Properties of Single-stranded RNA Synthesized by a Crude RNA Polymerase Fraction from Barley Leaves Infected with Brome Mosaic Virus

By J. KUMMERT AND J. SEMAL
Faculté des Sciences agronomiques 5800 Gembloux, Belgium

(Accepted 18 February 1972)

SUMMARY

The RNA polymerase activity of a cell-free particulate fraction of barley leaves infected with brome mosaic virus was investigated in the presence of actinomycin D and EDTA. The RNA products were fractionated according to their solubility in 2 M-LiCl. Both LiCl-soluble and LiCl-insoluble fractions of the RNA synthesized during a 2 min. pulse of [$^3$H]-UTP were largely resistant to RNase in high salt concentration; the radioactive RNA of the insoluble fraction had sedimentation properties expected for replicative intermediates. After a 2 min. chase, LiCl-insoluble radioactivity was essentially RNase-sensitive in high salt, and sedimented in sucrose gradients in association with all three components of brome mosaic virus RNA, provided protective exogenous RNA was added throughout the experimental procedure. The overall results suggest that the in vitro synthesis of the RNAs found in virus particles occurs by a continuous process resulting possibly from recycling activity of the polymerase.

INTRODUCTION

Incubation of a crude RNA polymerase fraction prepared from barley leaves infected with brome mosaic virus (BrMV) together with [$^3$H]-UTP, actinomycin D and the necessary factors for RNA synthesis, promoted the incorporation of the labelled precursor into RNA (Semal & Kummert, 1971 a). After a short pulse of [$^3$H]-UTP, followed by deproteinization with phenol and detergent, radioactivity was incorporated into RNA which was resistant to hydrolysis by pancreatic ribonuclease (RNase) in 2 × SSC (SSC = 0·15 M-NaCl, 0·015 M-sodium citrate, pH 7·0). This double-stranded RNA was identified as a segment of BrMV-RNA associated with a complementary strand, as characterized by interference of BrMV-RNA (purified from virus particles) with self-re-annealing of the pulse-labelled product (Semal & Kummert, 1971 b). When the incorporation mixture was incubated under pulse-chase conditions, part of the pulse-labelled RNase-resistant RNA was chased into single-stranded RNA, most of which sedimented more slowly than the small (S) component of BrMV-RNA (Semal & Kummert, 1971 a). Because endogenous nuclease activity was associated with the crude RNA polymerase fraction, the size of the single-stranded RNA was not considered significant for the characterization of the pulse-labelled product.

In further experiments, the suspected effect of endogenous nucleases was minimized by reducing the duration of the pulse-chase period; also it was found that pulse-chased single-stranded RNA of larger size was obtained when MgCl$_2$ was omitted from the extraction medium (Semal, Jacquemin & Kummert, 1972).
The present paper describes pulse-chase experiments performed in the presence of exogenous RNA during the preparation and incubation of the crude RNA polymerase fraction, in order to protect the RNA-synthesizing complex and the newly made products from the action of endogenous nucleases.

METHODS

Cell-free RNA synthesis. The techniques for preparation of the crude RNA polymerase fraction for cell-free synthesis of RNA in the presence of [3H]-UTP and actinomycin D, and for purification of the RNA product, were similar to those described previously (Semal & Kummert, 1971a, b) with the following modifications: (1) barley plants were grown in a cabinet with controlled temperature and constant light before and after inoculation; (2) infected leaves were harvested 66 hr after inoculation; (3) the concentration of mercaptoethanol was increased 2-fold; and (4) no MgCl₂ was added to the extraction medium.

Fractionation of the labelled RNA product. The final ethanol precipitate from the purification procedure was resuspended in 2 x SSC and the solution was mixed with 1 vol. of 4 m-LiCl; the mixture was kept for 16 hr at 4° and then centrifuged for 10 min. at 10,000g. The LiCl-insoluble fraction was suspended in 2 x SSC and layered on top of a 12 ml., 5 to 20 °/° linear sucrose gradient in 0.1 M-sodium acetate, 0.001 M-MgCl₂ and 0.001 M-CaCl₂ (Bockstahler & Kaesberg, 1965) containing 1 µg./ml. of polyvinyl sulphate. The material was centrifuged for 7 hr at 35,000 rev./min. in the 6 x 14 ml. rotor of an MSE 65 superspeed centrifuge. After the run, tubes were pierced at the base and the effluent was fractionated through a Uvicord flow-cell and the E₅₄₅ was recorded. In some experiments, the LiCl-soluble fraction was precipitated with 2 vol. of ethanol, resuspended in 2 x SSC, and fractionated in a sucrose density gradient as described above. The acid-insoluble radioactivity associated with aliquots of the fractions was determined before and after treatment with RNase in 2 x SSC, as described by Semal & Kummert (1971a).

