RNA Synthesis in Broadbean Leaves Infected with Broadbean Mottle Virus

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Tissue of broadbean leaves infected with broadbean mottle virus (BBMV) was infiltrated with actinomycin D followed by $[^{14}C]$uridine. As shown by velocity sedimentation analysis in sucrose gradients, two major classes of single-stranded RNAs distinct from RNAs of healthy leaf tissue were synthesized under those conditions. Part of the RNA product synthesized in infected leaves was incorporated into virus particles and was analogous to BBMV-RNA. Another class of labeled RNA product sedimented between the light ribosomal RNA and transfer RNA, and was not found in appreciable amounts in newly formed virus particles. This slower-sedimenting class of RNA was not an artifact due to actinomycin D treatment as it was formed also in untreated infected tissue.

INTRODUCTION

A virus-induced RNA-polymerase activity has been identified in cell-free extracts of broadbean leaves infected with broadbean mottle virus (BBMV) (Semal, 1970; Romero and Jacquemin, 1971).

In order to permit a comparison with the in vitro system, the present paper will present a study of in vivo RNA synthesis in tissue of broadbean leaves infected with BBMV. The effect of actinomycin D on RNA synthesis, and the amount and characteristics of the newly made RNAs have been investigated.

MATERIALS AND METHODS

Plant and virus. Broadbean seedlings (Vicia faba) var. "Maxime" were grown in a greenhouse. Very young plants were inoculated with BBMV by rubbing the lower epidermis of both unexpanded leaves of the first verticil with the juice of BBMV-infected broadbean mixed with Carborundum. Inoculated plants were placed in a cabinet with continuous light and constant temperature (23–24°C).

Treatment with actinomycin D and incubation with $[^{14}C]$uridine. All operations were performed under fluorescent light in a water-saturated chamber. In some experiments, actinomycin D and $[^{14}C]$uridine were supplied successively to detached leaves through the petioles; leaves were then washed with water and frozen at $-25^\circ$. However, this method of introduction of actinomycin D and $[^{14}C]$uridine through the petiole of intact leaves did not inhibit DNA-dependent RNA synthesis sufficiently for the purpose of this study. Therefore, a method using tissue-sliced samples with vacuum infiltration was developed in further experiments. One gram (fresh weight) of leaf tissue was cut into pieces (1 × 10 mm) with a McIlwain tissue Chopper (Mickle Laboratory Engineering Co.). Leaf pieces were immersed under vacuum in actinomycin D, and the tissue was then incubated in an aqueous solution of $[^{14}C]$uridine. After incubation, leaf fragments were washed thoroughly with water and kept frozen at $-25^\circ$.

Extraction of RNA from leaf tissue. One gram of frozen tissue was homogenized with a mortar and pestle in 3 ml of glycine buffer (0.1 M glycine, 0.1 M NaCl, 0.01 M EDTA, pH 9.5), and mixed with 0.3 ml 10% bentonite, 0.3 ml 10% sodium dodecyl
sulfate (SDS), and 5 ml water-saturated phenol. The emulsion was broken by centrifugation, and the water phase was reextracted once with phenol. The water phase was mixed with two volumes of cold ethanol and allowed to stand overnight at 4°C. The precipitate was dissolved in 2 ml of 0.01 M sodium acetate, pH 5.0.

Virus purification. Purification of BBMV from infected leaves was performed as previously described (Romero and Jacquemin, 1971), adding unlabeled virus as carrier. The purified virus was resuspended in 2 ml of sodium acetate 0.01 M pH 5.0, and the suspension was mixed with 4 ml of water-saturated phenol, 0.2 ml of 10% bentonite and 0.2 ml of 10% SDS. The emulsion was then treated as above to extract the RNA.

Density gradient centrifugation. Nucleic acid suspensions were layered on top of 20 ml gradients of 5–20% sucrose in 0.01 M acetate pH 5.0 containing 1 μg/ml of sodium polyvinylsulfate and 0.001 M EDTA. After centrifugation at 4°C in the swing-out rotor (23 ml) of an MSE centrifuge, tubes were pierced at the bottom and fractions of about 0.5 ml were collected after passage through an Uvicord flow cell analyzer.

