Subgenomic RNAs in Virions of Southern Bean Mosaic Virus

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ABSTRACT

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The proportion of subgenomic RNAs in southern bean mosaic virus bean strain (SBMV-B), relative to the infectious genomic (25S) RNA, is ~2.3 times greater in late- versus early-progeny virions. The specific infectivity of late-progeny SBMV also is lower than that of the early-progeny virions. Apparently, a proportion of late-progeny SBMV lacks 25S RNA; attempts to resolve them were unsuccessful. Subgenomic SBMV RNAs neither inhibit nor stimulate the infectivity of 25S RNA. When virions are exposed at 45 C for 2 hr, subgenomic components complex with genomic RNA, but this treatment causes no marked decline in the infectivity. Exposure at a somewhat higher temperature (65 C, 10 min) is necessary for SBMV inactivation with genomic compaction. Subgenomic RNAs were detected in SBMV-B virions synthesized in necrotized tissue of *Phaseolus vulgaris* 'Pinto' as well as in the 50S subviral nucleoprotein. Two additional SBMV strains, viz, cowpea and Nigerian, also encapsidate the subgenomic components.

Virions of southern bean mosaic virus bean strain (SBMV-B) contain heterogeneous small RNAs in addition to full-length (M_r 1.4×10^6; 25S) infectious genome (9,10,22). The small RNAs possess a 3'-nucleotide sequence (90 residues), which is identical with that of the 25S RNA (10) and are efficient in vitro messengers of viral capsid protein (9,22). The coat cistron on the 25S SBMV RNA, however, is not translated in vitro. A similar physical and functional genomic heterogeneity has been reported (14,17,19) for turnip yellow mosaic virus (TYMV).

This study was designed to obtain additional information on the biology and nature of small SBMV RNAs. Specifically, we wished to determine if the type of infection (necrotic versus non-necrotic) influences encapsidation of small RNAs; to compare the relative proportion of these components in early- and late-progeny virions; and, to ascertain if other SBMV strains also contain small RNAs. The effect of small RNAs on the infectivity of principal SBMV genome also was investigated.

MATERIALS AND METHODS

According to the criteria proposed by Atabekov and Morozov (1), 25S RNA was designated as genomic RNA while the term subgenomic RNAs was adopted for the small SBMV RNAs. SBMV-B was propagated in *Phaseolus vulgaris* L. 'Bountiful' (24) and assayed on opposite halves of primary leaves of *P. vulgaris* 'Pinto' (23). SBMV cowpea (SBMV-C) or Nigerian (SBMV-Nig) strains were propagated in *Vigna unguiculata* (L.) Walp. 'California Blackeye.' Unless stated otherwise, infected tissue was harvested 21 days postinfection and virions were isolated (24) either immediately or after storage for 3–4 wk at −17 C. The concentrations of virions or the viral components were estimated spectrophotometrically. The equipment used for containing or transferring virions or RNAs was heat-sterilized or soaked in 1% SDS (60 C), rinsed with sterile deionized water, and then oven-dried. Strict precautions were followed to prevent nuclease contamination of virions or the viral RNAs.

Several methods were used for RNA isolation and purification.

Nuclease inhibitors such as bentonite, sodium dodecyl sulfate (SDS), or sodium diethyl pyrocarbonate were included in the extraction media. The sodium perchlorate-SDS method (30) and the EDTA:LiCl:freeze-thaw procedure (7) were used most often for RNA isolation. Other methods followed were, phenol extraction (29), SBMV capsid disruption in the presence of EDTA and SDS at pH 7.5 (23) or pH 9.0 (5), and RNA recovery with sucrose gradient sedimentation. SBMV RNAs were used immediately after purification or were stored in 70% ethanol at −17 C. RNA from tobacco mosaic and bromegrass mosaic viruses and two from *Escherichia coli* (17S and 23S; Sigma Chemical Co., St. Louis, MO 63178) were employed as markers.

