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Highly active template-specific RNA-dependent RNA polymerase from barley leaves infected with brome mosaic virus

[RNA replicase/viral replication/RNA synthesis/RNA nucleotidyltransferase (RNA dependent)]

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ABSTRACT The extraction of a template-dependent and template-specific RNA-dependent RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from a eukaryotic source is described. The enzyme, extracted from barley leaves infected with brome mosaic virus (BMV), is capable of incorporating high levels of radioactivity into trichloroacetic acid-insoluble products. The purification procedure included solubilization with nonionic detergent and precipitation with polyethylene glycol. The enzyme was more than 50 times more active than was a comparable preparation from mock-inoculated leaves and was stimulated more than 15-fold by the addition of BMV RNA to the reaction. Other viral RNA templates were less than 25% as efficient as was BMV RNA in stimulating UMP incorporation; poly(A), tRNA, and mRNA gave little stimulation and rRNA was inactive. Autoradiographic analysis after electrophoretic separation of the radioactive products from reaction mixtures containing BMV RNA template revealed prominent bands that coelectrophoresed with replicative forms of BMV RNAs. When BMV RNA template was enriched in RNA3 or RNA4, larger proportions of the products were replicative forms of RNA3 or RNA4, respectively.

The rapid synthesis of viral RNA after infection of eukaryotic cells by viruses having an RNA genome requires the presence of an active replicase (RNA-dependent RNA polymerase; nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). Although a template-specific replicase has been isolated and well characterized from *Escherichia coli* infected with bacteriophage Qβ (1, 2), only limited success has been achieved in isolating a similar enzyme from virus-infected plant or animal tissues. A fairly active enzyme, showing a degree of template specificity, has been isolated from leaves of cowpeas infected with cowpea mosaic virus (3). Replicase has been extracted from smooth membranes of encephalomyocarditis virus-infected BHK-21 hamster cells (4), but BHK rRNA and Qβ RNAs were equally good templates. RNA-dependent RNA polymerase activity has been described for plant tissues infected with several viruses, including tobacco mosaic virus (TMV) (5, 6), turnip yellow mosaic virus (7), alfalfa mosaic virus (8), tobacco necrosis virus (9), cucumber mosaic virus (10), cowpea chlorotic mottle virus (CCMV) (11), and brome mosaic virus (BMV) (12–14). However, in most of these instances, stimulation of RNA polymerase activity can also be detected in mock-infected (wounded) tissue.

We describe here the extraction and high biological activity of an enzyme from BMV-infected barley leaves that is considerably stimulated by the addition of RNA and for which BMV RNA is a markedly better template than other RNAs tested thus far.

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MATERIALS AND METHODS

Plant Culture, Inoculation, and Polymerase Extraction. Barley (*Avena sativa* Linnaeus cv. Dickson) plants were grown in a mixture of equal parts of vermiculite and soil in a 25°C greenhouse having 24 hr of supplemental fluorescent light. The plants were watered daily except for the day of inoculation and the preceding day. Seven or 8 days after planting, the first leaves were inoculated with BMV (0.2 mg/ml of 10 mM Na acetate, pH 4.5, containing 1 mM Mg acetate). Four days after inoculation, when the first leaves showed abundant mosaic symptoms, the symptomless second leaves were excised and placed on ice; all subsequent procedures were at 0–4°C. Approximately 2 g of leaf tissue was ground in 25 ml of 10 mM Mg acetate/10 mM KCl/1 mM Na_2EDTA/10 mM dithiothreitol/50 mM Tris·HCl, pH 7.4, (buffer A) with glass used as an abrasive. The slurry was centrifuged for 10 min in a Beckman JA-20 rotor at 3000 rpm. The supernatant was made 10% (vol/vol) in glycerol and centrifuged for 15 min at 20,000 rpm. Five milliliters of buffer A, 1 ml of glycerol, and 0.1 ml of Nonidet P-40 were added to the pellet and stirred for 90–120 min until there was an even suspension. The preparation was centrifuged for 15 min at 20,000 rpm and the pellet was discarded. The supernatant was made 10% (vol/vol) in polyethylene glycol (6000–7500 molecular weight) and stirred for 30 min, divided into portions (0.75 ml), and frozen at −70°C. Immediately prior to use, the extract was thawed and centrifuged for 10 min at 10,000 rpm. The supernatant was discarded, and the bright green "oily" layer (fraction A) and the green pellet (fraction B) were combined. One milliliter of 10 mM $MgCl_2$/25 mM $(NH_4)_2SO_4$/10 mM dithiothreitol/50 mM Tris·HCl, pH 8.0, containing 1 mM each ATP, GTP, and CTP (buffer B) was added per gram of starting material. The enzyme preparations retained good activity for 1–2 weeks when stored at −70°C in the presence of glycerol after precipitation with polyethylene glycol.

