Synthesis of Virus and Virus-induced RNA in Southern Bean Mosaic Virus-infected Soybean Cell Cultures

By F. S. WU* AND H. H. MURAKISH†

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824, U.S.A.

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SUMMARY

The synthesis of 3H-uridine-labelled complete virus, virus RNA and presumed replicative form (RF) were studied during the replication of southern bean mosaic virus (SBMV) in soybean callus cells. The inoculated cells were pre-incubated at 6 °C for 4 days and then moved to 25 °C to improve the synchronization of virus multiplication. The synthesis of complete virus, measured by the incorporation of 3H-uridine into virions and by infectivity assays, rose rapidly during 0 to 34 h after transfer to 25 °C. An RNA with a mol. wt. approximating to that of the postulated RF of SBMV-RNA and exhibiting partial resistance to RNase digestion was synthesized in significant amounts. This occurred after a 16 to 24 h incubation, preceding the major period of virus RNA synthesis which reached maximum during 40 to 48 h. Pulse-chase experiments, within limits, suggested the possible precursor role of the postulated RF in the synthesis of virus RNA. Accumulation of an RNA with electrophoretic properties similar to the presumptive RF of SBMV-RNA, was found in inoculated cells incubated at 6 °C from 84 to 96 h, suggesting a possible blockage of virus replication at the double-stranded RNA stage in these cells.

INTRODUCTION

Southern bean mosaic virus (SBMV) has been proposed as a type member of a group of five small, isometric plant viruses, each with a single RNA component with a mol. wt. of about 1.4 × 10^6 (Hull, 1977). Studies on the biology, transmission characteristics, electron microscopy, physical and chemical properties of SBMV were summarized by Hull (1977). However, very little information pertaining to replication of SBMV-RNA was found. The reason for this may have been the unavailability of a suitable system in which SBMV replication would take place in a near-synchronous manner.

Recent studies have shown that differential temperature treatment greatly enhanced the synchrony of multiplication of tobacco mosaic virus in tobacco plants (Dawson & Schlegel, 1973). Likewise, low-temperature pre-incubation treatment of soybean callus resulted in near-synchronous synthesis of SBMV (White et al. 1977).

Our objective was to investigate the synthesis of SBMV virions, SBMV-RNA and virus-related RNAs using the soybean callus system.

* Present address: Biochemistry and Biophysics Section, University of Connecticut, Storrs, Connecticut 06268, U.S.A.
† To whom reprint requests should be addressed.
**METHODS**

*Virus.* A bean strain of SBMV was used throughout the investigation. The virus was extracted from bean leaves (*Phaseolus vulgaris* cv. Prince) 3 to 4 weeks after inoculation and purified as described by Wu & Murakishi (1978). 3H-uridine labelled virus was isolated from infected callus by homogenizing in 0.2 M-potassium phosphate buffer, pH 7.6, at a ratio of 3 ml buffer to 1 g callus using a Tenbroeck tissue homogenizer (Kontes Glass, Vineland, New Jersey, U.S.A.). One mg of unlabelled virus was added to the homogenate to serve as carrier. Subsequent steps were similar to those described by Wu & Murakishi (1978). The final pellet was suspended in distilled water.

*Culture and selection of callus for inoculation.* All operations were performed using sterile techniques in a laminar flow hood. Soybean (*Glycine max* cv. Harosoy 63) callus culture was initiated from hypocotyls of aseptically germinated seeds and maintained on a modified Linsmaier & Skoog (1965) medium, designated as R3 medium (Wu & Murakishi, 1978). Rapidly growing cultures, sampled every 2 days, were considered suitable for use when cells from 10 ml of suspension sedimented to approx. 1.5 ml after standing for 5 min in a 15 ml conical centrifuge tube. In order to obtain uniform infection, uniform-sized cell aggregates were selected. Callus cultures were filtered through stainless steel screens (864 and 406 μm; Cistron Corp., Lebanon, Pennsylvania, U.S.A.), those retained on the second screen being collected, cultured in R3 medium for 20 h and then inoculated with virus.