Quantitative estimation of the SBMV genomic components was as follows: RNA was heat-denatured (65 C for 5 min), chilled, sedimented in sucrose gradients, and the gradients were scanned at 254 nm (ISCO UA-5 monitor with a built-in recorder). The total RNA peak area was estimated (considered as 100%) from which the proportion of subgenomic RNAs and the principal genome was calculated. Separation of subgenomic RNAs from 25S RNA was achieved with repeated cycles of gradient centrifugation. Briefly, RNA was suspended in 0.01 M tris-HCl buffer (pH 8.0, 0.1 M NaCl, 1 mM EDTA, and 0.1% SDS) heated (65 C, 5 min), and sedimented in sucrose gradients. The desired fractions were obtained, diluted ×2 with deionized water, RNA precipitated with cold ethanol plus 0.15 M sodium acetate, recovered with centrifugation, and then dissolved in tris buffer containing the appropriate additives. The entire procedure was repeated two times and the purity and homogeneity of the resolved RNAs were assessed by gel electrophoresis.

RNA denaturation with neutralized formaldehyde was done according to Boedtker (4) whereas formamide (90%) denaturation according to Veerisetty et al (27). Sucrose (ribonuclease-free) density gradient columns were linear (5–25%) with centrifugation performed either at 149,000 *g* for 2.5 hr (SW 50.1 rotor, Beckman, Palo Alto, CA 94304) or at 43,000 *g* for 18 hr (SW 27 rotor). RNA electrophoresis was conducted in nondenaturing polyacrylamide disk gels (15) or in denaturing agarose methyl mercury slabs (3). RNA in acrylamide gels was stained with methylene blue, destained, and scanned at 580 nm whereas RNA in agarose slabs was stained with ethidium bromide and photographed under short-wave ultraviolet light.

Equilibrium centrifugation of SBMV in cesium sulfate (99% purity, Gallard-Schlesinger, Carle Place, NY 11514) followed the procedure of Hull (12). The 50S SBMV ribonucleoproteinaceous

complex (RNPC), which consists of SBMV RNA and approximately one-third of the total capsid protein, was isolated as described previously (24). Details of other procedures are described under the individual experiments.

RESULTS

Subgenomic SBMV RNAs appear as ill-defined entities (Fig. 1A, indicated with arrows) preceding or contiguous with the 25S component when total RNA (nondenatured) is analyzed with sucrose gradient sedimentation. Denaturation with heat (65 C, 5 min) or formamide (90%) dissociates subgenomic components from the 25S RNA, but the former still appear largely aggregated (Fig. 1B and D). Following denaturation with formaldehyde, the principal RNA sediments at 18S, as reported by Brakke and Van Pelt (5), with considerable amount of subgenomic RNAs also detected (Fig. 1C). Gel electrophoresis (Fig. 2) complemented results based upon the gradient sedimentation and also permitted a better resolution of the denatured SBMV genomic components. In additional tests it was determined that repeated alternate heating (at 65 C, 5 min) and cooling or heating to 80 C caused no change in the mass of 25S RNA or increased the proportion of small RNAs.

Comparable amounts of the subgenomic components and 25S RNA were recovered with a variety of RNA isolation procedures (described in the Materials and Methods). Further, virions pretreated with SDS, Triton X-100 or sodium diethyl pyrocarbonate or those isolated with chloroform-butanol procedure (29) yielded similar amounts of subgenomic RNAs. Moreover, freshly purified virions or those stored for 3 wk at 4 C yielded subgenomic components and 25S RNA in comparable proportions. These results indicate that SBMV genomic polydispersity is an inherent feature and is not due to any nuclease activity. We have shown previously (13) that no such enzyme is associated externally with the SBMV capsid, but the presence of a nuclease within the virions is possible.

SBMV (3 mg/ml) was exposed (37 C for 1 hr) to pancreatic RNase (50 μg/ml) and then sedimented through a 10% sucrose cushion to remove the enzyme. RNA isolated from the nuclease-treated and untreated SBMV yielded similar amounts of small RNAs. Additionally, treatment of SBMV RNA with Proteinase K (28), before or after heat denaturation, caused no change in the proportion of subgenomic RNAs relative to 25S RNA. These results demonstrate that small RNAs are located within the capsid and that genome-linked SBMV protein (28) plays no direct role in maintaining the overall conformation of the SBMV genomic components.

