Synthesis, Accumulation and Encapsulation of Individual Brome Mosaic Virus RNA Components in Barley Protoplasts

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(Accepted 25 October 1979)

SUMMARY

The rates of virus RNA synthesis and virion accumulation were investigated in brome mosaic virus-infected barley protoplasts. Single-stranded virus RNAs could be detected as early as 6 h after inoculation. Only RNA components 1 and 2 were detected at this time, suggesting that their synthesis is initiated relatively early in infection. The RNAs were synthesized at similar rates from 6 to 35 h post inoculation with maximal synthesis until approx. 25 h after inoculation. Double-stranded replicative forms of the four virus RNAs were observed. Their synthesis was first detectable at 6 h post inoculation and followed a time course similar to that of the single-stranded RNA species. Analysis of RNA encapsidation and infectivity assays of protoplast homogenates revealed that virion formation was greatest between 10 and 25 h after inoculation. All four RNAs were present in virions at 10 h post inoculation. Particles containing RNA 3 and RNA 4 accumulated at a faster rate than particles containing RNA 1 or RNA 2.

INTRODUCTION

Translation (Shih & Kaesberg, 1973, 1976; Davies, 1976), transcription (Hadidi & Fraenkel-Conrat, 1973; Kummert & Semal, 1977) and aminoacylation (Hall et al. 1972) of brome mosaic virus (BMV) RNAs have been extensively studied in vitro. However, there is much to be learned about how these observations relate to RNA function in vivo. Of particular interest is the role of the 3' terminal RNA sequence, since modifications affecting aminoacylation also affect infectivity (Kohl & Hall, 1977).

Transcription of BMV RNA has been investigated in intact plant tissue (Philipps et al. 1974; Bastin & Kaesberg, 1976), but the highly asynchronous nature of infection has imposed serious limitations on the acquisition and interpretation of data. These problems can be largely overcome by using protoplasts instead of intact tissue. Motoyoshi et al. (1974) investigated some aspects of virus RNA transcription in tobacco protoplasts using BMV variant V5. Unfortunately, the Russian isolate of BMV, whose properties have been most fully documented (Lane, 1974), was reported not to be infective in tobacco protoplasts. This observation prompted the development of a protoplast system from barley in order to study virus infection processes and to investigate aminoacylation in vivo. Under our conditions, barley protoplasts are easily isolated and routinely infected to high levels with RNA from the Russian isolate of BMV. A similar barley protoplast system which could be infected with BMV RNA was recently described by Okuno & Furusawa (1978). Inoculation with virus RNA rather than whole virus is advantageous in that the behaviour of individual component RNAs and chemically-modified RNAs can be studied. In this article we describe the synthesis and encapsidation of virus RNA in barley protoplasts.
METHODS

Isolation of barley protoplasts. Barley, *Hordeum vulgare* L. cv. 'Dickson', was grown in equal parts of peat moss and vermiculite in an environmental chamber at the University of Wisconsin Biotron. The seedlings were sprinkled once a day with half-strength Hoagland's solution and illuminated with approx. 20000 lux from cool white fluorescent and incandescent bulbs for 16 h per day. The temperature was 24°C while the lights were on and 16°C during darkness; the relative humidity was 75%.

All solutions used for protoplast preparations were autoclaved or filter sterilized and glassware was heat sterilized. Approximately 2 to 5 g of primary leaf tissue were sliced with razor blades into 1 to 2 mm pieces which were rinsed with 0.6 M mannitol. The slices were placed into 30 to 50 ml of enzyme solution containing 2% cellulase (Cellulysin, Calbiochem), 0.1% Macerozyme R-10 (Yakult Biochemicals Co., Ltd., Nishinomiya, Japan), 0.1% bovine serum albumin (Sigma, St. Louis, Mo., U.S.A.), and 0.6 M mannitol, pH 5.7. The tissue slices were incubated without shaking at 30°C for 3 to 3.5 h. The suspension was gently swirled and poured through small mesh polyester monofilament cloth (Tetko, Inc., Elmsford, N.Y., U.S.A.) to remove undigested tissue. The protoplasts were collected by sedimentation (50 g for 2 min), washed twice with 0.6 M mannitol and immediately inoculated with BMV RNA.

