Analysis of Sowthistle Yellow Vein Virus-Specific RNAs in Infected Hosts

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ABSTRACT

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Recombinant plasmids containing sequences derived from the genome of sowthistle yellow vein virus (SYVV) were constructed and used as probes in northern blots to analyze viral-specific RNAs extracted from infected plants and aphids. Recombinant plasmid probes hybridized to a 13-kb, genome-size RNA present in extracts from infected but not uninfected hosts. Four distinct size classes of polyadenylated RNAs were also detected in infected plant extracts. No sequence relatedness was detected between the genomic RNAs of SYVV and another plant rhabdovirus, *Sonchus* yellow net virus (SYNV), in northern blots by using plasmids containing SYVV or SYNV sequences, or DNA complimentary to SYNV RNA as probes. SYVV plasmid probes detected SYVV infection of individual aphids in dot-hybridization assays.

Additional keyword: mRNA.

The genomes of plant rhabdoviruses remain poorly characterized compared with rhabdoviruses that infect animals. Animal rhabdoviruses have genomes composed of a single 11- to 13-kb RNA species of negative sense that serves as a template for transcription of five or six polyadenylated mRNAs in infected cells (1,2,7,12,13,14,21,25). In contrast, *Sonchus* yellow net virus (SYNV) is the only plant rhabdovirus that has previously been examined with respect to genome properties. Although SYNV RNA encodes genes for five virion proteins analogous to those of animal rhabdoviruses (10,15,17,19), only minimal conservation of amino acid sequence between SYNV and animal rhabdovirus proteins has thus far been observed (11,29). Hence, potentially unique and interesting properties of plant rhabdoviruses might be best obtained by examining additional plant rhabdovirus genomes.

We selected the aphid transmissible plant rhabdovirus, sowthistle yellow vein virus (SYVV), for such a comparative analysis (6,20). SYVV consists of enveloped, bacilliform particles (18) containing five proteins (22,27,28) typical of other rhabdoviruses (5).

This report describes the molecular cloning and preliminary characterization of the genome of SYVV, only the second plant rhabdovirus for which cDNA clones have been constructed. We also describe the use of viral-specific recombinant plasmid probes for a comparison of the sequence relatedness of SYNV and SYVV RNAs, and for the analysis of viral-specific RNAs produced in infected plants. In contrast to SYNV, SYVV has been shown to replicate in an aphid vector, *Hyperomyzus lactucae* L. (3,6,20,24), thus providing the opportunity to study a plant rhabdovirus in both invertebrate and plant hosts. Toward this end, we report on the ability to detect SYVV infection of individual aphids by using the cloned plasmid probes.

MATERIALS AND METHODS

Maintenance and purification of virus isolates. SYNV (ATCC PV-263) was propagated and purified as described by Jackson and Christie (15). The SYVV isolate used was collected in Berkeley, CA (20), and propagated in sowthistle, *Sonchus oleraceus* L., that had been inoculated by using viruliferous *H. lactucae* reared under controlled conditions (20). SYVV was purified from sowthistle 14–35 days postinoculation as described for SYNV, except that the step gradient was eliminated from the procedure. The presence of rhabdovirus particles in purified preparations was determined by electron microscopy (24). Virus suspensions were stored frozen at −20 C.

Virion RNA purification. SYNV RNA was extracted from virions and purified by sucrose gradient centrifugation as described by Milner and Jackson (17), except that proteinase K digestion (10 $\mu g \cdot ml^{-1}$, 30 min, 37 C) was included during the disruption of virions. SYVV was disrupted as described for SYNV, and the disrupted suspension was extracted with chloroform. The RNA was subsequently precipitated from the aqueous phase with 70% ethanol, 0.1 M sodium acetate before sucrose gradient centrifugation. Gradient fractions containing 44 S rhabdovirus RNA were pooled, precipitated with ethanol, resuspended in TE (10 mM Tris-Cl, 1 mM ethylenediaminetetraacetic acid [EDTA]), pH 7.6, and stored at −70 C.