Chemicals. Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. Common chemicals and RNase-free sucrose were from Merck Co. Unlabelled nucleotides were obtained from Schwarz BioResearch Inc.; tricyclohexylamine salt of phosphoenol pyruvic acid, pyruvate kinase and pancreatic RNase A were from Sigma Chemicals Co. PPO (2, 5-diphenyloxazol) and POPOP (p-bis-2-(5-phenyloxazolyl)-benzene) were purchased from Packard Instrument Co. Uridine-5-T-5'-triphosphate ([³H]-UTP, 1 to 2 c/m-mole) was obtained from The Radiochemical Center, Amersham. Exogenous RNA was from Serva (8 s yeast RNA), from Mann Research Laboratories (bovine liver ribosomal RNA) or from Calbiochem Co. (wheat ribosomal RNA).

RESULTS

Kinetics of UTP incorporation by the crude RNA-polymerase fraction in the presence of exogenous RNA

The crude RNA polymerase fraction was prepared and incubated in the presence of 50 µg./ml. of exogenous RNA (yeast RNA or liver r-RNA). Fig. 1 and 2 show that incorporation of radioactivity into RNA was almost linear up to 16 min.; the early product was highly resistant to RNase in 2 x SSC, but the amount of RNase-resistant radioactivity levelled off after 2 min.
In vitro synthesis of BrMV-RNA

Fig. 1. Kinetics of incorporation of [³H]-UTP by the RNA polymerase preparation in the presence of actinomycin D; exogenous yeast RNA was present throughout the experiment. ○ — ○, acid-insoluble radioactivity; ● --- ●, acid-insoluble radioactivity after RNase treatment (5 µg./ml. RNase for 30 min. at 37° in 2 x SSC).

Fig. 2. See Fig. 1. Exogenous ribosomal RNA was present throughout the experiment.

Effect of exogenous RNA on sedimentation properties of the pulse-chased products

The crude RNA polymerase fraction was prepared and incubated either in the presence or absence of yeast RNA. After 2 min. pulse-labelling, followed by 2 min. chase with an excess of unlabelled UTP, the RNA was purified and treated with 2 M-LiCl; the LiCl-insoluble material was fractionated by centrifugation, using BrMV-RNA as internal marker. In the presence of exogenous RNA (Fig. 3), well defined peaks of radioactivity were associated with all three peaks of BrMV-RNA. In the absence of exogenous RNA (Fig. 4), a shoulder of radioactivity was associated with the large (L) component of BrMV-RNA, whereas the S component was increased. Radioactive RNA was essentially single-stranded in both cases, as shown by its sensitivity to RNase in 2 x SSC.

Sedimentation properties of the pulse-labelled RNA products before and after a chase

The LiCl-insoluble RNA products obtained after a 2 min. pulse of radioactive UTP, or after a 2 min. pulse followed by a 2 min. chase, were fractionated in sucrose density gradients. BrMV-RNA was centrifuged as an external marker in a companion tube. As shown in Fig. 5, the LiCl-insoluble pulse-labelled material sedimented heterogeneously and was resistant to RNase in 2 x SSC, with some RNase-sensitive RNA associated with the faster sedimenting fractions. After the chase (Fig. 6), radioactivity of the LiCl-insoluble fraction was associated with all three components of BrMV-RNA, and was sensitive to RNase in 2 x SSC. After pulse-labelling for 2 min., the LiCl-soluble product was essentially RNase-resistant and
most of it sedimented at about 14 s (Fig. 7); after the chase, there was a relative decrease in the 14 s peak, with the concomitant appearance of slow-sedimenting single-stranded RNA (Fig. 8).

