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In Vitro Replication of Tobacco Mosaic Virus RNA in Tobacco Callus Cultures: Solubilization of Membrane-Bound Replicase and Partial Purification[1]

JAMES L. WHITE* AND H. H. MURAKISHI

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

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A fraction containing membrane-bound tobacco mosaic virus RNA replicase was isolated from tobacco mosaic virus-infected tobacco callus cultures. The replicase activity reached a maximum 60 h after inoculation and then declined. The enzyme activity was insensitive to actinomycin D and DNase. The corresponding fraction from healthy callus contained essentially no activity. The viral RNA synthesis in vitro proceeded linearly for 30 min and required the four nucleotide triphosphates and Mg^{2+} ions. Mn^{2+} was a poor substitute for Mg^{2+}. During RNA synthesis the product was at least 70% resistant to RNase in 2X SSC (0.15 M NaCl plus 0.015 M sodium citrate), but completely digested by RNase in 0.1X SSC. Analysis of the product by polyacrylamide gel electrophoresis revealed a double-stranded RNA (4.0×10^6 daltons) that appeared to be replicative form and a partially RNase-resistant structure similar to replicative intermediate form. Washing the membrane-bound replicase with Mg^{2+}-deficient buffer solubilized the enzyme. The solubilized enzyme was further purified by DEAE-Sephadex column chromatography. The DEAE-purified enzyme was nearly completely dependent upon tobacco mosaic virus RNA for activity. Analysis of the product on a sucrose gradient revealed a double-stranded RNA with sedimentation of 16S and smaller heterogeneous RNase-sensitive products.

The study of phage-specific RNA-dependent RNA polymerase (replicase) has been aided by the success in purifying stable, template-free enzyme. Qβ replicase has been purified to a high degree of homogeneity (8, 9, 12, 13). The active enzyme complex consists of four polypeptides, one of which is the gene product of the replicase cistron of the phage genome (12, 13). A unique property of the enzyme is its template specificity. The only nucleic acids known to serve as templates are Qβ RNA, Qβ complementary strand, and variants of Qβ RNA, including 6S RNA present in Qβ-infected cells.

The investigation of eukaryotic viral RNA replication has been hampered by the inability to isolate stable, template-free replicase in a soluble form. Most RNA replicases isolated from infected plant or animal cells are associated with membranes (2, 11, 15, 20, 31) and possess endogenous template. Attempts to solubilize RNA replicases from membranes by using various detergents resulted in enzyme preparations that were quite unstable (2, 7, 10, 12, 23, 26, 32) or still contained template RNA (2, 7, 21, 26).

An RNA polymerase from tobacco mosaic virus (TMV)-infected plants was purified to an extent where its activity was completely dependent upon TMV RNA for its activity (23); however, the enzyme was quite unstable. Replicase from cowpea mosaic virus-infected tissue has been purified from membranes by using a Mg^{2+}-deficient buffer. This procedure, gentler than detergent disruption of membranes, resulted in soluble, stable, template-free enzyme (30).

During studies of viral replicases, an RNA-dependent RNA polymerase was detected in the soluble fraction of healthy tissue homogenates in Chinese cabbage (host for turnip yellow mosaic virus) (3) and tobacco (host for TMV) (6). The enzyme activity in tobacco leaves is stimulated upon TMV infection. Its function in healthy plants is unknown.

In this paper we report some properties of TMV replicase and soluble RNA-dependent RNA polymerase in TMV-infected callus; also described is a procedure to release TMV replicase from membranes, resulting in a stable, template-free enzyme. After DEAE-Sephadex chromatography, the enzyme is nearly completely dependent upon TMV RNA as a template.

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MATERIALS AND METHODS

Buffers. Buffer A contained: sucrose, 0.4 M; KCl, 10 mM; $MgCl_2$, 5 mM; dithiothreitol (DTT) or dithioerythritol (DTE), 2.5 mM; Tris, 150 mM, pH 8.2 at 4°C; and glycerol, 10% (vol/vol). Buffer B contained: KCl, 10 mM; DTT or DTE, 2.5 mM; NH_4Cl, 25 mM; Tris, 10 mM, pH 8.0 at 4°C; and glycerol, 10% (vol/vol). Buffer C contained: NH_4Cl, 10 mM, ethylene glycol-bis-(β-aminomethyl ether)N,N'-tetraacetic acid, 10 mM; DTT or DTE, 5 mM; Tris, 100 mM, pH 8.2 at 4°C; and glycerol, 10% (vol/vol). Buffer E contained: sodium acetate, 20 mM; sodium EDTA, 1 mM; and Tris, 40 mM, brought to pH 7.8 with glacial acetic acid containing 5% sucrose. SSC was 150 mM NaCl plus 15 mM sodium citrate, pH 7.0.