*Virus inoculation and low-temperature pre-incubation.* One gram batches of callus were placed in 50 ml test tubes containing 3 ml of liquid medium and virus (180 μg/ml) and inoculated as previously described (Murakishi et al. 1970). The inoculated cells were washed with fresh medium, incubated at 25 °C for 4 h and then at 6 °C for low-temperature, pre-incubation treatment on a rotary shaker (120 excursions/min) under white fluorescent light (900 lux). After 4 days at 6 °C, the inoculated cells were incubated at 25 °C under the same light and shaking conditions. Time of changing from 6 to 25 °C was taken as zero time in incubation.

*Actinomycin D (AMD) and 3H-uridine labelling.* At intervals after the shift to 25 °C, 5 g batches of callus cells were transferred to fresh medium. Forty μg/ml AMD (Calbiochem, Los Angeles, California, U.S.A.) was added to suppress host RNA synthesis and the culture was incubated in the dark for 2 h. 3H-uridine (5,6 8H-uridine, 44.9 Ci/mm, New England Nuclear Corp., Boston, Mass., U.S.A.) was then added to a final concentration of 200 μCi/ml and the incubation continued for the appropriate duration. Callus cells were collected on a funnel lined with Miracloth (Chicopee Mills, New York, U.S.A.), washed five times with 10 ml of fresh medium and then weighed. Nucleic acids were extracted immediately.

*Extraction of nucleic acids.* Callus cells were mixed with extraction buffer (0.1 M-glycine, 0.1 M-NaCl and 0.02 M-EDTA, pH 9.4, containing 0.4% bentonite and 1.5% SDS) and water-saturated phenol containing 10% m-cresol and 0.1% 8-hydroxyquinoline at a ratio of 1 g:4 ml:8 ml. The mixture was homogenized in an Omni-mixer (Sorvall Inc., Norwalk, Connecticut, U.S.A.) at 4 °C for 3 min at a speed setting of 6. Samples were taken from the homogenates for determination of trichloroacetic acid-insoluble radioactivity. The homogenate was then centrifuged at 15000 g for 10 min and the aqueous phase collected and stored on ice. The interface and the phenol phases were re-extracted with extraction buffer and the aqueous phases combined and centrifuged at 15000 g for 50 min to remove the bentonite. After addition of several drops of 3 M-sodium acetate, pH 6.0 and 2 vol. of 95% ethanol to the supernatant, the nucleic acids were precipitated at −20 °C overnight. The nucleic acids were collected by centrifugation at 20000 g for 30 min, dried with N2 gas briefly, suspended in 1× SSC (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0) and digested.
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with DNase (15 μg/ml, Calbiochem) in the presence of 0.01 M-MgCl₂ at 35 °C for 20 min. DNase was removed by phenol extraction, and the RNA was recovered by ethanol precipitation. The RNA was finally resuspended in 1× SSC and the absorption spectra were determined in a Cary 15 spectrophotometer (Applied Physics, Monrovia, California, U.S.A.). The absorbance ratio of nucleic acids at 240:260:280 nm was 1:2:1. The concentration of nucleic acid was determined assuming an A₂₆₀nm = 26. Precautions were taken to avoid RNase contamination on glassware and solutions (Sreevalsan, 1973).

Density gradient centrifugation. Rate zonal sucrose density gradient centrifugation (Brakke, 1960) was performed in 5 to 20% linear gradients prepared in SSC. The samples were centrifuged in a Spinco (Beckman Instruments, Inc., Palo Alto, California, U.S.A.) SW 25.1 rotor at 22,000 rev/min for 16 h at 4 °C. The fractions were collected following analysis with an ISCO (Instrumentation Specialties Co., Lincoln, Nebraska, U.S.A.) density gradient fractionator and u.v. monitor.

Determination of acid-insoluble radioactivity. RNA in samples from sucrose gradients were precipitated by adding trichloroacetic acid (TCA) to 8% and yeast RNA to 200 μg/ml. Total ³H-uridine incorporated into whole cells was determined by adding 1 ml of 20% TCA to 0.5 ml of homogenate from a mixture of callus, buffer and phenol during the extraction of RNA. After 30 min at 0 °C, the precipitates were collected on Whatman GF/C glass fibre papers and washed twice with 5 ml of 5% TCA and 95% ethanol. Filter papers were dried at 60 °C for 2 h and digested at 55 °C for 4 h with 0.8 ml of NWT mixture consisting of NCS solubilizer (Amersham Corp., Arlington Heights, Illinois, U.S.A.), water and tolune at ratio of 9:1:1. After digestion, 10 ml of scintillation fluid (6 g PPO and 75 mg POPOP per litre of toluene) was added to each vial and radioactivity determined in a Beckman LS-133 liquid scintillation counter.