Effect of formaldehyde on the sedimentation properties of the pulse-chased RNA product

The LiCl-insoluble fraction of the product of a pulse-chase experiment conducted in the presence of liver r-RNA was mixed with carrier BrMV-RNA and treated with formaldehyde as described by Boedtker (1968), to rupture the secondary structure. Treatment with formaldehyde (Fig. 10) decreased the sedimentation velocity of BrMV-RNA, but radioactivity remained quantitatively associated to extinction. A similar decrease in sedimentation velocity was observed when the product was centrifuged in a sucrose density gradient prepared with acetate buffer containing 10^{-8} M-EDTA in place of MgCl₂ and CaCl₂, and again radioactivity was associated with extinction.

Effect of exogenous RNA on the nucleotide sequence of the pulse-labelled RNase-resistant product

RNA was synthesized for 2 min. by the crude RNA polymerase fraction, either with or without addition of exogenous RNA throughout the preparation and incubation. The RNase-resistant RNA product was prepared for re-annealing studies according to Semal &
In vitro synthesis of BrMV-RNA

Fig. 5. Sedimentation profile in a 5 to 20% sucrose gradient (right to left) of the 2 M-LiCl-insoluble fraction of the RNA isolated from the RNA polymerase preparation after incubation with [3H]-UTP (2 min. pulse). Yeast RNA was present throughout the experiment. Centrifugation for 7 hr at 35,000 rev./min. L, M, S: large, middle and small components of BrMV-RNA used as external extinction marker. R₁ and R₂: ribosomal RNA markers. ○ --- ○ acid-insoluble radioactivity; ● --- ● acid-insoluble radioactivity after RNase treatment (5 μg./ml. of RNase for 30 min. at 37°C in 2 x SSC).

Fig. 6. See Fig. 5 Two min. pulse followed by 2 min. chase; 2 M-LiCl-insoluble RNA fraction.

Fig. 7. See Fig. 5. Two min. pulse; 2 M-LiCl-soluble RNA fraction.

Fig. 8. See Fig. 5. Two min. pulse followed by 2 min. chase; 2 M-LiCl-soluble RNA fraction.
Fig. 9. Sedimentation profile in a 5 to 20% sucrose gradient (right to left) of the 2 M-LiCl-insoluble fraction of the RNA isolated from the RNA polymerase preparation after incubation with [\(^{3}H\)]-UTP (2 min. pulse followed by 2 min. chase). Exogenous ribosomal RNA was present throughout the experiment. The RNA product was mixed with carrier BrMV-RNA and heated for 15 min. at 63°C in 1.1 M-formaldehyde, before layering on the gradient. Centrifugation for 7 hr at 35,000 rev./min. Peaks of extinction are indicated by arrows. ○ --- ○ acid-insoluble radioactivity.

Fig. 10. Sedimentation profile in a 5 to 20% sucrose gradient (right to left) of the 2 M-LiCl-insoluble fraction of the RNA isolated from the RNA polymerase preparation after 16 min. incubation with \([^{3}H]\)-UTP. Yeast RNA was present throughout the experiment. Centrifugation for 7 hr at 35,000 rev./min. L, M, S: large, middle and small components of BrMV-RNA used as internal extinction marker. ● --- ● acid-insoluble radioactivity after RNase treatment (5 μg./ml. of RNase for 30 min. at 37°C in 2×SSC). ○ --- ○ acid-insoluble radioactivity.

Kummert (1971b); this RNA was dissociated by heating and was re-associated in the presence of either BrMV-RNA, or exogenous RNA. Results of Table 1 show that re-association was completely inhibited by low concentrations of BrMV-RNA, while the homologous exogenous RNA had no effect on re-annealing. As shown in Table 2, at least 50% of the pulse-labelled product was resistant to RNase before deproteinization.

