$). The protected sequences are complementary to the 3′ terminus of SYNV genomic RNA and consist of three major fragments terminating at positions 140, 141, and 142. Faint bands at positions 139 and 143 are also evident, but the intensity of these bands, and other minor bands, varied from experiment to experiment depending on the extent of the nuclease reaction (data not shown). The bands at positions 140, 141, and 142 were always present, but the band at position 143 became stronger at lower nuclease concentrations, and a band at position 144 was sometimes present (data not shown). Experiments with unfractionated RNA from infected plants produced a pattern of nuclease-resistant bands (data not shown) similar to that obtained by hybridization with poly(A)$^+$ RNAs. Lane A$^-$ of Fig. 2 shows the result of hybridization of labeled SYNV genomic RNA with the poly(A)$^-$ RNA fraction of SYNV-infected tobacco leaves. The absence of hybridizing RNAs in this fraction indicates that the SYNV leader RNA fractionates almost exclusively with poly(A)$^+$ RNA. We believe this is due to the presence of regions in the leader RNA sequence containing numerous adenylate residues. The hybridizing RNA species are synthesized during SYNV infection, since no nuclease-resistant fragments could be detected in control reactions using RNA

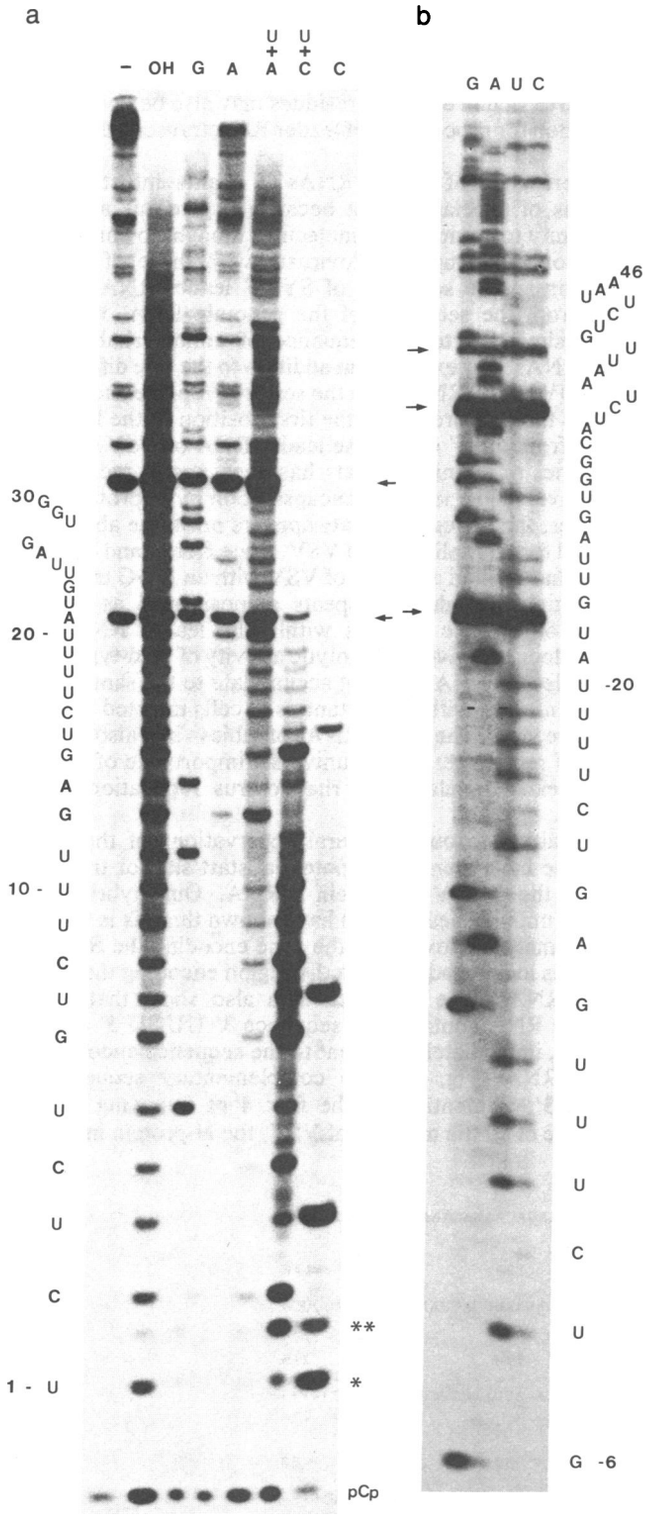

FIG. 1. (*a*) Autoradiogram of a 20% polyacrylamide sequencing gel, showing enzymatic sequencing of 3′-^{32}P end-labeled SYNV RNA. Lanes from left to right contain various amounts of SYNV RNA plus 1 μg of carrier tRNA: −, no enzyme (10,000 cpm); OH, alkali treatment in 0.5 M $NaHCO_3$ (pH 9.0) for 3 min at 100°C (30,000 cpm); G, RNase T_1 (0.4 u) (10,000 cpm); A, RNase U_2 (0.01 u) (10,000 cpm); U+A, RNase Phy M (0.75 u) (20,000 cpm); U+C, RNase *B. cereus* (0.5 u) (20,000 cpm); C, RNase CL3 (2.5 u), (10,000). All enzyme reaction mixtures, including the control without enzyme, were incubated for 15 min as described (20). The ultimate uridine residue (asterisk at right of autoradiogram), which could not be determined by the enzymatic procedure, was identified by exhaustive RNase T_2 