Preparation of SBMV-RNA. SBMV purified from sucrose density gradient centrifugation was adjusted to a concentration of 2.2 mg/ml and was mixed with an equal vol. of the extraction buffer containing 20 mg/ml purified bentonite (Fraenkel-Conrat, 1966) and 1.5% SDS. The mixture was shaken gently for 20 min at 25 °C and stored at 4 °C for 1 h. The insoluble material was removed by centrifugation at 15,000 g for 50 min at 0 °C. The supernatant was subjected to 5 to 20% sucrose gradient centrifugation in a SW 25.1 rotor as described above. The RNA was collected, dialysed against 1× SSC overnight, and precipitated with 2 vol. of 95% ethanol. The precipitates were then dried briefly with N₂ gas and resuspended in 1× SSC.

Enrichment of LiCl-soluble RNA. To enrich the RF, RNA was prepared from infected cells which had been incubated in ³H-uridine for 15 to 25 h after the temperature shift from 6 to 26 °C. The RNA was made 2 M with respect to LiCl (Baltimore & Girard, 1966) and stored at 4 °C overnight. After centrifugation, the supernatant was added to 2 vol. of 95% ethanol and the resultant precipitates were collected by centrifugation and resuspended in 1× SSC. The LiCl-soluble RNA was subjected to 5 to 20% sucrose density gradient centrifugation.

Polyacrylamide-agarose gel electrophoresis (PAGE). The methods described by Loening (1967) and Bourque & Naylor (1971) were adapted for the separation of high mol. wt. RNA on 2.25% polyacrylamide gels containing 0.5% agarose (Bausch & Lomb, Rochester, New York, U.S.A.). Electrophoresis was carried out at a constant current of 5 mA/gel. The gels were scanned with a Gilford Model 2000 spectrophotometer (Gilford Instruments Inc., Oberlin, Ohio, U.S.A.), frozen with dry ice and sliced into 1 mm thickness on a gel slicer (Mickel Lab. Eng. Co., U.K.). Each gel slice was digested in 0.8 ml of NWT mixture at 55 °C for 2 h. After digestion, 10 ml of scintillation fluid was added to each vial and radioactivity determined.

RNase, DNase and NaOH treatment of RNA. One ml fractions from the sucrose density
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Fig. 1. Time course of total $^3$H-uridine incorporation into acid-insoluble materials from 1 g of infected callus cells (○—○); into complete virus purified from 1 g of infected callus cells (■—■); and infectivity (▲—▲) associated with callus cells inoculated with SBMV. Incubated cells were incubated at 6 °C for 4 days then moved to 25 °C. The time when cells were moved to 25 °C represents zero time incubation. Cells were then treated with 40 µg/ml of AMD for 2 h after which $^3$H-uridine (200 µCi/ml) was added for 8 h. Complete virus was extracted and subjected to three cycles of differential centrifugation before radioactivity was determined. Infectivity and total $^3$H-uridine incorporation into acid-insoluble materials were determined as described in the Methods. Each point in the graph represent the mean value of two experiments except in the case of infectivity in which they were based on triplicate samples.

gradients were divided into five 0.2 ml aliquots. Three of these aliquots were adjusted to make the final concentration of 1 × SSC and served as samples for the control. DNase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.; 5 µg/ml) digestion and alkaline hydrolysis with 0.2 N-NaOH. The remaining two samples were adjusted to either 2 × SSC or 0.1 × SSC for treatment with RNase (Sigma; RNase A, 1.0 µg/ml plus RNase T1, 50 units/ml) at high and low salt, respectively. Enzyme treatments were done at 35 °C for 30 min. Duration of alkaline hydrolysis at 35 °C was 15 h. After incubation, the acid-insoluble radioactivity was determined.

Infectivity assays. Primary leaves of Pinto bean (Phaseolus vulgaris L) plants previously kept in the dark for 3 to 4 days were used for bioassay by the half-leaf method. Lesion numbers from triplicate samples were averaged for each point on the infectivity curve (Fig. 1).