Inoculation, incubation and labelling of protoplasts. The Russian isolate of BMV was propagated in barley and purified as previously described (Shih et al. 1972). RNA was isolated from the virus by phenol extraction according to Bockstahler & Kaeberg (1965) except that all manipulations were at 4°C. The RNA was suspended in water and stored at –70°C. Preparations were tested for infectivity by local lesion assay on *Chenopodium hybridum* and the integrity of the RNA was checked by polyacrylamide gel electrophoresis in non-denaturing gels as described below.

The protoplasts were inoculated with BMV RNA by direct suspension of pelleted protoplasts in inoculum at 4°C. The inoculum contained 10 mM-potassium citrate, pH 5.0, 0.7 M mannitol and 25 μg/ml protamine sulphate (Sigma). BMV RNA was added to give a final concentration of 5 μg/ml 3 to 5 min before use. For mock inoculation, BMV RNA was omitted from the inoculum. Following isolation, the protoplasts were collected by sedimentation (50 g for 2 min), suspended in the cold inoculum at 1 x 10^6 protoplasts/ml and incubated in an ice bath for 10 min. The protoplasts were again sedimented and washed twice with 10 mM-potassium citrate, pH 5.0, containing 0.7 M mannitol to remove unadsorbed RNA.

The protoplasts were suspended in culture medium at 0.5 to 1.0 x 10^6 viable protoplasts/ml. The culture medium was similar to that used by Aoki & Takebe (1969) and contained: 0.2 mM-KH₂PO₄, 1 mM-KNO₃, 1 mM-MgSO₄, 1 μM-KI, 0.1 μM-CuSO₄, 10 mM-CaCl₂, 0.7 M mannitol, 300 μg/ml Cephaloridine (Eli Lilly and Co., Indianapolis, Ind., U.S.A.) and 10 μg/ml gentamicin sulphate (Sigma), pH 6.5.

RNA was labelled by adding 50 μCi of carrier free ^32P-orthophosphoric acid and 10 μg of actinomycin D per ml of culture medium 1 h after inoculation. The protoplasts were incubated without shaking at 26°C under constant illumination of approx. 1800 lux. After incubation, the concentration of the protoplasts was determined using a haemocytometer. Viability was defined as the ability of the protoplast membrane to exclude Evans' Blue dye 0.01%, w/v. The protoplasts were collected by centrifugation, washed once with 0.7 M mannitol and frozen.

Infectivity assay. The frozen protoplast pellet was thawed, suspended in 30 mM-potassium phosphate buffer, pH 7.5, at 1 x 10^6 protoplasts/ml and homogenized using a ground glass tissue grinder. Half-leaves of *C. hybridum* were used in a randomized design to assay the
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infectivity of the protoplast homogenate. If necessary, several dilutions of the homogenate were inoculated in order to produce discrete countable lesions.

Fluorescent antibody staining. BMV antigen was detected in infected protoplasts by an indirect staining procedure (Goldman, 1968). Antiserum to BMV was produced in rabbits (Ball, 1974). The gamma globulin fraction was isolated by ammonium sulphate precipitation (Shepard, 1972) and any antibodies to healthy plant proteins were removed by absorption using a powder of acetone-extracted healthy barley leaves (0.3 g powder/ml of gamma globulin). The final gamma globulin preparation had a titre of 1:160 in microprecipitin tests with BMV. Protoplasts were fixed on to slides and were treated with anti-BMV gamma globulin at 1:8 dilution as described by Otsuki & Takebe (1969) except that the slides were kept at room temperature for 1 h. The protoplasts were then treated for 1 h at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit IgG (0.3 mg antibody protein/ml) which was purchased from Cappel Laboratories, Cochranville, Penn. 19330. The slides were viewed under epi-illumination using a Zeiss fluorescence microscope with a halogen lamp. The percentage of fluorescent protoplasts and the percentage of viable protoplasts at harvest were used to calculate the percentage of surviving protoplasts that were infected.