Polymerase Assays. Ethanol was removed from radioactive nucleotide substrates by lyophilization (for ^3H-labeled compounds) or evaporation under a stream of N_2 (for ^{32}P-labeled compounds). The radioactive materials were immediately dissolved in buffer B to give solutions containing 5 mCi/ml (1 Ci = 3.7×10^{10} becquerels). Protein was measured by the technique of Lowry *et al.* (15). Plant viral RNAs used as templates were isolated from virion preparations by phenol extraction and ethanol precipitation (16). mRNA and rRNA were prepared from bean seed (17); Qβ RNA was a generous gift from A. Palmenberg; tRNA from *Bacillus subtilis* was purchased from General Biochemicals (Chagrin Falls, OH).

Abbreviations: BMV, brome mosaic virus; CCMV, cowpea chlorotic mottle virus; TMV, tobacco mosaic virus; NaCl/Cit, standard saline citrate.

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Glass fiber filter assay. This assay was used for reactions involving [^{3}H]UTP as the radioactive substrate. Each reaction contained 25 μCi of [^{3}H]UTP (10 or 46 Ci/mmol), actinomycin D at 25 μg/ml, BMV RNA at 36–380 μg/ml, and 50–90 μl of enzyme (in buffer B); the final volume was adjusted to 100 μl with buffer B. Typically, incubation was for 1 hr at 30°C. The reaction was stopped by the addition of 3 ml of 10% trichloroacetic acid containing 4% $Na_4P_2O_7$ and 4% NaH_2PO_4. The tubes were placed on ice for about 10 min. Initially, 100 μg of bovine serum albumin was added as a carrier, but it was found to be unnecessary and was omitted in later experiments. The reaction mixtures were filtered under reduced pressure through Whatman GF/C filters (24-mm diameter) that had been wetted with 6 ml of 5% trichloroacetic acid containing 2% NaH_2PO_4 and 2% $Na_4P_2O_7$ (solution D). The retained material was sequentially washed with 30 ml of solution D, 30 ml of 100 mM $Na_4P_2O_7$ in 1 M HCl (solution E), 10 ml of 80% (vol/vol) ethanol, and 6 ml of diethyl ether. The filters were dried under an infrared lamp and radioactivities were measured in toluene scintillator (18) at 23% efficiency.

Paper disc assay. This assay was used for reactions involving [^{32}P]UTP (250 μCi/ml, ≈410 Ci/mmol) as the labeled substrate. The reaction mixtures were similar to those described above but were scaled down to 50 μl and contained 0.5 μM UTP (in buffer B). At the end of the reaction, a 40-μl sample was spotted onto a 2.25-cm Whatman 3 MM paper disc that had been prewetted with 20% trichloroacetic acid containing 8% NaH_2PO_4 and 8% $Na_4P_2O_7$. The sheets of discs (18) were washed (5 min per wash) three times with solution D, twice with solution E, twice with a 1:1 mixture of ethanol and diethyl ether, and twice with diethyl ether. After drying, the radioactivities of the discs were measured in toluene scintillator at ≈100% efficiency.