Figure 3 shows electrophoretic analyses of SBMV RNA and selected markers under stringent denaturing conditions; ie, treatment with methyl mercury hydroxide and separation in
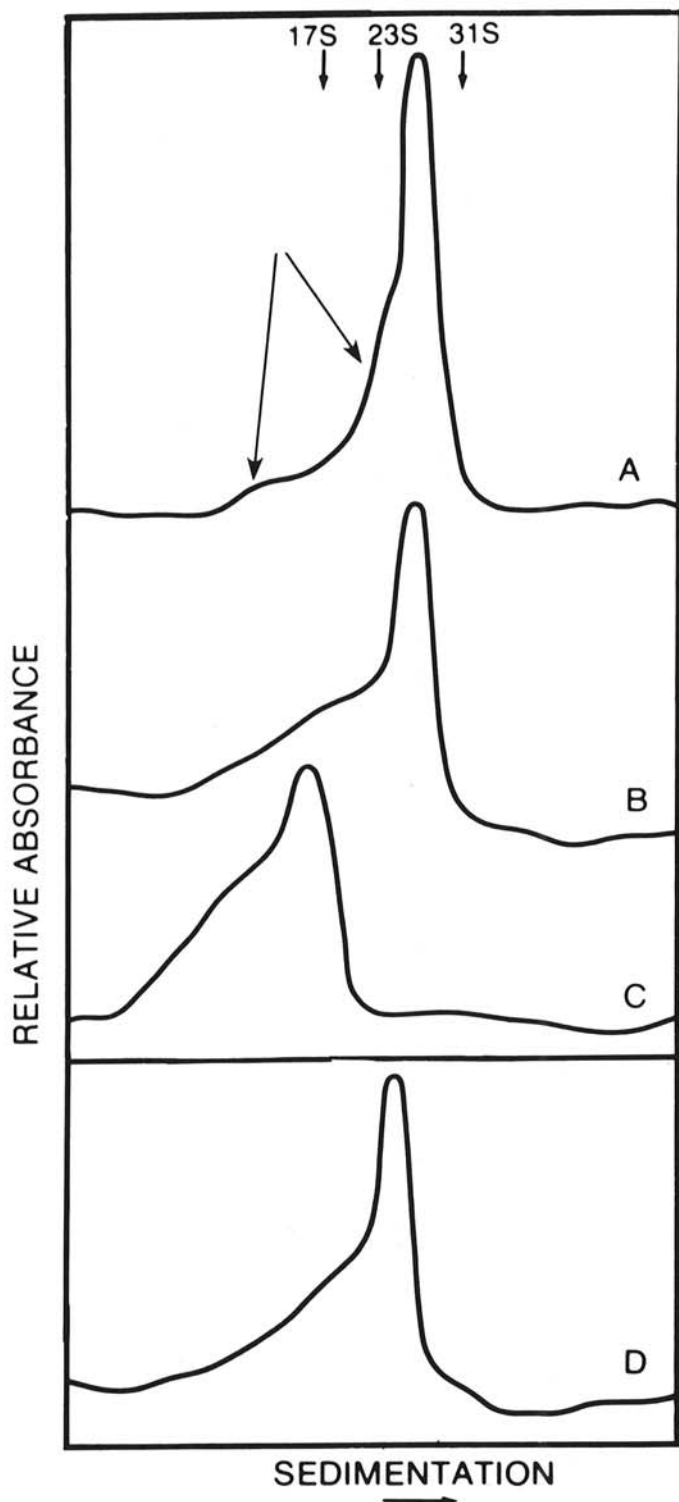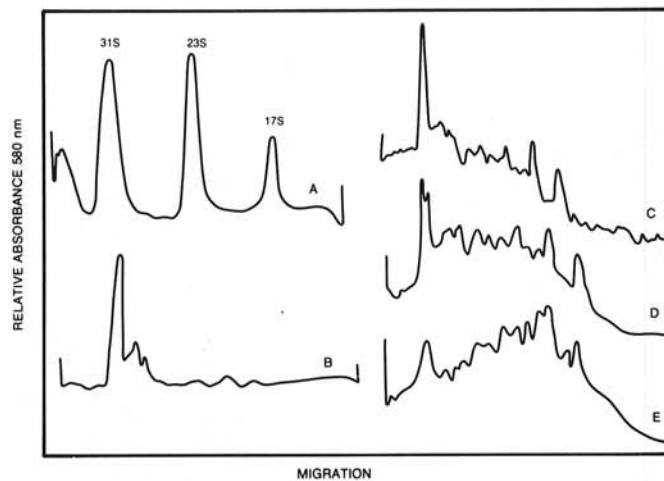