RESULTS

Synthesis of virions

The incorporation rate of $^3$H-uridine into virions closely paralleled the increase in infectivity during the initial period of virus synthesis (Fig. 1). The synthesis of virions rose rapidly from 0 to 34 h and at a slightly lower rate from 34 to 80 h.

The total $^3$H-uridine incorporated into TCA-insoluble materials increased slightly during the initial 15 h period, but decreased thereafter reaching a minimum during the 45 to 56 h incubation period. Incorporation then remained at a minimum level until an abrupt increase occurred during the 69 to 80 h incubation. Thus, while the synthesis of complete virus was in its most rapid phase, $^3$H-uridine incorporation into TCA-insoluble materials dropped sharply.
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Fig. 2. Sedimentation profile of RNAs. Acid-insoluble radioactivity of RNA was determined following rate zonal sucrose density gradient centrifugation in an SW 25·1 rotor at 22,000 rev/min for 16 h. RNAs were extracted from: (a) healthy cells incubated for 14 to 24 h without AMD; (b) SBMV-infected cells incubated for 14 to 24 h without AMD; (c) healthy cells incubated 38 to 48 h without AMD; (d) SBMV-infected cells incubated for 38 to 48 h without AMD; (e) healthy cells incubated for 38 to 48 h with AMD; (f) SBMV-infected cells incubated for 38 to 48 h with AMD.

Density gradient analysis of RNA

Since SBMV-RNA had the same sedimentation rate as the 25S ribosomal RNA, they could not be separated by sucrose density gradient centrifugation. Without AMD, RNA profiles of healthy and infected cells incubated for 14 to 24 h were similar in the 18S and 25S RNA regions but slowly sedimenting RNA was greater in amount in infected (Fig. 2b) than in healthy (Fig. 2a) cells.

During the 38 to 48 h period without AMD, infected cells showed increased radioactivity in the 25S region (Fig. 2d) in comparison to healthy cells (Fig. 2e). Adding AMD to healthy cells completely inhibited 18S and 25S RNA (Fig. 2e). AMD appeared to stimulate the synthesis of low mol. wt. RNAs in both healthy (Fig. 2e) and infected (Fig. 2f) cells. The SBMV-RNA peak was noticeable in this treatment period as was a heterodisperse radioactivity between the 4S and 25S regions (Fig. 2f).

PAGE analysis of virus-induced RNA

PAGE profiles of RNAs extracted from infected callus during the initial 2 to 6 h after the temperature shift to 25 °C, revealed the presence of a small amount of slow migrating RNA (Fig. 3b, slices 16 to 23). After 16 to 24 h, the slow migrating peak (SMP) RNA was the
Fig. 3. Electropherograms of RNA extracted from healthy and from SBMV-infected soybean callus. Callus cells after inoculation with virus were pre-incubated for 4 days at 6 °C and then incubated at 25 °C for various intervals. At various times after the transfer to 25 °C (0-time), cells were treated with AMD (40 μg/ml) for 2 h and then labelled with ³H-uridine (200 μCi/ml) for 6 h. Nucleic acids were extracted and treated with DNase prior to electrophoresis for 2 h at 5 mA/gel. Markers used were 25S and 18S rRNA extracted from unlabelled, healthy callus. (a) RNA extracted from control, healthy cells labelled at 16 to 24 h. (b) to (e) RNA extracted from SBMV-infected cells labelled at: (b) 2 to 6 h; (c) 16 to 24 h; (d) 40 to 48 h; and (e) 76 to 84 h. The RNA extracted from control, healthy callus labelled during intervals earlier or later than 16 to 24 h gave similar distributions of radioactivity in gels as (a).

major peak (Fig. 3c). In addition, there were smaller peaks at the 25S and 18S regions. During the same period in comparable healthy cells, RNA synthesis was nearly completely inhibited (Fig. 3a). When the mol. wt. of the SMP was extrapolated from its mobility in PAGE, it was estimated to be 2.8 × 10⁶ (Fig. 4) or twice the mol. wt. of single-stranded SBMV-RNA (Diener, 1965). Although this would be in agreement with the expected mol. wt. of the theoretical RF of SBMV, it is not possible to determine accurately the mol. wt. of dsRNA using ssRNA as mol. wt. markers (Aoki & Takebe, 1975). Relative molecular radii are perhaps revealed rather than relative mol. wt. (Fisher & Dingman, 1971).