Sedimentation properties of the labelled RNA product after a long period of incubation in the presence of radioactive UTP

As shown in Fig. 1, the product of incubation of the crude RNA polymerase for 16 min. was largely sensitive to RNase when yeast RNA was present throughout the procedure. The LiCl-insoluble part of this product was fractionated by centrifugation in a sucrose gradient, using BrMV-RNA as an internal marker (Fig. 10). Radioactivity was sensitive to RNase, and was mostly associated with the middle (M) and the S components of BrMV-RNA, together with a shoulder in the region of the L component. The sedimentation profile of the LiCl-soluble fraction of the 16 min. product (Fig. 11) showed that it was made largely of slow-sedimenting RNase-sensitive RNA, together with most of the RNase-resistant RNA found in the total 16 min. product.
Table 1. Re-association of the double-stranded pulse-labelled RNA product in the presence of various RNA's

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Wheat r-RNA</td>
<td>Yeast RNA</td>
</tr>
</tbody>
</table>

* The RNA polymerase fraction was prepared and incubated either without addition of exogenous RNA, or in the presence of wheat r-RNA or yeast RNA.
† RNase treatment was performed by incubating the final RNA products with RNase (5 μg/ml) for 30 min. at 37°C in 10 × SSC.
§ The RNA polymerase fraction was incubated for 2 min. with [3H]-UTP. The double-stranded pulse-labelled RNA product was prepared, heated and reassociated in the presence of various RNAs, and acid precipitable radioactivity was measured as described by Semal & Kummert, 1971b.
‡ BrMV-RNA was prepared as described by Semal & Kummert, 1971b.

Table 2. RNase resistance of the labelled product before deproteinization

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>2 × SSC</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>No RNase</td>
<td>RNase</td>
</tr>
<tr>
<td>1</td>
<td>1014</td>
<td>866</td>
</tr>
<tr>
<td>2</td>
<td>1271</td>
<td>1041</td>
</tr>
</tbody>
</table>

The particulate fraction (Semal, 1970) was isolated after a 2 min. pulse of [3H]-UTP and was incubated with 50 μg/ml of RNase. RNA was then isolated and acid-insoluble radioactivity was expressed as counts/min. for two independent samples.

DISCUSSION

In our previous work (Semal & Kummert, 1971a, b) the double-stranded pulse-labelled RNA synthesized by a crude RNA polymerase fraction prepared from leaves infected with BrMV was shown by hybridization to have nucleotide sequences in common with the RNAs extracted from BrMV particles. However, no clear labelling of single-stranded RNA of the size of the two larger (large = L and middle = M) components of BrMV-RNA were chased from this double-stranded pulse-labelled RNA precursor. In fact, the presence of endogenous nuclease activity in the crude RNA polymerase preparations precluded the identification of single-stranded RNA products on the basis of size.

Our present results (Fig. 4) indicate that avoiding MgCl2 in the medium used for grinding
the leaves, and using short periods of pulse-and-chase, resulted in a definite labelling of the M and S components of BrMV-RNA, together with the association of some radioactivity with the L component. It is possible that when extraction is performed with the modified medium (containing $10^{-3}$ M-EDTA and no MgCl$_2$), the replicative complex is partially deproteinized and the nuclease activity is lowered accordingly. It may be also that treatment with EDTA has a more specific effect, such as dissociation of ribosomes from the replicative complex (Kolakofski & Weissmann, 1971).

Addition of exogenous RNAs during preparation and incubation of the crude RNA polymerase fraction resulted in the labelling of all three BrMV-RNAs, including component L which is probably a mixture of acrylamide RNA species 1 and 2 (Lane & Kaesberg, 1971); we do not presently know, however, whether both RNA species were labelled. As expected from size, the L component was more sensitive in our hands to various agents (including traces of RNase) than the M and S components. Thus it is possible that the L component was also synthesized in the absence of exogenous RNA, but was rapidly degraded to slower-sedimenting material.

The addition of protective exogenous RNA during incorporation of RNA precursor by a crude RNA polymerase preparation raises the question of the possible role of exogenous RNA as a template for RNA synthesis by endogenous polymerases. Our experiments on self-re-annealing of the pulse-labelled double-stranded RNA product, which is the precursor of the single-stranded RNA, indicate that the early product made in the presence of exogenous RNA is entirely virus-specific. The fact that liver ribosomal RNA and 8 s yeast RNA have identical effects on the size of the single-stranded product, also points to the protective function of exogenous RNA.
All three components of BrMV-RNA were labelled \textit{in vitro} after 6 to 24 hr incubation of infected leaves with radioactive precursors (Hiruki, 1969); however, component M was not labelled \textit{in vitro} after 1 hr pulse (G. Philips, personal communication). Our results \textit{in vitro} indicate that under suitable conditions of pulse-chase, all three classes of single-stranded BrMV-RNA are labelled at comparable rates. Data are consistent with $[^3H]$-UTP being covalently linked to virus RNA (Fig. 9).