Fig. 1. Sucrose density gradient sedimentation profiles of southern bean mosaic virus ribonucleic acid after treatment with selected denaturants. RNA was isolated according to Denloye et al (7). A, untreated RNA; B, exposed for 10 min at 65 C and then rapidly chilled; C, treated with 1.1 M formaldehyde; and D, treated with 90% formamide for 5 min at 63 C. Approximately 15 μg RNA (A, B, and C) was layered on 5–25% sucrose gradients (0.1 M tris-HCl buffer, pH 7.5) and centrifuged for 2 hr at 149,000 g and gradients monitored at 254 nm. In D, RNA (30 μg) was sedimented (149,000 g, 4 hr) in sucrose gradients containing 50% formamide and scanned at 280 nm. Sedimentation markers used were, Escherichia coli rRNAs (17S and 23S) and TMV-RNA (31S).

Fig. 2. Gel electrophoretic migration patterns of southern bean mosaic virus ribonucleic acid (SBMV-RNA) following denaturation. Approximately 30 μg RNA was electrophoresed in 2.2% polyacrylamide gels at 5 mA/gel for 3.5 hr at 5 C. RNA was stained with methylene blue and then scanned at 580 nm. A, mixture of TMV-RNA (31S) and Escherichia coli rRNAs (17S, 23S); B, SBMV-RNA, untreated; C, SBMV-RNA, heated for 10 min at 65 C and then rapidly cooled; D, SBMV-RNA, treated at 25 C with 5.6 M urea; and E, SBMV-RNA, extracted with phenol.

agarose containing denaturant. These observations lead to the following conclusions: First, with repeated cycles of sucrose gradient sedimentation 25S RNA can be separated effectively from small RNAs; further, 25S RNA is a homogeneous genetic element while subgenomic components are physically heterogeneous. Second, virions generated in the necrotized Pinto bean leaf tissue encapsidate subgenomic RNAs. Third, the 50S SBMV RNPC contains small RNAs as well as 25S RNA. Finally, SBMV-C and SBMV-Nig strains, like SBMV-B, encapsidate the subgenomic components. These results, however, are largely qualitative in nature and at best only provide a rough quantitative estimation of the various SBMV genomic components.

Table 1 shows that the subgenomic SBMV RNAs are noninfectious per se and neither stimulate nor inhibit the infectivity of 25S RNA. Similar results have been reported for TYMV (18).

Virions resolved with isopycnic cesium sulfate centrifugation (12) were examined for RNA composition and infectivity. Figure 4 shows that SBMV is resolved into two principal fractions banding at densities of 1.215 and 1.275 g/ml. These virions were separated on a preparative scale and purified further by resedimentation in cesium sulfate. No differences were observed either in the specific infectivities or the genomic composition of such resolved virions. SBMV prefixed with formaldehyde banded largely at a density of 1.275 g/ml. These results suggest that SBMV heterogeneity in cesium sulfate reflects capsid instability or conformational variations of particles contained in a population, as proposed by Hull (12), rather than actual differences in their RNA content. In the isopycnic cesium chloride gradients SBMV bands homogeneously at a density of 1.36 g/ml (25).