The synthesis of SBMV-RNA reached a maximum between 40 and 48 h (Fig. 3d), at the time when synthesis of complete virus was at its most rapid phase (Fig. 1). Incorporation into SMP, the presumed RF, was greatly reduced at this time but that into the 18S RNA persisted. During 76 to 84 h, the synthesis of virus RNA decreased but it was still the major RNA synthesized. The amount of the presumptive RF remained similar to that during the 40 to 48 h period. This is probably related to a later cycle of virus replication which is thought to occur through division of infected cells after maximum virus RNA synthesis had been reached (Pelcher et al. 1972; Beachy & Murakishi, 1973).
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Fig. 4. Plot of log mol. wt. versus distance of migration during polyacrylamide-agarose gel electrophoresis. Electrophoresis was for 3 h at 5 mA/gel. The 25S and 18S rRNA were extracted from healthy soybean callus. The 23S and 16S chloroplast rRNA were extracted from healthy Prince bean leaves. Numbers in parentheses represent approximate mol. wt. x 10^-8.

Fig. 5. Electropherograms of RNA isolated in pulse-chase experiment. Soybean callus cells inoculated with SBMV were subjected to 4 °C for 4 days and then incubated at 25 °C for 16 h. The cells were then treated with AMD (40 μg/ml) for 2 h and pulse-labelled for 1 h (a) in medium containing 3H-uridine (200 μCi/ml). This was followed by washing away the 3H-uridine and chasing with fresh medium containing 40 μg/ml AMD and excess unlabelled uridine for (b) 3 h or (c) 18 h. Electrophoresis was for 100 min at 5 mA/gel on 2-25% polyacrylamide-agarose gels.

Pulse-chase experiment

In order to determine the possible precursor role of the SMP RNA found in PAGE, a pulse-chase experiment was conducted. Twenty-six h after incubation at 25 °C, the cells were treated with AMD (40 μg/ml) for 2 h and then pulse-labelled for 1 h in fresh medium containing 200 μCi/ml of 3H-uridine. The cells were washed with fresh medium to which cold uridine had been added at the rate of 1 mg/ml. After washing, the cells were divided into three equal portions. One portion was frozen with dry ice immediately (pulse); the...
Fig. 6. Sedimentation profile of LiCl-soluble RNA and its degraded intermediates. Infected cells were incubated in \(^3\)H-uridine for 15 to 25 h after the temperature shift to 25 °C. 2 M-LiCl-soluble RNA was precipitated with ethanol, resuspended in 1 × SSC and centrifuged in a Spinco SW 50.1 rotor at 50,000 rev/min for 3 h. Acid-insoluble radioactivity was determined as described in the Methods. (a) LiCl-soluble RNA from healthy (□ --- □) and from infected (○ ©) callus cells. Absorbance at 254 nm of LiCl-soluble RNA from healthy and from infected cells were virtually identical and are represented as a single solid line (---). (b) Acid-precipitable radioactivity of LiCl-soluble RNA extracted from infected cells after treatment with 0.2 N-NaOH for 16 h at 35 °C (□ --- □), and after digestion with pancreatic DNase (10 µg/ml) for 30 min at 25 °C (○ --- ○). (c) RNase-resistant radioactivity in LiCl-soluble RNA from SBMV-infected callus cells. Fractions were treated with RNase A (1.5 µg/ml) and RNase T₁ (40 units/ml) for 30 min at 35 °C in 2 × SSC (○ --- ○) or with tenfold RNase A (15 µg/ml) and tenfold RNase T₁ (400 units/ml) in 2 × SSC (● --- ●), and 0.1 × SSC (□ --- □). TCA-precipitable radioactivity was then determined. (d) As for (c) except results are from healthy callus.

Others (chases) were further incubated in the washing solution for 3 and 18 h, respectively. The nucleic acids were then extracted and analysed by PAGE (Fig. 5a to c). During the 1 h pulse, SMP RNA with its major peak migrating into the expected position of SBMV-RF was found (Fig. 5a). The amount of its radioactivity was reduced to less than one-half during the 3 h chase (Fig. 5b) but no significant decrease of the radioactivity was shown after chasing for 18 h (Fig. 5c). The synthesis of virus RNA was not obvious during the 1 h pulse. The 3 h chase resulted in an increased 25S peak (Fig. 5b) and after the 18 h chase, it further increased as did the 18S peak (Fig. 5c).