Assay of RNA Products. Products, labeled with ^{32}P in a 250-μl reaction mixture, were extracted with equal volumes of 100 mM NaCl/10 mM Na_2EDTA/0.5% sodium dodecyl sulfate in 100 mM Tris·HCl, pH 7.2, and water-saturated phenol. The RNA was precipitated with ethanol from the aqueous phase at −20°C. Single-stranded RNA was digested at 30°C for 30 min in 0.3 M NaCl/0.03 M sodium citrate, pH 7.0, [2× standard saline citrate (NaCl/Cit)] containing RNase A (20 μg/ml, Sigma) and RNase T1 (30 units/ml, Sigma). Subsequently, the RNase was digested with proteinase K (500 μg/ml, Beckman) at 37°C for 1 hr. The RNA products were subjected to electrophoresis in 12.5 cm × 15.5 cm × 1.5 mm slab gels containing 2.4% acrylamide, 0.1% methylenebisacrylamide, and 0.45% agarose in Tris/borate buffer (19). Just before electrophoresis, the RNA samples were heated to 60°C for 5 min in 5 M urea. The gels were run at 25–30 mA for 2 hr at 4°C. Replicative forms of BMV and viral RNAs synthesized in barley protoplasts 16 hr after inoculation with BMV RNA were used as markers.

RESULTS

Comparison of Endogenous and BMV Polymerase Activities. One of the difficulties in detecting RNA replicase activity in many plant tissues is the presence of an endogenous RNA polymerase that is stimulated by traumatization (14, 20). Romaine and Zaitlin (6) found that although healthy tobacco leaves had little RNA-dependent RNA polymerase activity, mock inoculation resulted in the production of measurable levels of activity. Higher activities were detected after inoculation with TMV. However, there was a great difference in the activity of the enzyme extracted from second leaves of barley from mock-inoculated or from BMV-infected plants (Table 1). Although fraction A had greater activity than fraction B, the latter fraction contained approximately 40% of the total activity for extracts from infected plants. For this reason, fractions A and B were combined for use as polymerase in all subsequent experiments. For typical preparations, the activity of UMP incorporation into trichloroacetic acid-insoluble material by the "wound" polymerase is negligible in comparison with that of the virus-induced polymerase. As a further control to the experiments shown in Table 1, extracts from directly traumatized second barley leaves (in contrast to extracts of second leaves after inoculation or mock inoculation of the first leaf) were assayed. The activity of such extracts was very small (14,000–19,000 cpm by the paper disc assay) compared with that of polymerase from BMV-infected leaves (over 500,000 cpm in a similar assay).

Table 1. Comparison of polymerase activity from BMV-infected and mock-inoculated barley plants

Fraction	[^{3}H]UMP incorporation, cpm	
	BMV-infected	Mock-inoculated
A	71,300	1700
B	43,800	1400

Each reaction mixture contained 3.6 μg of BMV RNA and 50 μl of enzyme in a standard 100-μl glass fiber filter assay.

Characteristics of BMV Polymerase. The kinetics of the reaction and its dependence on added BMV RNA are shown in Fig. 1 *A* and *B*. The incorporation of [^{32}P]UMP into tri-