When TYMV virions are exposed at 45 C for 50 min in 1.0 M KCl (pH 7.0), subgenomic RNAs complex with the genomic RNA (18). This is evident from the observation that RNA isolated from treated TYMV sediments uniformly at 38S while RNA from unheated virus is polydisperse with a major component sedimenting at 28S. No data is available on the biologic consequence of this in situ RNA complexing process. A similar experiment was performed with SBMV. For comparison, virions were exposed at 65 C for 10 min, a treatment that inactivates SBMV and induces an intimate complexing of the viral RNAs with no other change in the virus structure (9,27). RNA from the heated and unheated SBMV was examined with gradient sedimentation and gel electrophoresis (Fig. 5). SBMV exposed at 45 C (in the absence or presence of KCl) yielded monodisperse RNA that sedimented at ~28–30S. From virions exposed at 45 C and then at 65 C, a uniformly sedimenting 32S RNA complex was recovered which comigrated with RNA isolated from SBMV heated at 65 C (27) but without a prior incubation at 45 C. The data presented in Table 2 indicates that if exposed at 45 C, no appreciable infectivity
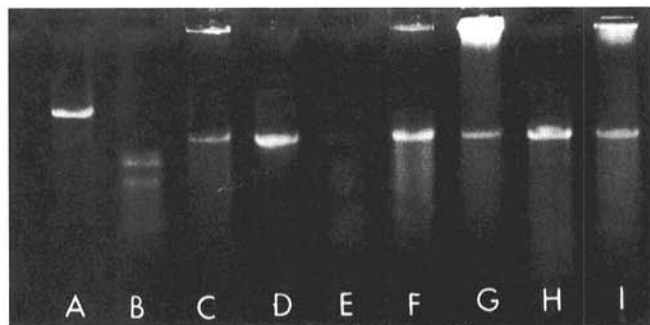
decline occurs for the virions or their RNAs. However, if exposed at 45 C and then at 65 C the virions or isolated RNA proved poorly infectious, like SBMV exposed at 65 C only. These results indicate that exposure at a moderate temperature (ie, 45 C) induces in situ complexing of the SBMV genomic components without any attendant loss in the infectivity. But, for SBMV inactivation with genomic compaction (9,27) exposure at somewhat higher temperatures is necessary. Exposing SBMV at 65 C causes no genomic degradation since RNA isolated from such virions can be transformed from a lowly infectious to the highly infectious state with denaturation (9,27). The precise mechanism of the temperature-induced in situ complexing of subgenomic components with the genomic RNA is unknown. Additionally, it remains to be determined if any specific sites exist on the principal SBMV or TYMV RNAs where the subgenomic RNAs are attached in the heat-inactivated virions.

The following study was conducted in assessing if the proportion of subgenomic components, relative to 25S RNA, varies with the infection periods. Four, 8, 16, and 21 days postinfection leaf tissue of cultivar Bountiful was harvested and stored at −17 C. Following virus purification, RNA was isolated and analyzed with electrophoresis in methyl mercury agarose slabs (Fig. 6).

TABLE 1. Infectivity of southern bean mosaic virus ribonucleic acids (RNAs) following repeated heat denaturation and separation by sucrose gradient sedimentation[a]

RNA samples	RNA concentration (μg/ml)	Number of local lesions[b] induced
Subgenomic RNAs	50	0.5[c]
versus	versus	versus
Total (unfractionated) RNA	20	92.1
Full-length (25S) RNA	15	60.4
versus	versus	versus
25S RNA plus subgenomic RNAs	15 + 15	64.2