Most of the radioactivity was present in the less-than-4S region during the 1 h pulse and the amount of radioactivity in this region was reduced following the 3 h chase. During the 18 h chase, the major RNA synthesized was 4 to 5S RNA which agrees with the results from density gradient sedimentation analysis of RNA extracted from AMD-treated cells during the 38 to 48 h incubation (Fig. 2f).
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Analysis of LiCl-soluble RNA

After sucrose density gradient centrifugation of the LiCl-soluble RNA, two peaks of 260 nm absorbance were observed: a major one from fractions 3 to 10 and a minor one from fractions 11 to 17 (Fig. 6a). Each fraction was treated with TCA and the acid-soluble radioactivity was determined. The results showed a single large peak (Fig. 6a, fractions 3 to 10) which was insensitive to DNase treatment but sensitive to alkaline hydrolysis (Fig. 6b). In order to determine whether RF was present in LiCl-soluble RNA from infected cells, each fraction from the gradients was treated with RNase at high salt (2 × SSC) and low salt (0.1 × SSC) concentrations. RNase treatment in high salt solution revealed two RNase-resistant RNAs with their highest radioactivity peaks at fractions 3 and 10 (Fig. 6c). Increasing RNase concentration to 10-fold at high salt disclosed the same peaks with less acid-insoluble radioactivity, especially in fraction 3. Fraction 10 remained the major peak and was less influenced by the higher RNase concentration (Fig. 6c). However, at low salt solution, 10-fold RNase treatment eliminated both peaks and no acid-insoluble RNA could be detected in the gradient (Fig. 6c). Edy et al. (1976) reported that dsRNA was resistant to RNase T1 in low-salt media but its resistance was dependent upon the concentration of both salts and enzyme. The RNA in fractions 7 to 12 appears to be double-stranded based on its high RNase resistance at high salt and RNase susceptibility at low salt, its sedimentation rate in sucrose gradient, and its presence only in infected cells. It is possible that the fraction 3 RNA could represent 4S, tRNA which has some double-stranded regions in its structure and is less sensitive to AMD than rRNA. However, significant radioactivity remained in this fraction after the high-salt, 10-fold RNase treatment, and infected cells had higher radioactivity (Fig. 6c) than healthy cells (Fig. 6d). Therefore, it seems more likely that fraction 3 RNA might represent dsRNA rather than tRNA.

No infectivity associated with cells incubated at low temperature

During the 4-day, pre-incubation period at 6 °C, no virus replication could be detected either by testing for infectivity of virions or infectious RNA following phenol-bentonite extraction (Fraenkel-Conrat, 1966) or by the purification method used for SBMV isolation. Likewise, inoculated cells which had been incubated at 6 °C continuously for 4 to 15 days
Slow migrating RNA

A slow migrating RNA, with a major radioactive peak with an estimated mol. wt. of $2.8 \times 10^6$ accumulated in infected cells incubated at 6 °C from 84 to 96 h (Fig. 7). Healthy cells under the same conditions showed a much smaller RNA peak (Fig. 7). Scanning the gels at 260 nm did not reveal any differences between healthy and infected cells but the difference in radioactivity between them was pronounced.

The migration distance of this polydisperse RNA in PAGE was similar to the SMP RNA synthesized in 1 h pulse-labelled RNA (Fig. 5a) and the presumed RF synthesized during the 16 to 24 h incubation period after transfer to 25 °C (Fig. 3c). A small amount of radioactivity was present in healthy cells. The reason for this has not been investigated but it is possible that the precursor of rRNA was not completely suppressed by AMD when callus cells were held at low temperature for 4 days.

DISCUSSION

$^3$H-uridine was taken up by soybean callus and incorporated into virion RNA as well as other high mol. wt. RNAs. In infected cells treated with AMD, the amount of $^3$H-uridine incorporated into complete virus increased with time while the total radioactivity into TCA-insoluble material decreased rapidly (Fig. 1). SBMV infection did not inhibit cellular RNA synthesis (Fig. 2) but suppression occurred after AMD was added. This is similar to the finding of Jackson et al. (1972) that synthesis of virus-related RNAs in TMV-infected tobacco cells were insensitive to AMD while host RNA was inhibited.