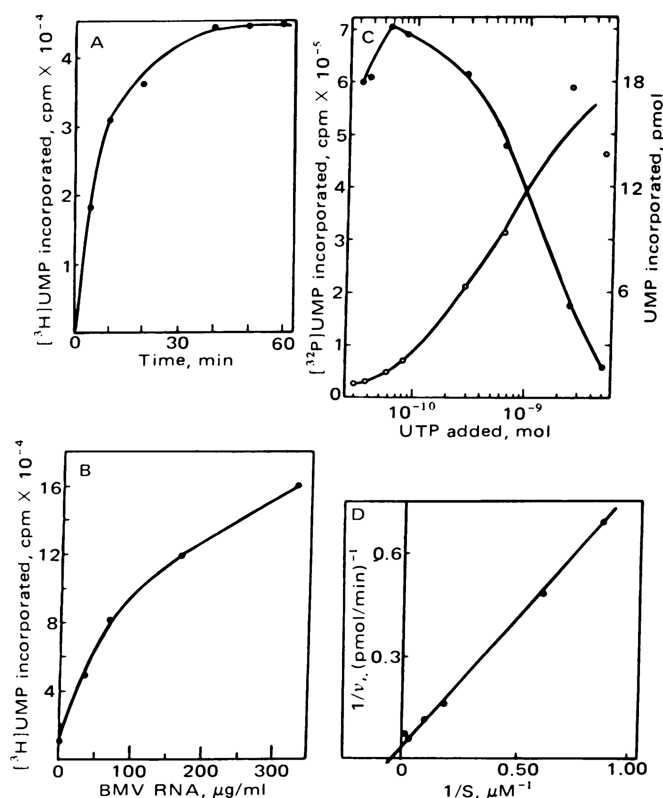

FIG. 1. Characteristics of BMV RNA polymerase. Standard glass fiber filter assays were used to follow the kinetics of [^{3}H]UMP incorporation into trichloroacetic acid-insoluble material (*A*) and dependence on added BMV RNA (*B*). The reaction mixtures in *A* contained 38 μg of BMV RNA and 90 μl of enzyme; those in *B* were incubated for 60 min and contained 50 μl of enzyme. A comparison of optimal conditions for incorporation in terms of radioactivity (●) and number of moles (○) is shown in *C*. Each 50-μl reaction mixture contained 33 μg of BMV RNA, 12.5 μCi of [^{32}P]UTP, and additional UTP to give the values shown. Incorporation into acid-insoluble material was determined by the paper disc assay for 40-μl samples and is corrected for zero time values of 20,000 cpm; at low incorporation levels the experimental error is high. A double reciprocal plot of the data from *C*, yielding a K_m for UTP of 15.3 μM, is shown in *D*.

chloroacetic acid-insoluble material was directly proportional to the volume of enzyme in the reaction mixture and varied from 35,500 cpm for 5 μl (14 μg of protein) of enzyme in the reaction mixture to 317,400 cpm for 40 μl of enzyme. Electrophoretic analysis of the polymerase preparation showed it to contain many polypeptides (data not shown). The reaction was sensitive to the omission of CTP or GTP and very sensitive to the omission of ATP (Table 2). The latter sensitivity may reflect a requirement for ATP as an energy source in addition to its being a substrate for RNA synthesis. The reaction was dependent on UTP concentration, which in practice enforced a choice between maximizing the reaction for incorporation of radioactivity or for molar levels of UMP incorporated. Maximal incorporation of radioactivity was obtained with 1.14 μM UTP (0.57 pmol of UTP added per 50 μl), and at least 50 μM UTP (2.5 nmol of UTP added per 50 μl) for maximal molar incorporation (Fig. 1*C*). If all of the UTP could be added as [^{32}P]UTP, then maximal radioactive incorporation would, of course, correspond to the value for maximal molar incorporation. The K_m for UTP-dependent activity was 15.3 μM (Fig. 1*D*).

A broad plateau for activity with respect to Mg^{2+} was obtained, with an optimal level of 8–10 mM (data not shown). The addition of ethylenediaminetetraacetic acid in the absence of added Mg^{2+} did not entirely eliminate activity. K^+ was inhibitory, a proportional decrease in activity being noted as the level of added K^+ was increased from 0 to 56 mM. The amount of UMP incorporated into acid-insoluble material changed only slightly as the incubation temperature was increased from 25°C to 35°C, and 30°C was used for all experiments shown.

The data of experiment 2 of Table 2 indicate that the activity observed for BMV replicase is not due to any of several DNA-dependent polymerases or phosphorylases known to be present in normal plant tissues (12, 14). The retention of maximal activity in the presence of high levels of actinomycin D indicates that DNA is not the preferred template of this enzyme and hence that it is not a DNA-dependent RNA polymerase. The insensitivity to α-amanitin makes it unlikely that DNA-dependent RNA polymerase II of nuclear origin is active; rifampicin insensitivity indicates that DNA-dependent RNA polymerases from organelles are not responsible for the incorporation. The inhibition shown by low levels of pyrophosphate but not by low levels of orthophosphate confirms the absence of polynucleotide phosphorylase activity.