Bockstahler & Kaesberg (1965) proposed that components M and S arise \textit{in vivo} by a single cleavage of component L. Terminal labelling studies (Glitz & Eichler, 1971) led to the conclusion that component L may be cleaved to from components M and S, while component M may further be cleaved to give two fragments sedimenting as component S. Lane & Kaesberg (1971) suggested that component S was derived from component M. Our \textit{in vitro} studies indicate that when the labelling of component L was low compared to that of component M (Fig. 4, and unpublished results), the radioactivity of peak S was increased accordingly, and this probably reflects the degradative transformation of component L into material which happens to sediment as component S, with component M remaining unaffected. Further studies are needed to establish the relationship among the classes of BrMV-RNA synthesized \textit{in vivo} or \textit{in vitro}.

The major part of the radioactive RNA product of a 2 min. pulse was soluble in 2 M-LiCl, was entirely resistant to RNase in 2 x SSC, and sedimented as a major peak at 14 s and minor peaks of slower-sedimenting material. The other part was insoluble in 2 M-LiCl, was partially resistant to RNase in high salt, and sedimented heterogeneously between 14 s and 28 s.

After 2 min. chase, followed by deproteinization, the following modifications were observed; (1) the LiCl-soluble 14 s peak decreased sharply, while the minor peaks were quantitatively unchanged but displayed increased sensitivity to RNase; (2) the LiCl-insoluble fraction increased quantitatively and was composed essentially of the 3 classes of single-stranded (SS) BrMV-RNA.

These results suggest that LiCl-insoluble pulse-labelled material which displays the properties of replicative intermediate (RI), and LiCl-soluble 14 s product, both contribute to the formation of the LiCl-insoluble RNase-sensitive RNA which is generated during the chase. They differ from results obtained with RNA polymerase from several animal virus systems as illustrated by poliovirus (Girard, 1969). In the latter system, the pulse-labelled product was largely LiCl-insoluble RI, and was transformed during a chase into SS virus RNA and RF, causing an increase in LiCl-soluble RF.

Little is known of the mechanism of replication of virus RNA in plants, mainly because of the difficulties of performing pulse-chase experiments in whole tissue. In the case of BrMV, the \textit{in vitro} synthesis of SS-RNA from the 2 min. pulse-labelled RNase-resistant precursor proceeds along the main scheme of RI $\rightarrow$ SS-RNA. This is coupled with the accessory scheme of RNase-sensitive RNA being generated from the 14 s LiCl-soluble RNase-resistant RNA.

If the RNase-sensitive product of the 16 min. incubation (Fig. 11) consists of BrMV-RNA generated from RNase-resistant precursors in a similar way to the 2 min. pulse-chased RNA product, then the \textit{in vitro} synthesis of BrMV-RNA is a continuous process resulting from recycling activity of the RNA polymerase. Such a system is compatible with the cumulative synthesis of SS-RNA from a limited amount of an RNase-resistant intermediate of replication (Fig. 1 and 2).

Results essentially similar to those of BrMV have been obtained with the \textit{in vitro} synthesis of broadbean mottle virus RNA by J. M. Jacquemin (personal communication).
This work was supported in part (J.K.) by the ‘Institut pour l’Encouragement de la Recherche Scientifique dans l’Industrie et l’Agriculture’ Brussels and in part (J.S.) by the ‘Fonds National de la Recherche Scientifique’ Brussels. We express our sincere appreciation to Anne-Marie Pollart and Paulette Janssens for able technical assistance. Thanks are expressed also to Prof. C. Duculot and Dr Ph. Drèze, CAMIRA, Gembloux; and to Messrs E. François and A. Riga, Station de Physique et Chimie agricoles, Gembloux, for help with the radioactivity measurements.

REFERENCES


(Received 12 January 1972)