The appearance of the SMP RNA as the major RNA synthesized during the 16 to 24 h incubation period suggests that it could be the RF of SBMV-RNA. The presence of RF and RI in infected cells is well-known in a number of small RNA plant viruses (Siegel & Hariharasubramanian, 1974). In SBMV-infected callus cells, the presence of a putative RF and its involvement in SBMV-replication was indicated by: (i) the presence of RNase-resistant RNA at 2 × SSC in the LiCl-soluble RNA fraction of nucleic acids extracted from infected cells but not from healthy ones; (ii) its susceptibility to RNase at 0.1 × SSC; (iii) its resistance to DNase treatment and sensitivity to degradation by alkaline hydrolysis (Fig. 6); (iv) the rapid synthesis of SMP RNA which preceded the period of maximum synthesis of virus RNA and the decrease in its synthesis when the amount of virus RNA was at maximum (Fig. 3c, d); (v) the pulse-chase experiment which, within limits, revealed a precursor-product relationship between this RNA and virus RNA (Fig. 5); (vi) and by the fact that its synthesis was insensitive to AMD.

Although the appearance of the 25S RNA followed the pattern of the time course of virus synthesis, the concomitant occurrence of the 18S RNA (Fig. 3c, 5c) did not. As noted, the 18S peak was found only in infected and not in uninfected (Fig. 3a) cells which had been treated during the 16 to 24 h period. In addition, we found that when phenol was added to the extraction mixture during the isolation of RNA from SBMV virions, a similar 18S peak was observed (F. S. Wu & H. H. Murakishi, unpublished data). Thus, it is possible that the 18S peak may be virus- rather than host-related products. However, the possibility, although remote, also exists that the AMD may be less efficient in suppressing host RNA synthesis in infected than in uninfected cells.

An RNase-resistant low mol. wt. RNA (4 to 6S), fraction 3 of Fig. 6(c), (d), was found in extracts from both infected and healthy cells following rate zonal sucrose density gradient centrifugation. This may possibly represent dsRNA normally synthesized by cells. However,
its exact nature and relation to infection remain to be resolved. Other in vitro studies of extracts isolated from healthy plant cells have revealed an RNA-dependent RNA polymerase activity which synthesized low mol. wt. double-stranded products (Duda et al. 1973; Le Roy et al. 1977). RNA-dependent RNA polymerase isolated from both healthy and virus-infected tobacco leaves was reported to behave similarly (Ikegami & Fraenkel-Conrat, 1978). They therefore postulated that the enzyme was not coded for by virus RNA but instead resulted from a stimulated synthesis of host enzyme upon virus infection. It is possible that a similar situation exists in the soybean cell-SBMV system.

The pulse-chase experiment did not definitely show the precursor role of the SMP RNA in the synthesis of SBMV-RNA. The radioactivity peaks of SBMV-RNA increased during the 3 and 18 h chases but the amount by which the radioactivity decreased in the supposed RF peaks was somewhat less than the amount which it increased in the virus RNA peaks (Fig. 5a to c). An analogous situation was found in separated leaf cells (Jackson et al. 1972) as well as in protoplasts (Aoki & Takebe, 1975) in which the RNA precursor pool in plant cells was too large to permit the label to be completely chased from precursor to product. It seems probable that the RNA precursor pool in soybean callus cells could also have contributed to the increase in radioactivity of the SBMV-RNA peaks.

The occurrence of the heterodisperse, slow migrating RNA (Fig. 7) in infected cells incubated at 6°C for 4 days suggests that it might represent the putative RF. With the temperature shift to 25°C, the presumed RF which had accumulated would be available to synthesize virus RNA in a nearly synchronous manner after a brief lag period. After the low-temperature treatment, during the first 8 h of incubation at 25°C, the synthesis of SBMV-RNA was not discernible but the presumed RF was synthesized. It seems reasonable to assume that these cells were still affected by the low-temperature treatment so that active virus replication had not actually begun.

Purification and further characterization of the putative RF are necessary to verify its role in the replication of SBMV. Cold-temperature pre-treatment of infected soybean callus cells may offer a means to further investigate SBMV replication.

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