Analysis of RNA from protoplasts. A frozen pellet containing approx. 10^5 to 10^6 protoplasts was solubilized with 1 to 2 ml of buffer containing 100 mM-glycine, 10 mM-EDTA, 100 mM-NaCl, pH 9.5 (Babos & Shearer, 1969) to which 1% sodium dodecyl sulphate, 3 mg/ml bentonite and an equal volume of water-saturated phenol were added. The mixture was shaken for 2 min at room temperature and the phases separated by centrifugation. The phenol phase was extracted once with the glycine buffer. The combined aqueous phases were extracted twice more with an equal volume of water-saturated phenol and three times with cold ether. The RNA was ethanol-precipitated and collected by low speed centrifugation. The ethanol was removed by a stream of nitrogen and the RNA was dissolved in sterile distilled water and stored at -70°C. Approximately 2 × 10^3 to 2 × 10^4 ct/min of ^32P were incorporated in RNA per 10^4 protoplasts.

The RNA was subjected to electrophoresis in 12.5 × 15.5 cm × 1.5 mm slab gels containing 2-4% acrylamide, 0.1% methylenebisacrylamide and 0.45% agarose, using tris-borate buffer (Peacock & Dingman, 1968). Just before electrophoresis, the RNA samples, adjusted to contain equal ct/min, were heated to 60°C for 5 min in 5 M-urea. Electrophoresis was at 25 mA for 2 to 2.5 h at 4°C. Following electrophoresis, the RNA bands were stained with toluidine blue o and the gel was dried on to paper. Kodak XRP-5 or Kodak No-Screen Medical X-ray film was exposed to the gel. Usually a 2 to 5 day exposure to a gel containing 10^4 ct/min sample was sufficient to detect radioactive bands. If necessary, however, longer exposures were used to detect bands of low radioactivity.

The relative amounts of radioactivity in each RNA species were determined by measuring the absorbances of the bands in the autoradiograph with a double-beam recording microdensitometer (Joyce-Loebl, Model MK III C). Areas of the RNA peaks in tracings from inoculated samples were measured with a compensating planimeter using the tracings of RNA from mock-inoculated protoplasts as baselines. The peak areas were converted to relative number of moles by dividing the areas by the respective RNA mol. wt.

RESULTS

Detection of virus infectivity

The percentage of protoplasts infected with BMV was determined by staining preparations with fluorescent antibody. The percentage increased rapidly between 10 and 21 h after inoculation, when nearly all viable protoplasts were fluorescent (Fig. 1). Routinely, the inoculation procedure resulted in 90 to 100% infection of viable protoplasts. Virus antigen
could be detected as early as 7 h post inoculation (p.i.) although only a small percentage of protoplasts fluoresced. The amount of virus per protoplast increased rapidly from 7 to 20 h p.i. and then levelled off (Fig. 1). In this experiment, at 38 h p.i., each protoplast contained 33 pg BMV which is equivalent to $4 \times 10^6$ particles.

**Synthesis and accumulation of single-stranded BMV RNA**

Nucleic acid was isolated from inoculated and mock-inoculated protoplasts after various periods of incubation in the presence of $^{32}P$-orthophosphate and actinomycin D. Immunofluorescent assay showed that the percentage of infected protoplasts was not significantly affected by actinomycin D, whereas incorporation of $^{32}P$ into ribosomal RNA was inhibited by approx. 65%. RNA samples, adjusted to contain equal ct/min were analysed by polyacrylamide-agarose gel electrophoresis (Fig. 2). Virus RNAs were clearly detectable 8 h after inoculation and lengthy exposure of the X-ray film revealed trace amounts of virus RNAs at 6 h. In addition to the genomic RNAs, bands were present that migrated between BMV RNA 3 and RNA 4. Some of these bands, including ribosomal RNA bands, were also present in lanes containing samples from mock-inoculated protoplasts. When protoplasts were cultured with $^{32}P$-orthophosphate in the absence of actinomycin D, ribosomal RNAs were labelled but the other bands were absent (Fig. 2b). RNA from protoplasts which had been irradiated with u.v. light before inoculation (5 min at an intensity of 32 erg/mm²/s, 30 cm from a 15 W germicidal lamp G15T8, Westinghouse Corporation, Bloomfield, N.J., U.S.A.) to inhibit ribosomal RNA labelling and cultured without actinomycin D, likewise did not contain the small RNAs (Fig. 2c). The absence of these RNAs from protoplast samples cultured in the absence of actinomycin D suggests that they may be virus or cellular RNA degradation products and that they do not directly participate in the virus infection process.