Table 2. Effect of nucleotide triphosphate omission or antibiotic addition on BMV RNA polymerase activity

Component omitted from or added to reaction	Radioactivity incorporated, cpm	%
Exp. 1, omissions		
None	200,000	100
ATP	6,000	3
CTP	85,000	42
GTP	67,000	33
Exp. 2, additions		
None	176,000	100
Actinomycin D (70 μg/ml)	198,000	113
Rifampicin (100 μg/ml)	176,000	100
Rifampicin (5 μg/ml)	186,000	106
α-Amanitin (1 μg/ml)	189,000	107
α-Amanitin (0.1 μg/ml)	193,000	110
Na_2HPO_4 (2 mM)	163,000	93
$Na_4P_2O_7$ (2 mM)	81,000	46

Each series of reaction mixtures contained [^{32}P]UTP. Radioactivity incorporated into trichloroacetic acid-insoluble material was measured by standard 50-μl paper disc assays. For Exp. 1, 40-μl samples were taken from reaction mixtures containing 7.2 μg of BMV RNA; for Exp. 2, 30-μl samples of reaction mixtures containing 25 μg of BMV RNA were taken. Different enzyme preparations were used for Exps. 1 and 2. In a separate experiment, it was shown that Na^+ is not inhibitory in concentrations of up to 15 mM.

Template Dependency and Specificity. The incorporation of [^{3}H]UMP was stimulated more than 15-fold by the addition of BMV RNA (Fig. 1*B*). The reaction was notably template specific: the addition of rRNA gave no stimulation of activity and even RNA from the closely related CCMV had only 20–22% of the activity of BMV RNA (Table 3). A poly(A)-containing 16S RNA known to be an active messenger template for protein synthesis (17) had negligible activity as a template for BMV polymerase.

Product Characterization. The total reaction products were found to be 73% resistant to ribonuclease digestion in 2× NaCl/Cit. RNA was extracted from the reaction mixture, adjusted to 2 M in LiCl, and centrifuged to obtain a predominately double-stranded RNA supernatant containing 90% of the radioactivity. Characteristics of this RNA (Table 4) imply that some of the double strands consist of small segments that are soluble in trichloroacetic acid.

The electrophoretic separation of products synthesized in reactions to which no RNA, TMV RNA, or BMV RNA was added as template is shown in Fig. 2*A*. The reaction mixture containing added BMV RNA incorporated much higher levels of [^{32}P]UMP (cf. Table 3), but the volumes of samples added to the gel were adjusted so that similar amounts of radioactivity were added to each lane. This procedure greatly accentuates the apparent endogenous activity, and it is clear that BMV RNA present in the enzyme preparation serves as template for the lower levels of incorporation seen in the absence of added RNA. No products were seen at a position corresponding to 4×10^6 daltons, which is where the replicative form of TMV RNA would be expected to appear. Ribonuclease treatment of the products before electrophoresis did not cause any significant changes to the product profiles (Fig. 2*A*, lanes 4–6), indicating that the majority of products were double-stranded. A direct comparison of the electrophoretic mobility of single-stranded

Table 3. Template specificity of BMV polymerase

Exp.	Enzyme, μl	RNA, μg	Source or type of RNA	[^{3}H]UMP incorporation, cpm	%
1	50	3.6	BMV	18,000	100
			CCMV	4,000	22
			TMV	2,900	16
			TEV	4,000	22
2	80	16.8	BMV	64,900	100
			CCMV	13,000	20
			mRNA	2,900	4
			rRNA	250	0
3	90	25	BMV	36,900	100
			AMV	5,600	15
			Poly(A)	5,400	14
			Qβ	700	2
			tRNA	2,800	8