Molecular cloning of SYVV RNA. Gradient-enriched SYVV genomic RNA (1 μg) was used as a template for reverse transcription (23) of first-strand complementary DNA (cDNA) utilizing M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in the presence of DNase I digested calf thymus DNA random primers (50 μg). Second-strand DNA was synthesized by the RNase H-DNA Pol I procedure (9), and homopolymeric oligo-dC tails were added to the 3′ termini of both DNA strands by using terminal deoxynucleotidyl transferase (4). C-tailed DNA and *Pst*I-digested, G-tailed plasmid pUC 9 were annealed (16) and transformed into *Escherichia coli* strain DH5-α. Transformants containing recombinant plasmids were selected (26) and screened by colony, dot, and northern hybridizations.

Cellular RNA extractions. Total single-stranded (ss) RNA was isolated from plants or aphids as described by Carrington and Morris (4), except that the volumes were increased to accommodate 100 g of plant material. Total ssRNA from plants was fractionated into polyadenylated (poly A+) and nonpolyadenylated (poly A−) fractions by a single cycle of oligo-dT cellulose chromatography (16). Fractionated RNAs were precipitated with ethanol and stored at −70 C. Individual aphid samples were prepared for dot hybridization by grinding single aphids, previously frozen at −20 C, in 200 μl of extraction buffer (50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, and 0.5% sodium dodecyl sulfate) and processed as described for total RNA samples except for the omission of the ethanol and LiCl precipitations.

Plasmid isolation. Recombinant plasmids were isolated from broth cultures by using a boiling lysis procedure, followed by purification on CsCl-ethidium bromide gradients (16). Insert sizes were determined by electrophoresis of *Pst*I-digested plasmids. Plasmid pSYNV-GL9, containing a 1,750-bp insert of SYNV sequence, was a gift supplied by L. A. Heaton (10).

Electrophoresis. Nondenatured rhabdovirus genomic RNAs or plasmid DNA were electrophoresed in agarose gels by using Tris-phosphate-EDTA buffer (4). RNA samples used for northern transfers were denatured with glyoxyl-formamide and electrophoresed in agarose by using Tris-acetate-EDTA buffer (19).

RNA size standards (Bethesda Research Laboratories) used for northern blots were applied to one lane of each gel and removed for ethidium bromide staining before northern transfer.

Hybridizations. Colony hybridizations were performed as described by Grunstein and Hogness (8). Dot and northern hybridizations, including the preparation of cDNA probes, were conducted as described previously, except that M-MLV reverse transcriptase was used (23). Recombinant plasmid probes were labeled with ^{32}P by nick translation (16). SYVV virion RNA probe was prepared by end labeling partially degraded genomic RNA with polynucleotide kinase (19).

RESULTS

Isolation of rhabdovirus genomic RNA. The yield of SYNV genomic RNA was approximately 30 $\mu g \cdot Kg^{-1}$. The corresponding yield of SYVV genomic RNA was generally less than 1 $\mu g \cdot Kg^{-1}$. RNA extracted from SYVV virions contained a high molecular weight species indistinguishable in size to the 13-kb RNA of SYNV, as determined by electrophoresis under nondenaturing conditions (Fig. 1). Although our protocol involved a sucrose gradient centrifugation step to remove most of the host ribosomal RNA (rRNA) species present in the virion RNA preparations, such contaminants were still evident upon gel analysis even after gradient fractionation. This necessitated devising a cloning strategy to deal with rRNA sequences as a major contamination problem. Comparable SYNV genomic RNA preparations contained much less host RNA contamination.

Screening of recombinant clones. Six-thousand ampicillin-resistant colonies lacking β-galactosidase activity were screened for the presence of SYVV sequences by colony hybridization by using end-labeled SYVV genomic RNA as a probe. Because the RNA used for both cloning and for probe was contaminated with rRNA, the colony hybridization procedure was modified by hybridizing the colony blots for 18 hr with 10 $\mu g \cdot ml^{-1}$ of unlabeled rRNA extracted from uninfected sowthistle before adding the labeled RNA probe. From 1,038 colonies that hybridized to the SYVV RNA probe, 361 were screened for insert size. Eighty-four plasmids were identified that contained cDNA inserts ranging in size from 450 to 1,100 bp.