From microdensitometer analysis of the autoradiograph shown as Fig. 2a, the relative rates of synthesis of each RNA were determined. If the specific radioactivity of each virus RNA is assumed to be constant throughout infection as is the case with tobacco mosaic virus infection in tobacco protoplasts (Aoki & Takebe, 1975), and if the specific radioactivities of RNA 1, 2, 3 and 4 are assumed to be the same, the rates of synthesis of each
Fig. 2. Detection of virus RNA synthesis in protoplasts by autoradiography. Mock-inoculated (M) and inoculated (I) protoplasts continuously cultured in the presence of 32P-orthophosphate were harvested at the indicated times after inoculation. RNA was isolated and subjected to electrophoresis in a 2.4 % polyacrylamide-agarose gel at 25 mA for 2 h. Samples were adjusted to contain equal cts/min. (a) Time course of virus RNA synthesis in the presence of actinomycin D. (b) RNA from protoplast samples 16 h after inoculation cultured without actinomycin D. (c) RNA 35 h after inoculation of protoplasts which had been irradiated with u.v. light prior to inoculation and cultured without actinomycin D.

can be expressed on a molar basis (Fig. 3). RNAs 1 and 2 could be detected at 6 h p.i., RNAs 3 and 4 at 10 h p.i. The virus RNAs were rapidly synthesized until 25 h after inoculation and more slowly thereafter. The rates of synthesis of the four virus RNAs were alike from 16 to 35 h after inoculation (Fig. 3, inset). At this time, for each mole of RNA 1, there were approx. 2 moles of RNA 2, 3 moles of RNA 3 and 4 moles of RNA 4. It is surprising that at 10 h p.i. there were over six times the number of moles of RNA 4 as there were of RNA 1. It is possible, however, that the amount of RNA 4 was overestimated due to the presence of other RNAs of similar mol. wt. in the same area of the gel. RNA from virion preparations of this isolate of BMV contain approx. 1 mole of RNA 1: 1 mole of RNA 2: 3 moles of RNA 3: 3 moles of RNA 4 (Pyne & Hall, 1979).

Synthesis and accumulation of double-stranded BMV RNA

Portions of the RNA samples shown in Fig. 2a were treated with RNase 10 μg/ml RNase A and 15 units/ml RNase T1 in 300 mM-NaCl, 30 mM-sodium citrate, pH 7.0) at 30 °C for 30 min. Subsequently, the RNase was inactivated by digestion with Proteinase K (Beckman) at 500 mg/ml at 37 °C for 1 h.

Polyacrylamide gel analysis revealed four RNase-resistant bands (Fig. 4a). These bands were never found in mock-inoculated protoplasts; they migrated in the gel as expected for the replicative forms of BMV RNA and they co-migrated in gels with the products produced in vitro with BMV polymerase (Hardy et al. 1979). The bands shown in Fig. 4a probably represent both replicative forms and replicative intermediates from which the single-stranded tails have been removed by RNase digestion.

The relative rates of synthesis of the double-stranded (ds) RNAs determined by micro-
Fig. 3. Increase in the amounts of virus RNAs with time. Microdensitometer tracings of the gel autoradiograph shown in Fig. 2(a) were used to determine the relative number of moles of each virus RNA species present in the protoplast samples collected at selected times after inoculation. Values on the y-axis are arbitrary. ●—●, RNA 1; ▲—▲, RNA 2; ○—○, RNA 3; △—△, RNA 4. The inset shows the molar ratio of RNA 2 (▲—▲), RNA 3 (○—○) and RNA 4 (△—△) to that of RNA 1.