[a] As described in the Materials and Methods.
[b] Bioassays were performed on primary leaves of *Phaseolus vulgaris* 'Pinto.' Infectivity of the two samples was compared on the opposite halves of the same leaf. All inocula contained 50 mg of Celite per milliliter and were maintained in an ice-bath during inoculation.
[c] Represents average number of lesions induced on Pinto bean half-leaves. Each experiment was repeated twice using six leaves.
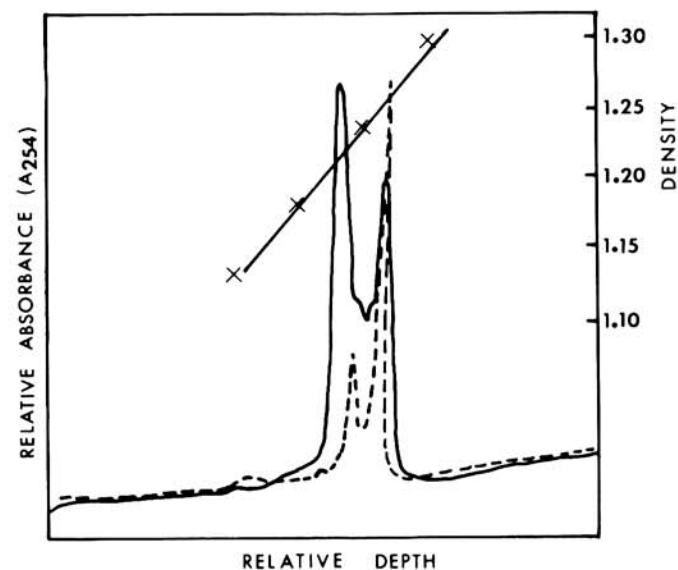

Fig. 3. Electrophoresis of viral RNAs (~1.5 μg) in agarose methyl-mercury gels. Lane **A**, tobacco mosaic virus RNA; **B**, bromegrass mosaic virus RNAs; **C**, total RNA from the bean strain of southern bean mosaic virus (SBMV-B); **D**, purified 25S RNA, SBMV-B; **E**, purified subgenomic RNAs, SBMV-B; **F**, total RNA, SBMV Nigerian strain; **G**, total RNA, SBMV cowpea strain; **H**, SBMV-B purified from the necrotized tissue of *Phaseolus vulgaris* 'Pinto'; **I**, RNA isolated from the 50S ribonucleoproteinaceous complex of SBMV-B. The subgenomic RNAs appear as 'hazy' entities migrating ahead of the distinctly banded genomic RNA.

Fig. 4. Sedimentation of southern bean mosaic virus (SBMV) in isopycnic cesium sulfate gradients. Approximately 25 μg of virus was centrifuged at 15 C for 18 hr at 149,000 g. Untreated (——) and formaldehyde-treated (----) SBMV.

Additionally, RNA was heat-denatured, sedimented in sucrose gradients and the proportion of subgenomic RNAs and the genomic RNA estimated (Fig. 7). Identical quantities of virions were used in these experiments which yielded comparable amounts of total RNA. For SBMV isolated 4–8 days postinfection, subgenomic RNAs comprised ~15% of the total RNA. Late progeny SBMV (16–21 days postinfection), however, had encapsidated ~2.0–2.3 more subgenomic RNAs as compared to the early progeny SBMV. Results presented in Table 3 demonstrate that the specific infectivity of SBMV virions recovered during early infection period was 4–6 times greater than those recovered during the late infection.

DISCUSSION

SBMV has been long considered as a typical example of a virus containing a single RNA strand as its sole genetic element (8,26) but this study as well as the other reports (10,22) show that its genome is considerably more complex. The heterogenous subgenomic SBMV RNAs appear aggregated and/or loosely attached to the principal genome in the native state. Obviously, these components possess certain inherent features for an effective integration with the 25S RNA into a macromolecular complex. This situation could facilitate encapsidation. As specific coat protein:RNA interactions are necessary for SBMV assembly (24) the subgenomic RNAs may play a role in viral morphogenesis. There is evidence suggesting that a small RNA (4S) component is involved directly in the assembly of RNA tumor viruses (2).

That any minor host nucleic acids are contained in a population of subgenomic SBMV RNAs remains to be established. In several animal oncogenic viruses, minor components possessing tRNA or rRNA features have been identified (2) but whether these are virus-specific products is unknown.