Size-selected plasmids were screened for the presence of host RNA sequences by dot hybridizations, in which heat-denatured plasmids were spotted onto nitrocellulose and probed with DNA complementary to rRNA isolated from uninfected sowthistle. Only one of 84 size-selected plasmids hybridized to the host-specific cDNA probe. Ten plasmids that failed to hybridize with the host-specific probe were then used as probes in dot hybridization assays of RNA extracted from plants and aphids. All 10 plasmids hybridized only to RNA extracted from infected plants and aphids, but not to RNA extracted from uninfected plants and aphids (Fig. 2). The infection-specific plasmids, designated pSYVVg, were then used for subsequent analyses of viral-specific RNAs.

Detection of genome-size RNA in host extracts. A genome-size 13-kb RNA was detected by northern blot analysis (Fig. 3) of poly A− RNA samples isolated from plants at 7, 10, 13, 16, and 19 days postinoculation. RNA of similar size was also detected in unfractionated ssRNA extracted from infected aphids. RNA extracted from uninfected plants or aphids lacked the 13-kb RNA. The intensity of the genome-size band in poly A− RNA extracted

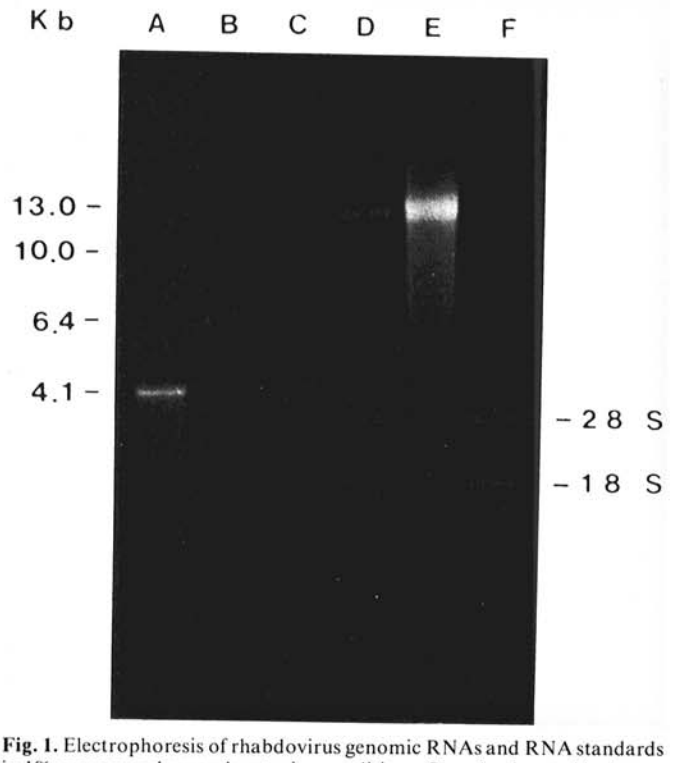

Fig. 1. Electrophoresis of rhabdovirus genomic RNAs and RNA standards in 1% agarose under nondenaturing conditions. Samples denoted by letters are: A, turnip crinkle virus RNA; B, tobacco mosaic virus RNA; C, western equine encephalitis virus RNA; D, sowthistle yellow vein virus RNA; E, *Sonchus* yellow net virus RNA; and F, ribosomal RNAs extracted from uninfected sowthistle. Sizes of viral RNAs are indicated at left in kilobases. Mobility of 28 S and 18 S ribosomal RNAs are indicated on the right.