For each experiment, an entirely different preparation of enzyme was used. Radioactivity (cpm) is shown for [^{3}H]UMP incorporation into trichloroacetic acid-insoluble material for 100-μl samples of standard glass fiber filter assays containing the indicated amounts of enzyme and is corrected for reactions without added RNA template (approximately 4000 cpm). With the exception of Qβ and TEV (tobacco etch virus) RNA, each viral RNA was confirmed to be intact by electrophoretic analysis, and CCMV RNA was shown to be infectious. AMV, alfalfa mosaic virus. tRNA was from *Bacillus subtilis;* rRNA was from bean seed; the mRNA was poly(A)-containing RNA coding for a bean seed protein (19).

Table 4. Characteristics of double-stranded RNA synthesized by BMV polymerase

Sample	Treatment	Trichloroacetic acid-precipitable material recovered, cpm	Significance
A	None (kept on ice)	15,700	Quantity of acid-precipitable radioactivity present in each sample of LiCl-soluble product
B	RNase in 2× NaCl/Cit	16,200	RNA is resistant to RNase before heating
C	Heated to 100°C for 10 min in 0.1× NaCl/Cit, then cooled quickly	7,900	Maximum amount of acid-precipitable radioactivity recoverable after heating
D	Same as C, then RNase in 2× NaCl/Cit	1,900	RNA becomes RNase-sensitive after heating
E	Heated to 100°C for 10 min in 0.1× NaCl/Cit, cooled slowly, then RNase in 2× NaCl/Cit	3,900	Some of the heated RNA will reanneal into a RNase-resistant product

RNA was extracted from a 200-μl reaction mixture containing [^{32}P]UTP. The ethanol-precipitated RNA was dissolved in 89 mM Tris/borate buffer (pH 8.9) containing 3 mM ethylenediaminetetraacetic acid, and an equal volume of 4 M LiCl was added. After 18 hr at 0°C, the LiCl-soluble nucleic acids were recovered by ethanol precipitation and dissolved in 0.1× NaCl/Cit. Samples of this material (20 μl) were used for each of the above treatments. A control sample of BMV [^{3}H]RNA (labeled *in vivo*) containing 30,825 cpm was subjected to RNase digestion under conditions identical to those for sample D; only 910 acid-precipitable cpm was recovered, showing the efficient digestion of single-stranded RNA under the conditions used.

BMV RNA, double-stranded BMV RNA, and products of the polymerase reaction (Fig. 2*B*) established that the *in vitro* products corresponded to the replicative forms.

To confirm that initiation of replication on the added template actually took place during the reaction, samples that were highly enriched in individual BMV RNA components were added to the polymerase reaction mixture. Samples containing enriched RNA components were prepared by collecting individual zones from sucrose density gradients after centrifugation. Several cycles of density gradient centrifugation resulted in a preparation containing approximately 99% RNA3 and in another containing >99% RNA4 as analyzed by polyacrylamide gel electrophoresis. Electrophoretic analysis of products from reaction mixtures containing these templates clearly showed (Fig. 3) enhancement of radioactive UMP incorporation into RNA that migrated as expected for the replicative form of the supplied BMV RNA component. Densitometer analysis of the four bands representing BMV replicative forms indicated that when template RNA enriched in RNA3 was added to the reaction, 74% of the label occurred in RNA3 replicative form and 13% in RNA4 replicative form. When unfractionated BMV RNA was added to the reaction mixture, 23% of the label was in RNA3 replicative form and 53% was in RNA4 replicative form. Similarly, when template RNA enriched in RNA4 was added to the reaction mixture, 84% of the label occurred in RNA4 replicative form and 10% in RNA3 replicative form.