Fig. 4. Double-stranded BMV RNAs. Portions of the RNA samples (Fig. 3) were treated with RNase to digest ssRNAs and then subjected to electrophoresis in a 2.4 % polyacrylamide-agarose gel at 25 mA for 2 h. (a) Autoradiograph of RNA samples. Lane 1 contains the RNase-treated RNA. The designation of the dsRNAs is given at the left. Lane 2 contains untreated RNA; the position of the ssRNA species is given to the right. (b) Increase in the amount of dsRNAs with time. Microdensitometer tracings of the gel autoradiograph were used to determine the relative number of moles of each virus dsRNA species present in the protoplast samples collected at selected times after inoculation: ●—●, dsRNA 1; ▲—▲, dsRNA 2; ○—○, dsRNA 3; △—△, dsRNA 4.
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Fig. 5. Changes in the relative amounts of RNAs present in virions with time. Protoplasts were cultured in the presence of $^{32}$P-orthophosphate and harvested at various times after inoculation. Virus particles were isolated from the samples, RNA was extracted from the virus preparations and subjected to electrophoresis in a 2.4% polyacrylamide-agarose gel at 25 mA for 2 h. Radioactive bands were detected by autoradiography. (a) Autoradiograph of RNA samples from virion preparations at the indicated times after inoculation (I) or mock-inoculation (M). (b) Microdensitometer tracings of the gel shown in (a) were used to determine the relative number of moles of each RNA present in virions at selected times after inoculation: RNA 1; RNA 2; RNA 3; RNA 4. The inset shows the molar ratio of RNA 2, RNA 3 and RNA 4 to that of RNA 1.

densitometer analysis of the autoradiograph is shown in Fig. 4b. DsRNAs 1 and 2 were first detectable at 6 h p.i. and dsRNAs 3 and 4 at 8 h p.i. The increase in amounts of these RNAs with time was similar to that of genomic BMV RNAs (Fig. 3) except that much smaller amounts of dsRNAs were synthesized. Early in infection (8 h p.i.), 38% of the label was in dsRNAs while at 22 h after inoculation, only 2% of the label was in these RNAs.

Encapsidation of BMV RNAs

In order to investigate the rate of virus RNA encapsidation, protoplasts were incubated with $^{32}$P-orthophosphate as for analysis of total RNA. Samples were collected at various times after inoculation and frozen. Unlabelled carrier BMV (1-0 mg) was added to each thawed sample and virus was purified as previously described (Shih et al. 1972) except that chloroform was omitted during homogenization and the polyethylene glycol precipitation step was omitted. The virus pellets following ultracentrifugation were resuspended in 50 mm-sodium acetate, pH 4.5, and the RNA extracted as described for RNA analysis. The nucleic acid preparations were analysed by polyacrylamide-agarose gel electrophoresis and autoradiography of the dried gel (Fig. 5a). Nucleic acid preparations from mock-inoculated protoplasts produced no radioactive bands, which indicated that labelled ribosomal RNA did not contaminate the virus preparations. At 10 h after inoculation, which was the earliest time investigated, encapsidated forms of all four RNAs were detected. The relative rates of encapsidation of the virus RNAs as determined by microdensitometer analysis are shown in
Fig. 5b. The rates of encapsidation of individual RNAs appear to be quite different from rates of their synthesis (Fig. 3); amounts of individual RNAs present in virions did not reflect the amounts present in total RNA preparations at similar stages of infection. More RNA 2 than RNA 1 was encapsidated. The rate of increase in encapsidated RNAs 3 and 4 was much faster than the rate of increase in encapsidated RNA 1 or RNA 2, so that at 35 h after inoculation, RNA components 3 and 4 were in excess (Fig. 5b, inset). Encapsidated forms of RNAs 3 and 4 were present throughout infection in equimolar amounts in agreement with Pyne & Hall (1979) and Zagorski (1978) who found nearly equimolar amounts of these RNAs in virion preparations from whole tissue. It is not certain whether all RNAs 3 and 4 are encapsidated in a single particle as postulated by Lane & Kaesberg (1971) or whether a small proportion of each is packaged without the other as suggested by Hull (1976). Nevertheless, because we observe equimolar amounts of RNA 3 and RNA 4 from virions, it appears that the specific radioactivities of these RNAs are equal.