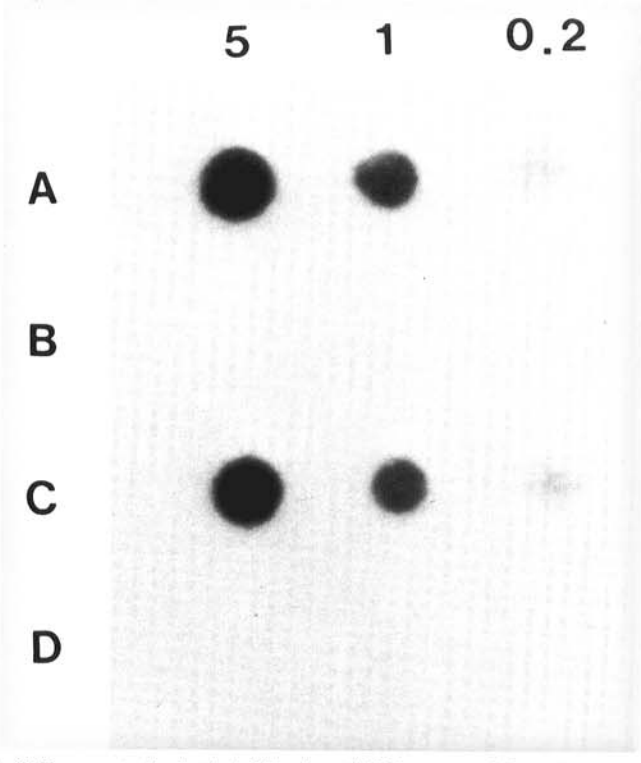

Fig. 2. Representative dot hybridization of RNA extracted from plants and insects probed with pSYVVg 1-23 (specific activity 1×10^7 cpm $\cdot \mu g^{-1}$, 2.5×10^5 cpm $\cdot$ ml^{-1}). Samples applied to nitrocellulose were: Poly A− RNA extracted from SYVV-infected sowthistle (A) or from uninfected sowthistle (B); and total RNA extracted from SYVV-infected aphids (C), or from uninfected aphids (D). Numbers at top indicate μg of RNA applied from each sample.

from plants increased between 7–10 days postinoculation, a time which corresponded with onset of symptoms. The intensity of the hybridizing band subsequently remained constant for up to 19 days postinoculation.

Hybridization analysis of SYVV and SYNV RNAs. To examine the relatedness of SYVV and SYNV RNAs, the poly A− RNA fraction containing genome-size RNA was isolated from infected plants and analyzed by northern hybridization. Plasmids containing SYVV or SYNV sequences, or cDNA synthesized by using SYNV virion RNA as template, were used as probes (Fig. 4). A sufficient amount of high-quality virion RNA was not available, however, for generating a comparable cDNA reagent with SYVV RNA for use in these experiments. When the poly A− RNA samples were probed with the recombinant plasmids, only the homologous RNA was detected under stringent hybridization and washing conditions (hybridized in 50% formamide at 42 C, washed at 65 C in 0.1× saline sodium citrate). Further, no heterologous hybridization was detected in northern blots also probed with SYNV cDNA under the same stringent conditions, as well as relaxed stringency conditions (hybridized in 20% formamide at 37 C, washed in 2× saline sodium citrate at room temperature).

Analysis of SYVV-specific polyadenylated RNAs. Four distinct size classes of SYVV-specific RNAs were detected by northern blot analysis of poly A+ RNAs by using the pSYVVg plasmid probes (Fig. 5). Comparable signals were absent in poly A+ RNA samples extracted from uninfected plants. The pSYVVg plasmids detected different sets of poly A+ RNAs, as outlined in Table 1. Six of the pSYVVg plasmids hybridized to a 6.6-kb RNA, one plasmid hybridized to both the 6.6-kb RNA and also to a 2.2-kb RNA, one plasmid hybridized to both a 1.6- and 1.3-kb RNA, whereas two plasmids hybridized to a 1.3-kb RNA. As expected, all 10 plasmids also hybridized to the genome-size RNA that was present in poly A+ fractions at lower levels relative to that in poly A− fractions. For unknown reasons, hybridization of pSYVVg 2-1 with the genome-size RNA of the poly A+ fractions was consistently weaker than that observed for the other plasmids.

Detection of SYVV RNA in individual aphids. To evaluate the utility of the plasmid probes for monitoring virus infection in the insect host, aphids were reared for 6 days on an SYVV-infected source plant and individually assayed for infection by dot hybridization. Before harvesting, individual aphids were serially transferred to healthy test seedlings for 48-hr inoculation-access periods on days 6, 8, and 10. Control aphids were treated similarly, after a 6-day period on uninfected sowthistle. Individual aphids were harvested after 12 days and stored at −20 C before extraction.