digestion and thin-layer chromatography. Note that the sequence positioned along the alkali ladder should be read from the enzymatic reactions that appear one band below the indicated nucleotide. This discrepancy results with 3′-end-labeled RNA because the enzymes cut to the 3′ side of the recognized base to liberate that base from the labeled fragment. The double asterisk indicates a sequence artifact thought to be caused by a cyclic nucleotide. The two arrows indicate nucleotides that could not be resolved by RNase sequencing. These two nucleotides were, however, clearly resolved by chemical sequencing (see *b*) and by dideoxynucleotide sequencing of recombinant DNA clones and of RNA with the specific primer (data not shown). (*b*) Autoradiogram of a 20% polyacrylamide sequencing gel, showing chemical sequencing of 3′-^{32}P end-labeled SYNV RNA. Lanes from left to right contain 20,000 Cerenkov cpm of SYNV RNA. Chemical reactions G, A, U, and C were conducted essentially as described (19). The sequence starts at position 6 from the 3′ end of SYNV RNA. Arrows indicate nucleotides that could not be clearly determined.

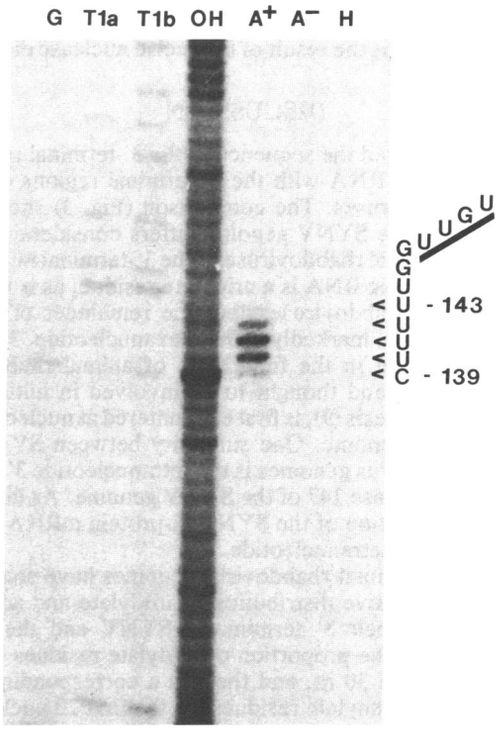

FIG. 2. Detection and sizing of leader RNA(s) of SYNV by electrophoresis in a 5% polyacrylamide gel. Lanes A^+, A^-, and H: RNA from SYNV-infected tobacco leaves, separated into a poly(A)$^+$ and a poly(A)$^-$ RNA fraction, and RNA from healthy tobacco plants, respectively, were hybridized with 3′-^{32}P end-labeled SYNV viral RNA, followed by RNase treatment prior to gel electrophoresis. Lane G: untreated 3′-^{32}P end-labeled SYNV viral RNA (the majority of the label was at the top of the gel). Lanes T1a and T1b: two different RNase T_1 concentrations (0.15 and 0.40 u/μl, respectively) were used for partial digestion of 3′-^{32}P end-labeled SYNV viral RNA. Lane OH: partial alkali degradation of 3′-^{32}P end-labeled SYNV viral RNA. Numbers (143 and 139) at right show the position of individual RNase-resistant bands deduced from the alkali ladder. Letters at right indicate the corresponding genomic sequence. The underlined sequence indicates the putative start for the N-protein mRNA.