**DISCUSSION**

The synthesis, accumulation and encapsidation of BMV RNA in protoplasts was followed by autoradiography of radioactive RNA after electrophoretic separation. We found it impossible to detect virus RNAs by scanning the u.v. absorbance of gels after electrophoresis because several unlabelled barley ribosomal RNAs co-migrate with virus RNAs. Because the specific radioactivities of RNA 3 and RNA 4 isolated from virions appear to be equal, it seems reasonable to assume that the specific radioactivities of each of the four virus RNAs were similar in the experiments described.

RNA 1 and RNA 2 were detected relatively early (6 h p.i.) in infection, similar to the situation found in intact barley leaves (Philipps et al. 1974). At 10 h p.i., components 3 and 4 were detected (Fig. 3). By this time, autoradiographic analysis showed that substantial amounts of RNA 4 were present; also, at this stage of infection, coat protein could be detected by immunofluorescence (Fig. 1). It is likely, therefore, that coat protein is translated from RNA 4 throughout infection. This suggestion is supported by evidence obtained from studies of translation of BMV RNAs in vitro which clearly establish that BMV coat protein is translated only from RNA 4, despite the presence of a coat protein cistron in RNA 3 (Shih & Kaesberg, 1973).

A small, rapidly-labelled, RNA (mol. wt. $0.56 \times 10^6$) has been described in tobacco protoplasts infected with BMV V5 (Bancroft et al. 1975) and in wild-type BMV infections of whole plants (Philipps et al. 1974). In both of these investigations, actinomycin D was used to inhibit the labelling of ribosomal RNA. In the present experiments, RNA preparations from infected barley protoplasts cultured in the presence of actinomycin D were found to contain several radioactive RNA species of low mol. wt. These RNAs were not present when infections took place in the absence of actinomycin D. It is possible, therefore, that the $0.56 \times 10^6$ mol. wt. species is not specific to BMV infection.

A replicative form was detected for each of the BMV RNA components. This agreed with the results of Lane & Kaesberg (1971) and Bastin & Kaesberg (1976). A replicative form of RNA 4 was not detected in tobacco protoplasts infected with BMV V5 (Bancroft et al. 1975), a variant that has reduced amounts of RNA 3 and RNA 4 (Bancroft & Lane, 1973) compared to the Russian isolate. Conceivably, only small amounts of dsRNA 4 were synthesized by this variant; hence, they were not detected. In our experiments, dsRNA 4 was present early in infection. This, together with the evidence that in reactions with BMV polymerase in vitro, ssRNA 4 initiates the formation of its own replicative form (Hardy et al. 1979), strongly suggests that RNA 4 is synthesized via its own replicative form.
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Encapsidation of the RNAs into virions began early in infection and occurred at rates different from those of virus RNA synthesis. Encapsidated forms of all four RNAs were isolated at 10 h p.i. The rates of accumulation of encapsidated forms of RNA 3 and RNA 4 were similar (to be expected if they occur in a single virion) and were greater than the rates of accumulation of encapsidated forms of RNA components 1 and 2. This agrees with the in vitro data of Herzog & Hirth (1978) who found, in studies of the reconstitution of BMV protein and RNA, that particles containing RNA 3 and RNA 4 accumulated at a faster rate than did particles containing RNA 1 or RNA 2.

This study was supported by N.I.H. Grant AI 115702 and by the College of Agricultural and Life Sciences, U. W. Madison. We gratefully acknowledge advice on protoplast isolation from Dr R. H. A. Coutts and G. E. Edwards and on infection of protoplasts with virus RNA from Dr J. Watts. Drs H. L. Shands and R. H. Forsberg generously provided barley seed. We thank Ms P. Kiberstis for providing the BMV RNA used for inoculation of protoplasts.

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(Received 30 August 1979)