The SYVV probes readily detected viral sequences in RNA extracted from individual aphids given access to SYVV-infected source plants, but failed to hybridize with samples extracted from aphids given access to uninfected plants (Fig. 6). Although all of the aphids (25/25) given access to the SYVV-infected source plant tested positive for SYVV infection by dot hybridization, no

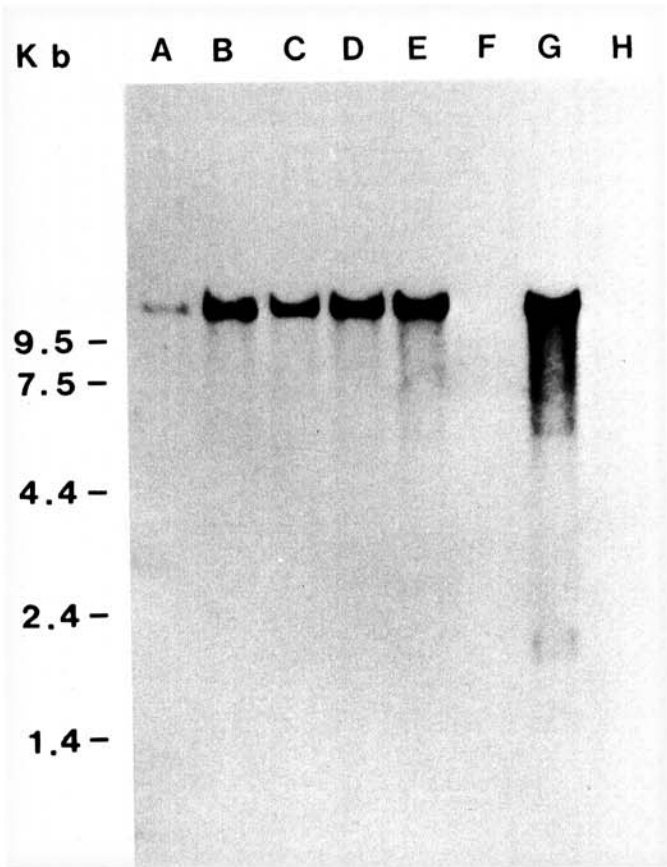

Fig. 3. Northern blot hybridization of RNA (10 μg) extracted from plants and aphids that were probed with a mixture of pSYVVg 8-92 (specific activity 1.3×10^7 cpm · μg^{-1}, 5×10^5 cpm · ml^{-1}) and pSYVVg 14-20 (specific activity 0.8×10^7 cpm · μg^{-1}, 5×10^5 cpm · ml^{-1}). Samples denoted by letters are: poly A− RNA from SYVV-infected sowthistle extracted 7 (A), 10 (B), 13 (C), 16 (D), and 19 (E) days postinoculation, poly A− RNA extracted from uninfected sowthistle (F), and total RNA extracted from SYVV-infected (G) or uninfected (H) aphids. Mobility and sizes (kb) of RNA markers (Bethesda Research Laboratories) are indicated at left.

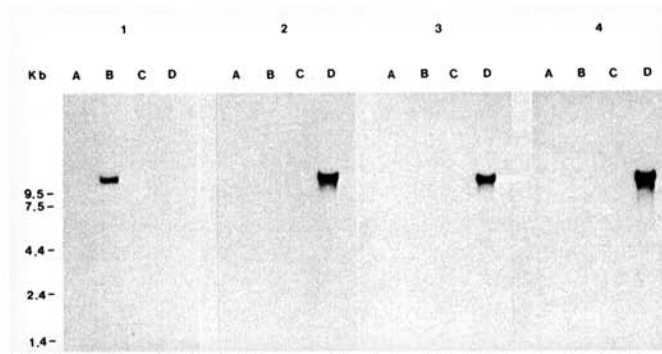

Fig. 4. Northern blot hybridization of poly A− RNA (10 μg) extracted from uninfected (A) or SYVV-infected (B) sowthistle, and uninfected (C) or SYNV-infected *Nicotiana edwardsonii* (D) probed with 5×10^5 cpm · ml^{-1} of (1) pSYVVg 2-59 (specific activity 0.7×10^7 cpm · μg^{-1}); (2) pSYNV-GL9 (specific activity 0.9×10^7 cpm · μg^{-1}); or (3 and 4) cDNA synthesized by using SYNV-genomic RNA template (specific activity 0.4×10^7 cpm · μg^{-1}). Hybridizations were conducted in 50% formamide at 42 C (1–3) or in 20% formamide at 37 C (4), and washed in 0.1× saline sodium citrate (SSC) at 65 C (1–3) or 2× SSC at 24 C (4). Autoradiography was for 22 hr (1), 5 hr (2), or 3 hr (3 and 4).