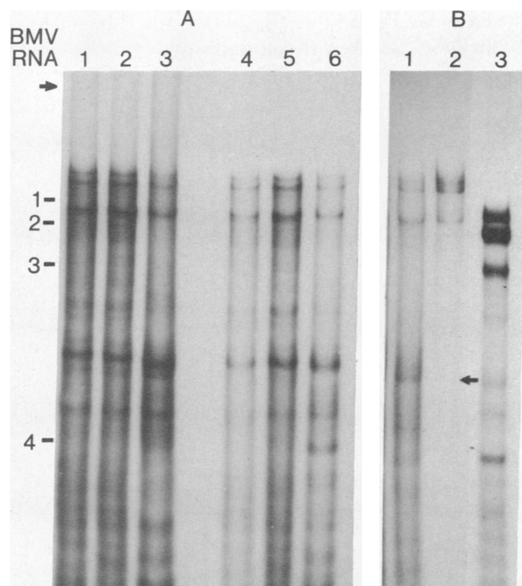

FIG. 2. Autoradiography of polynucleotide products of BMV polymerase. Labeled nucleic acids were phenol-extracted from reaction mixtures containing [^{32}P]UTP. A portion of the RNA products was incubated with RNase A at 20 μg/ml and RNase T1 at 30 units/ml in 2× NaCl/Cit and then with proteinase K at 0.5 mg/ml. Samples were subjected to electrophoresis through 2.4% polyacrylamide/agarose gels at 27 mA for 2 hr. (*A*) Comparison of untreated products (lanes 1–3) with RNase-incubated products (lanes 4–6). The products were synthesized in reaction mixtures containing no added RNA (lanes 1 and 4), added TMV RNA (lanes 2 and 5), or added BMV RNA (lanes 3 and 6). For comparison, the samples were adjusted to contain similar levels of radioactivity. Positions of single-stranded (genomic) BMV RNAs run in the same gel are indicated by the numbers on the left. The arrow indicates the position where a band of replicative form of TMV RNA (4 × 10^{6} daltons) would be expected. (*B*) Electrophoretic migration of RNase-incubated products formed by using BMV RNA as template (lane 1) and of replicative forms of BMV RNAs synthesized in BMV-infected barley protoplasts (lane 2); the arrow indicates the position of a faint band corresponding to the replicative form of RNA4. Lane 3 contains single-stranded RNAs prepared from BMV-infected barley protoplasts; the viral RNA components appear as intense bands.

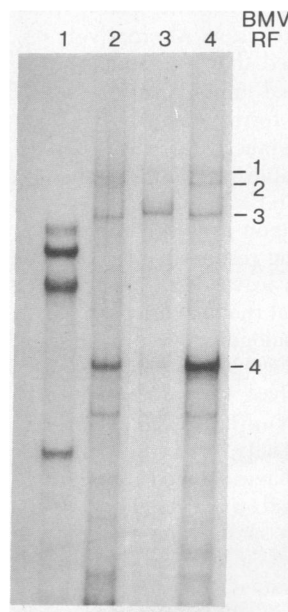

FIG. 3. Autoradiograph of polynucleotide products of BMV polymerase. Labeled nucleic acids were phenol-extracted from reaction mixtures containing [^{32}P]UTP. The products were synthesized in reaction mixtures containing unfractionated BMV RNA (lane 2); BMV RNA highly enriched in RNA3 (lane 3); or BMV RNA highly enriched in RNA4 (lane 4). The samples were adjusted to contain similar levels of radioactivity. Lane 1 contains single-stranded genomic RNA isolated from BMV-infected barley protoplasts. Designation of BMV replicative forms (RF) is indicated at the right.