isolated from healthy tobacco leaves (Fig. 2, lane H). Partial alkali digests (Fig. 2, lane OH) and partial RNase T_1 digests (Fig. 2, lanes T1a and T1b) of labeled genomic RNA were used to position the termination (or processing) sites of the leader RNA transcripts. Colonno and Banerjee (11) described *in vitro* VSV leader RNAs 47 and 48 nt long but were uncertain whether the 3′ heterogeneity resulted from termination at one of two adjacent residues or from processing by a cleavage mechanism that recognizes two sites. Similarly, in

the case of SYNV it is unclear whether the heterogeneity occurs *in vivo* or is the result of imprecise nuclease digestion.

DISCUSSION

We have compared the sequence of the 3′-terminal region of SYNV genomic RNA with the 3′-terminal regions of three animal rhabdoviruses. The comparison (Fig. 3) shows that this region of the SYNV genome differs considerably from those of the animal rhabdoviruses. The 3′-terminal nucleotide of SYNV genomic RNA is a uridylate residue, as is the case for the other rhabdoviruses, but the remainder of the sequence diverges markedly. The hexanucleotide 3′-UUUGGU 5′, present in the first 20 nt of animal rhabdovirus genomic RNAs and thought to be involved in initiation of VSV RNA synthesis (9), is first encountered at nucleotide 143 of the SYNV genome. One similarity between SYNV and animal rhabdovirus genomes is the tetranucleotide 3′ UUGU 5′ beginning at base 147 of the SYNV genome. As discussed below, transcription of the SYNV N-protein mRNA may be initiated at this tetranucleotide.

SYNV and animal rhabdovirus genomes have some similarity in the relative distribution of uridylate and adenylate residues near their 3′ termini. In SYNV and the animal rhabdoviruses, the proportion of uridylate residues exceeds 50% for the first 30 nt, and there is a correspondingly low proportion of adenylate residues for the first 20 nucleotides. The significance of this distribution is not known, but the uridylate residues may provide an extended structure, free of secondary interactions, that has a high binding affinity for the N protein (12).

We have shown that tobacco infected with SYNV contains short transcripts complementary to the 3′ terminus of the SYNV genome. We presume that these transcripts are coterminal with the 3′ terminus of the genome of SYNV, as is the case with animal rhabdoviruses. However, since we have not directly examined the leader RNAs, we cannot eliminate the remote possibility that the leader RNA contains 5′ residues that extend beyond the 3′ terminus of the genome. Nevertheless, these transcripts are similar to the plus-strand leader RNAs of animal rhabdoviruses (8) both in their location on the genome and in their possible heterogeneity at the 3′ termini, even though they differ considerably in their size and sequence. Termination (or processing) of transcription appears to occur near a uridine-rich region of the SYNV genomic RNA. A similar region of the genomes of VSV and rabies virus rich in uridylate residues may also be involved in termination (or processing) of leader RNA transcription (Fig. 3).

The presence of leader RNAs in plants infected with SYNV is of special interest because it gives us a unique opportunity to compare the molecules thought to control the replication of animal rhabdoviruses with those of a plant rhabdovirus. The sequence of SYNV leader RNA, as deduced from the sequence of the genomic RNA, has only limited similarity to the sequence of animal rhabdovirus leader RNAs. For example, in addition to the size difference, the SYNV leader RNA lacks the series of repeated adenylate residues that are present at the first position of the first five triplets from the 5′ end of the leader RNA of VSV (Fig. 3). This series of adenine repeats has been postulated to be a signal involved in selective encapsidation by N protein (11). However, the series of repeats appears not to be absolutely essential for multiplication of VSV, since Keene and coworkers (31) have found a mutant of VSV with an A→G transition interrupting the adenine repeats at position 4 as well as several other base changes within the leader RNA. The mutant does not have the cytolytic activity of wild-type VSV, and the leader RNA does not accumulate to the same extent in cells infected with the mutant as in cells infected with the wild-type virus. The leader RNAs of rabies virus also lack the series of repeats (8), so the universal importance of specific sequences in regulation of rhabdovirus replication is still obscure.