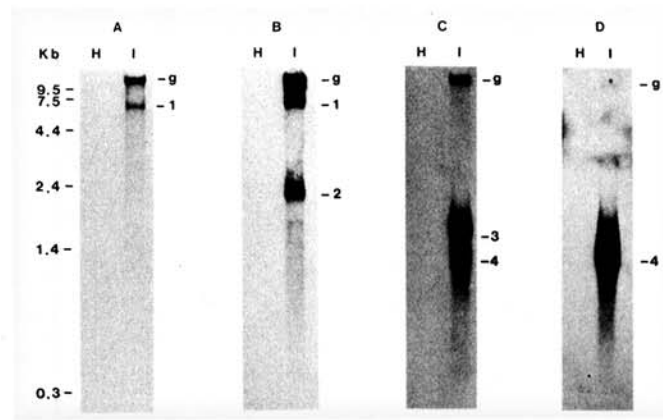

Fig. 5. Northern blot analysis of poly A+ RNA (5 μg) extracted from uninfected sowthistle (H) or SYVV-infected (I) sowthistle 10 days postinoculation. Blots were probed with pSYVVg plasmids 2-65 (A), 8-92 (B), 14-20 (C), or 2-1 (D) at 2.5×10^5 cpm · ml^{-1} (specific activity $5\text{-}10\times10^6$ cpm · μg^{-1}), washed in 2× SSC, 0.1% sodium dodecyl sulfate at room temperature, and autoradiographed for 23 hr (A), 52 hr (B), 37 hr (C), or 28 hr (D). Mobility of RNA size standards in kilobases (Bethesda Research Laboratories) are indicated at left. Mobility of SYVV genomic size RNA (g) and poly A+ RNA size classes (1–4) are indicated at right.

transmissions occurred between 6–8 days after nymph deposition (0 out of 25), only 2 out of 25 (8%) transmitted the virus to assay plants between days 8 and 10, and 13 out of 25 (52%) transmitted the virus between days 10 and 12. A cumulative total of 14 of 25 infected aphids (56%) aphids transmitted at least once during the inoculation test period. No transmissions were recorded for 25 aphids fed on uninfected plants, and samples extracted from 15 of these aphids were also negative when tested by dot hybridization.

DISCUSSION

SYNV remains as the only plant rhabdovirus genome to be characterized at the molecular level, principally because of difficulties in obtaining sufficient quantities of highly purified virions for the majority of plant rhabdoviruses that have been studied. SYNV replicates to high titre in a host suitable for purification, permitting the isolation of genomic RNA containing little host contamination (15). A similar purification protocol applied to SYVV permitted the isolation of genomic RNA preparations that were not satisfactory for making probes because they contained significant amounts of host rRNA, but which were sufficiently enriched in viral sequences to permit cDNA cloning. Construction of cDNA clones containing SYVV sequences ensured that probes used in subsequent hybridization assays were free of host contamination. The infection-specific nature of the signals observed with the pSYVVg plasmids in dot hybridizations of plant and insect RNA provided suggestive evidence that the plasmids contained virus sequences. Northern analyses in which a genome-size RNA was consistently detected only in infected plants and aphids provided additional evidence that the pSYVVg cDNA inserts were derived from SYVV RNA sequence. These results support the contention that the pSYVVg plasmids described here represent legitimate clones of the SYVV genome.

TABLE 1. Summary of size and specificity of pSYVVg cDNA plasmids

Plasmid	Insert size[a]	Poly A+[b]
1-23	820	1
1-24	880	4
1-34	790	1
2-1	900	4
2-15	650	1
2-59	1,100	1
2-65	820	1
8-92	860	1,2
10-13	450	1
14-20	700	3,4

[a] Base pairs determined from *Pst*I digest.
[b] Polyadenylated RNA size class detected in northern blot: 1 = 6.6 kb, 2 = 2.2 kb, 3 = 1.6 kb, and 4 = 1.3 kb.