Resistance of the RNA product to RNase. Ribonuclease (RNase) resistance was assayed by incubating RNA samples in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0) for 30 min at 37°C with 5 μg/ml of pancreatic RNase.

Radioactivity measurements. Acid-insoluble radioactivity was determined by precipitating aliquots of each fraction with trichloroacetic acid to a final concentration of 5% in the presence of carrier protein. The precipitates were filtered on Millipore membranes; the filters were dried and placed in scintillation fluid; the radioactivity was determined with a Packard liquid scintillation spectrometer.

Actinomycin D was a gift from Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey; [14C]uridine (specific activity 62 mCi/m mole) was purchased from the Radiochemical Centre, Amersham, England; and pancreatic RNase A was from Sigma Chemicals.

RESULTS

RNA Synthesis in Healthy or BBMV-Infected Broadbean Leaf Fragments.

Fragments of healthy or BBMV-infected leaves (harvested 4 days after inoculation) were immersed in 2 ml of an actinomycin D solution (20 μg/ml) for 2 hr under vacuum before labeling for 2 hr with [14C]uridine (0.5 μCi/ml) in the presence of actinomycin D. RNA was extracted and analyzed by sucrose density gradient centrifugation. With healthy tissue RNA synthesis was inhibited 80–90% by actinomycin D treatment (Fig. 1). With actinomycin D-treated infected material, two broad classes of radioactivity were obtained (Fig. 2). The slower-sedimenting peak was not a result of actinomycin D treatment as it was found also with untreated material. All RNAs synthesized in either healthy or BBMV-infected leaf fragments were almost completely hydrolyzed by treatment with RNase in 2× SSC (Figs. 1 and 2).

Incorporation of [14C]Uridine into BBMV Virions

To learn whether the RNAs synthesized in the infected leaf tissue of broadbean
were encapsulated into BBMV virions, leaf fragments (excised from broadbean at 6 days after inoculation with BBMV) were incubated for 12 hr in the presence of actinomycin D and [\textsuperscript{3H}]uridine. After incubation BBMV was purified and viral RNA was extracted and analyzed in sucrose density gradient; the total RNA of leaf fragments treated in the same way was also analyzed (Fig. 3). The radioactivity associated with the RNA from virus particles sedimented in the same position as the faster-sedimenting class of radioactive RNA obtained from actinomycin D-treated infected tissue while little if any appeared in the region of the slower-sedimenting class.

**DISCUSSION**

The in vivo labeling of RNA in infected leaf tissue was partly resistant to actinomycin D, and the products were essentially single-stranded, as shown by their sensitivity to RNase in 2 X SSC. Two major classes of RNA, with some heterogeneity in each class, were synthesized by the actinomycin D-treated infected leaf tissue. One class of labeled RNA sedimented between both ribosomal RNAs in the same position as RNA extracted from BBMV particles; the other class of labeled RNA sedimented around 12–14 S and was not encapsulated into virions. The latter RNA was not an artifact due to actinomycin D treatment, as it was present in comparable amounts in untreated infected tissue. Thus the faster sedimenting class of radioactivity may be equated to BBMV-RNA, while the nature and function of the slower-sedimenting radioactive RNA are unknown. The synthesis of virus-induced, actinomycin D-resistant, RNase-sensitive, unencapsulated RNA shown here in BBMV-infected tissue has been described also for tobacco infected with tobacco mosaic virus (TMV) (Babos and Shearer, 1969a, b) and for barley infected with bromegrass mosaic virus (Philipps, personal communication). Also a small virus-specific single-stranded RNA has been reported in TMV-infected tobacco cells (Zaitlin, et al., 1971).

It is not presently known whether the slow-sedimenting labeled RNA in our experiments is single-stranded, or if it represents an RNase-sensitive type of double-stranded RNA.
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