DISCUSSION

The term "replicase" has been used for RNA-dependent RNA polymerases from prokaryotic cells that selectively copy the genomic RNA of bacteriophages such as Qβ. In plants and other eukaryotes that are infected by viruses having an RNA genome, enzymes having a replicase function must exist because large quantities of viral RNA are synthesized after infection. These enzymes apparently do not copy tRNAs, mRNAs, or rRNAs present in the cell. Hence, like Qβ replicase, they must be capable of selecting the RNA templates to be copied. Thus far, the polymerase activity detected in virus-infected plants (by assay of UMP incorporation into trichloroacetic acid-insoluble material) has only rarely been shown to exhibit any template specificity. Starting with Chinese cabbage leaves infected with turnip yellow mosaic virus, Mouchès *et al.* (7) prepared a replicase that showed a degree of template specificity but had activity considerably lower than that reported here. Zabel *et al.* (3) used a competition assay for the binding of cowpea mosaic virus replicase to nitrocellulose filters to provide evidence for template recognition. An RNA polymerase preparation from BMV-infected barley leaves yielded RNA products that, on electrophoretic separation, showed a heterodisperse profile containing peaks of radioactivity in regions close to the positions of BMV RNA components (13). The activity of this preparation was low, and addition of BMV RNA resulted in only a 1.7- to 3.3-fold increase in UMP incorporation. A limited template specificity was shown for a Triton X-100-solubilized RNA polymerase from BMV-infected barley leaves (12). In this case, although CCMV RNA was found to have nearly 90% of the template activity of BMV RNA (compared with 20–22% for CCMV RNA in the experiments shown in Table 3), TMV RNA showed only 20% of the BMV RNA activity, a value similar to our present studies. However, the overall activity of the Triton-solubilized enzyme was very low.

Apart from the above exceptions, the lack of template specificity and generally low activity of RNA polymerases isolated from plant tissues to date demands that caution be taken in deciding if these enzymes play a major role in the replication of viral pathogens having an RNA genome. In contrast, the enzyme described in the present study exhibited a template specificity similar to that expected for a virus-specific replicase. Viral RNAs other than BMV RNA were inefficient templates (Table 3), and although TMV RNA stimulated incorporation of radioactive label, no evidence of synthesis of TMV replicative form could be discerned after electrophoretic analysis of the products (Fig. 2A); conceivably, the TMV RNA served to protect endogenous BMV RNA in the reaction. Alternatively, if TMV specific products were synthesized, they were small and heterodisperse and therefore migrated much faster upon electrophoresis than BMV replicative forms. Cellular RNAs such as tRNA, rRNA, and mRNA were inactive, or very poor, templates for the BMV polymerase (Table 3). These data provide convincing evidence that, at least in BMV-infected barley leaves, replication does not depend upon a ubiquitous host polymerase like that suggested as being responsible for viral replication in tobacco (21) or cowpea leaves (22). The poor template function of poly(A) shows that the enzyme we have isolated is not a poly(U) polymerase analogous to that isolated from HeLa cells infected with poliovirus (23).

A combination of factors appears to have been important in contributing to the extraction of an enzyme having a high level of viral RNA-specific polymerase activity. (*i*) Symptomless second leaves from plants whose first leaves showed abundant symptoms of BMV infection were used. (*ii*) Techniques requiring the use of high ionic strength such as ion-exchange chromatography, ammonium sulfate precipitation, and washing pellets with high salt were avoided. Instead, Nonidet P-40 (a nonionic detergent) was used for solubilization and polyethylene glycol was used to precipitate the protein. (*iii*) Ethanol was removed from the radioactive nucleotide triphosphate substrate. As evidenced by the data of Table 3, different preparations had various levels of activity. Nevertheless, all preparations had good activity, and the variation appears to be attributable mainly to differences in the intensity of infection due to slight changes in the environment of the host plants, in the quality of the viral inoculum, and in inoculation procedures.

Electrophoretic analysis of the reaction products revealed major bands of RNase-resistant radioactive RNA that comigrated with double-stranded forms of BMV RNA (Fig. 2). Of particular interest is the appearance of a double-stranded RNA migrating in the expected position for the replicative form of BMV RNA4. This evidence supports the report by Bastin and Kaesberg (24) of a possible replicative form of RNA4 in BMV-infected barley leaves. Because RNA4 is not required for infection and its sequence is present in BMV RNA3 (25), RNA3 could have served as the template for double-stranded RNA4. However, RNA4 replicative form was synthesized from RNA4 template, but not from RNA3 template. These data established that initiation of replication took place on the supplied template.

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