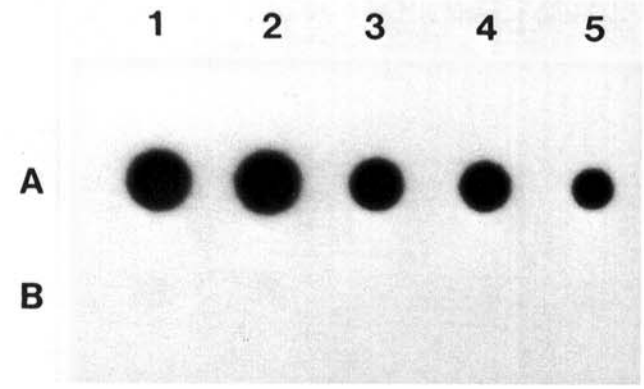

Fig. 6. Dot hybridization of extracts from 12-day-old individual aphids (1–5) reared on SYVV-infected sowthistle for 6 days (A) or uninfected sowthistle (B). Aphid extracts were probed with pSYVVg 1-34 (specific activity 1.2×10^7 cpm · μg^{-1}, 2.5×10^5 cpm · ml^{-1}) in 50% formamide at 42 C, washed in 2× SSC at 37 C, and autoradiography was for 21 hr. Individual aphids represented by extracts A1, A3, and A4 transmitted SYVV to test plants during a 6-day inoculation-access period between days 6 and 12, whereas the remaining aphids did not transmit during the inoculation-access test period.

The lack of detectable hybridization between SYVV and SYNV sequences indicated that the two viruses are distinct. Although only a portion of the SYVV genome was represented by the plasmid probes used, the lack of hybridization between SYVV RNA and SYNV cDNA suggests that the two viruses share no extensive regions of high sequence relatedness. The availability of a cDNA library to SYVV RNA should now facilitate more detailed comparisons between SYVV and other rhabdoviruses isolated from both plant and animal hosts.

The viral-specific polyadenylated RNA species detected in northern blots suggest that SYVV gene expression is similar to other rhabdoviruses. Because these poly A+ RNAs were selectively enriched by oligo-dT cellulose chromatography, they probably are mRNAs transcribed from the negative sense genomic RNA. The genome-size RNA also observed in these northern blots was most likely genomic RNA present in the poly A+ fractions as a contaminant.

The genes encoding SYVV poly A+ RNAs 1 and 2 appear to be adjacent on the genomic RNA because a single plasmid probe (pSYVVg 8-92) hybridized to both RNAs. The genes encoding poly A+ RNAs 3 and 4 also appear to be linked on the genome, as pSYVVg 14-20 hybridized to both RNAs. The apparent size and linkage of the genes encoding poly A+ RNAs 1 and 2 are similar to the size and arrangement of the L and G genes of SYNV (10). Poly A+ RNAs 3 and 4 are also similar in size and linkage to the N and M2 genes of SYNV (10,11,19,29).

Despite these apparent similarities in genome organization, further evidence is required before definitive coding assignments can be made for any of the SYVV poly A+ RNAs. Moreover, additional poly A+ RNAs produced for other viral genes may not have been detected because the plasmid probes used to identify the SYVV poly A+ RNAs represented only a portion of the genome. The use of end-labeled virion RNA as a probe to detect poly A+ RNAs (17,19) would have aided in the detection of all viral encoding poly A+ RNAs. However, the quantity and purity of SYVV virion RNA was not sufficient for this purpose.

The recent identification of a sixth SYNV gene (10) attests to the uncertainty of gene assignments based solely on size estimates. Clearly, a more extensive evaluation of the poly A+ RNAs and more definitive mapping of the clones will be necessary before a tentative map of the SYVV genome will be available.

Dot hybridization analyses of individual aphids given access to SYVV-infected source plants indicated that acquisition of the virus is very efficient. Although only 56% of the aphids given access to SYVV transmitted the virus during the inoculation-access period, the latent period of SYVV in aphids is variable (6). Therefore, it is likely that the inoculation-access test period was not sufficient for all of the infected aphids to become viruliferous. Although these experiments were designed simply to evaluate the utility of the cloned probes for virus detection in insect vectors, these results and the northern analysis in Figure 3 suggest that such specific and sensitive DNA probes may well facilitate studies on the transmission and replication of rhabdoviruses in aphids because the hybridization assays were a reliable and sensitive means of detecting SYVV in individual aphids. This further suggests that recombinant DNA probes may also be useful in epidemiological studies to determine the spatial and temporal dispersion of rhabdoviruses in both aphid and plant host populations.

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