In addition to our structural observations of the leader RNA, we have identified a potential start site for transcription of the SYNV N-protein mRNA. Our hybridization studies (unpublished results) have shown that, as is the case with animal rhabdoviruses, the gene encoding the SYNV N protein is located adjacent to the region encoding the SYNV leader RNAs. The sequence data also show that SYNV genomic RNA contains the sequence 3′ UUGU 5′ at bases 147–150, immediately adjacent to the sequence encoding the leader RNA (Fig. 3). The complementary sequence, 5′ AACA 3′, is identical to the first 4 nt following the cap structure of all the mRNAs of VSV, the N-protein mRNA of

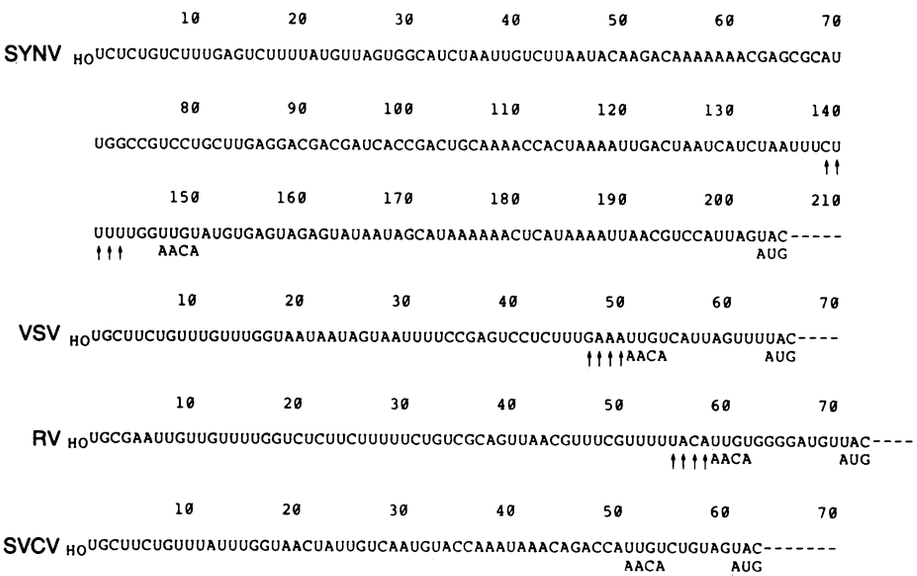

FIG. 3. Comparison of the genomic templates encoding the plus-strand leader RNAs of SYNV and animal rhabdoviruses. The leader-RNA "genes" start at the 3′ termini of their respective genomes, and their respective ends are indicated by arrows. The sequence AACA appears to be common to the 5′ end of the mRNAs of all four rhabdoviruses. Data are from McGeoch *et al.* (30) for VSV (strain Indiana), Kurilla *et al.* (8) for rabies virus (RV), and Roy *et al.* (10) for spring viremia of carp virus (SVCV).

rabies virus, and probably the N-protein mRNA of spring viremia of carp virus (8, 10, 32). Use of synthetic oligonucleotides in primer-extension experiments on poly(A)$^+$ RNAs of SYNV-infected tobacco has indicated that the gene encoding the N-protein mRNA begins at position 147 (unpublished results). These results provide circumstantial evidence that the 5′-terminal 4 nt of plant and animal rhabdovirus N-protein mRNAs have been conserved during evolution. In contrast, the untranslated leader sequence on the N-protein mRNA that precedes the open reading frame appears to differ considerably from the untranslated leader sequences of the N-protein mRNAs of several animal rhabdoviruses (Fig. 3). The most striking difference is the length of the leader sequence of the N-protein mRNA. This sequence is 56 nt long in the SYNV N-protein mRNA, whereas the leader sequence of the N-protein mRNA of animal rhabdoviruses is less than 13 nt long (Fig. 3). In SYNV, the first AUG initiation codon (beginning at position 203) precedes an open reading frame capable of encoding at least 270 amino acid residues.

We thank Dr. Brenda G. Hunter for helpful technical advice, Mr. Howard Laffoon for excellent technical assistance, Mrs. Caroline Logan for photography, and Ms. Cymber Sitchler for typing the manuscript. This work was supported by a grant from the National Science Foundation (DMB-83180070). This is journal paper no. 10,529 of the Purdue University Agricultural Experiment Station.

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