The subgenomic components in several animal viral infections arise by a specific splicing of the proviral genome (21) or with a preferential transcription (or amplification) of selected segments of the negative RNA strand (6). The latter possibility also has been considered for plant viruses (1). Preliminary evidence that small SBMV RNAs are derived from 25S RNA is indicated by our observation (*unpublished*) that when rigorously purified 25S RNA was used as an inoculum the progeny virions contained abundant

TABLE 2. Infectivity of variously heated samples of southern bean mosaic virus (SBMV) and the RNA isolated[a] from these virions

Treatments	Entities assayed	Infectivity[b] (%)
SBMV, 25 C (control)	Virions	100.0
	RNA (25S)	100.0
SBMV, 45 C, 2 hr	Virions	95.0
	RNA (28–30S)	80.5
SBMV, 45 C, 2 hr	Virions	6.8
then 65 C, 10 min	RNA (32S)	5.0
SBMV, 65 C, 10 min	Virions	1.0
	RNA (32S)	0.0

[a] Virions (1 mg/ml) were heated as indicated in 0.1 M tris-HCl buffer, pH 7.5. RNA was isolated with the EDTA:LiCl:freeze-thaw method (7). The concentrations of virions and the isolated RNA during assay were, respectively, 0.1 and 20 µg/ml.
[b] Infectivity of heated SBMV and its RNA was estimated relative to the infectivity of unheated SBMV and its RNA. The results represent average values from two different experiments employing 10–15 primary cultivar Pinto bean leaves.
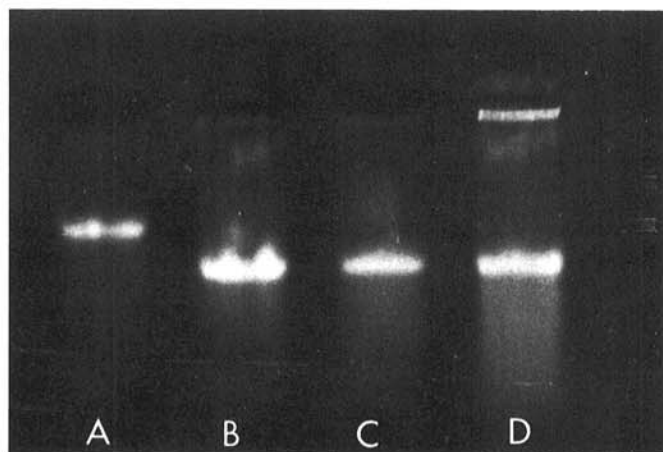

Fig. 6. Agarose methylmercury gel electrophoretic analyses of southern bean mosaic virus (SBMV) ribonucleic acid (~2 µg) isolated from virions after the various periods of infection in *Phaseolus vulgaris* 'Bountiful.' **A,** marker TMV RNA; **B,** RNA from SBMV recovered 4 days; **C,** 8 days; and **D,** 16 or 21 days postinfection.
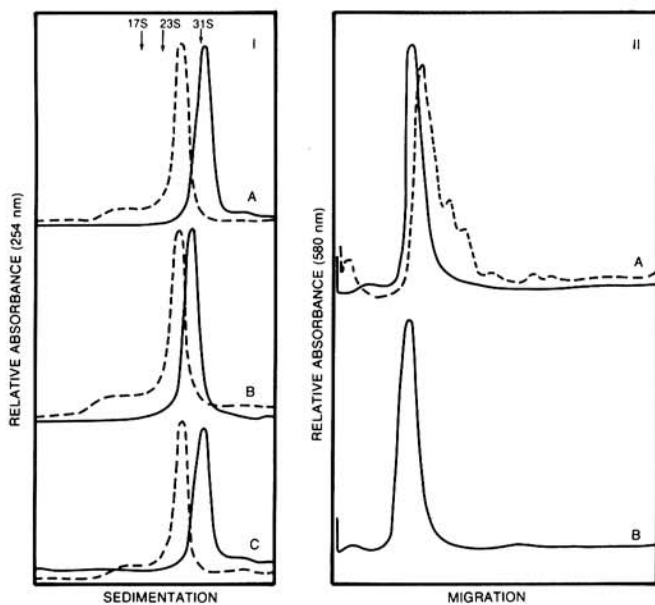

Fig. 5. Sucrose density gradient sedimentation profiles (I) and gel electrophoretic migration patterns (II) of southern bean mosaic virus (SBMV) ribonucleic acids isolated from unheated (----) and heated (——) virus. I. **A,** control RNA, and RNA isolated from SBMV exposed at 65 C; **B,** RNA isolated from SBMV exposed for 2 hr at 45 C; and **C,** RNA from SBMV exposed for 2 hr at 45 C and then for 10 min at 65 C.
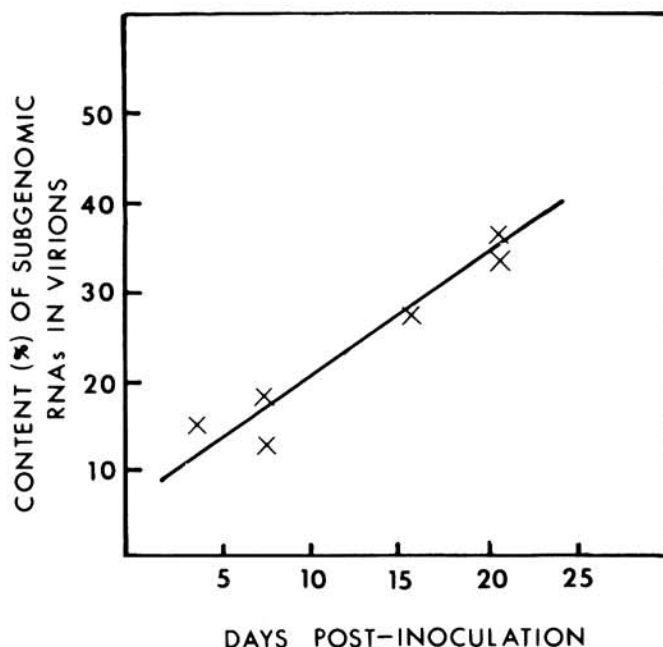

Fig. 7. Percent content of subgenomic RNAs in southern bean mosaic virus isolated from *Phaseolus vulgaris* 'Bountiful' leaves after the various infection periods.

TABLE 3. Specific infectivities of southern bean mosaic virus (SBMV) purified 8 and 22 days postinfection[a]

Experiment	Virus purification (days postinfection)	Local lesions induced[b] (no.)	Relative infectivity of early- vs late-progeny SBMV
1	8	70.8	×6.0
	22	11.8	
2	8	29.2	×4.0
	22	7.2	
3	8	58.5	×3.7
	22	15.7	

[a] Progeny virions isolated 8 and 22 days after infection contained, 15 and 40%, respectively, subgenomic RNAs.
[b] Infectivity assays were performed as described in the footnote of Table 1. The number of lesions represent average values induced on a total of 12 half-leaves. SBMV concentration was 0.35 μg/ml.

quantities of small RNAs. In view of the above considerations, the following possibilities can be considered for the observed differences in the proportion of subgenomic RNAs recovered from virions after the various infection periods. During early stages of pathogenesis the amplified cistrons (coat and others) may actively be involved in synthesizing their respective products and, consequently, are excluded from encapsidation. The probability of their encapsidation, however, increases during late infections, either due to an accumulation of such components, or due to a lesser involvement in translational events. Alternatively, the small RNAs may preferentially be generated during the later stages of viral pathogenesis by postsynthetic modifications of the genomic RNA.

The physical distribution of subgenomic RNAs in the individual virions remains unresolved. Depending upon their genomic content TYMV can be separated into particles containing varying amount of RNAs (17). Such studies with SBMV were unsuccessful indicating that particles with gross differences in their RNA content did not exist in a population. With subfractionation of the putative monodisperse SBMV virions sedimented in a heavy salt gradient (25) it may be possible to isolate particles of varying RNA content, as has been achieved with cucumber mosaic virus (16). Since the late progeny SBMV possesses a reduced specific infectivity, a distinct possibility exists that a proportion of these virions lack genomic RNA. Alternatively, late progeny SBMV may contain virions comparable to the defective interfering particles which are widespread in animal virus infections (11).

The subgenomic components, from an evolutionary perspective, may represent an intermediate developmental form of multipartite to single genomic units or vice versa (20). That such components contain additional information of as yet unknown nature which leads to a better virus survival is speculative. For an understanding of the possible regulatory steps involved in SBMV life cycle, the subgenomic RNAs offer attractive possibilities.

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