Preparations of tissue cultures and inoculation. A pigmented cell culture derived from pith of tobacco (*Nicotiana tabacum* L. var. Havana 38) was used throughout this study. The cells were maintained and inoculated as previously described (17, 18). Cell suspensions (approximately 500 mg, fresh weight) in log phase of growth were aseptically inoculated by dispersion in a TMV solution (150 μg/ml) using a Vortex mixer. Inoculated cells were pooled, stirred gently, and washed with fresh medium (17). One-gram samples were spread over the surface of agar and incubated at 22 to 25°C under fluorescent lights (3.7 kergs/cm^2 per s).

Preparation of the replicase and RNA-dependent RNA polymerase. All operations were carried out at 4°C. The glassware used to prepare the extracts and enzyme assays was autoclaved. Five grams of healthy or TMV-infected callus was soaked for 2 min in buffer A. The cells were then drained on a filter and gently disrupted in 8 ml of buffer A with the aid of a mortar and pestle. The homogenate was filtered through Miracloth, and the filtrate was centrifuged at 31,000 × *g* for 30 min. The resultant supernatant was brought to pH 4.7 with 0.05 N HCl, allowed to sit at 4°C for 15 min, and then centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in buffer B (0.05 ml per g of tissue). This solution was used as the source for the RNA-dependent RNA polymerase (6). The 31,000 × *g* pellet was resuspended in buffer B with the aid of a conical tissue grinder and centrifuged at 31,000 × *g* for 30 min. This procedure was repeated twice. The final pellet was resuspended in 0.5 ml of buffer B for each 5 g of tissue used. This suspension was used as the source of the crude bound replicase.

Replicase and RNA-dependent RNA polymerase assay. The standard assay mixture contained 100 mM Tris (pH 8.0 at 33°C), 10 mM $MgCl_2$, 7.5 mM DTT or DTE, 25 mM $(NH_4)_2SO_4$, 10 μg of actinomycin D (AMD) per ml, 0.5 mmol each of ATP, CTP, and GTP per ml, and 1 nmol of [^{3}H]UTP per ml (specific activity, 43 Ci/mmol). Samples with template-free enzyme RNA were added to the reaction mixture at a concentration of 50 μg/ml. The standard reaction mixture was 0.1 ml; the reaction was begun by adding the enzyme. Incubation was carried out at 33°C for designated periods of time in a reciprocal shaking water bath. The reaction was terminated by transferring two 50-μl samples onto separate 2.3-cm Whatman 3MM disks, which were then placed into cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and 0.02% uracil. The disks were washed as described by Zaitlin et al. (32). The radioactivity on the disk was determined as described by Pelcher et al. (19). Zero-time values for assays with bound replicase were significant and were subtracted from the values obtained for incubated samples. The results are expressed as picomoles of [^{3}H]UMP incorporated per milligram of protein per 30 min of incubation time for bound replicase and soluble RNA polymerase. With template-free enzyme reaction mixtures, the incubation time was increased to 120 min. Protein was determined by the technique of Lowry et al. (14).

Preparation of solubilized replicase. Solubilized replicase was prepared by adding bound replicase to buffer C and stirring the mixture for 30 to 60 min at 4°C. The solution was centrifuged at 31,000 × *g* for 30 min. The resulting supernatant was removed, brought to 60% saturation with $(NH_4)_2SO_4$, allowed to mix slowly for 30 min, and then centrifuged at 20,000 × *g* for 20 min. The resulting pellet, which contained the majority of the replicase activity, was resuspended in buffer B (without glycerol), layered on a linear 7.5 to 20% glycerol gradient, and finally centrifuged in a Spinco SW50L rotor at 175,000 × *g* for 15 h. Sedimentation markers were ovalbumin (45,000 daltons) and aldolase (158,000 daltons). After sedimentation, 0.5-ml fractions were collected and enzyme activity was determined on 75-μl samples. The fractions comprising the enzyme activity peak were pooled and immediately applied to a DEAE-Sephadex column (0.6 by 9.0 cm). The protein (in buffer C with 50 mM KCl) was layered on the column. The column was then washed with buffer C containing 50 mM KCl until unabsorbed material passed through the column. The protein was eluted in buffer C with a linear 0.05 to 0.3 M KCl gradient.

Extraction of RNA. RNA was extracted from scaled-up reaction mixtures (0.5 to 2.0 ml) with phenol or perchloric acid. Assay mixtures were diluted with a small buffer volume and phenol extracted as described by Bradley and Zaitlin (4). RNA was extracted from reaction mixtures by using a modification of Wilcockson's (28) perchloric acid method. Five percent (wt/vol) sodium dodecyl sulfate (SDS) and 70% (wt/vol) $NaClO_4$ were added to the reaction mixture. This mixture was vortexed for 2 min and then centrifuged at 1,000 × *g* for 10 min. The protein-SDS complex formed a pellicle, and the liquid phase containing RNA was removed and placed into cold ethanol. After standing overnight at −20°C, the precipitated RNA was removed by centrifugation and washed with 75% ethanol. The final precipitate was resuspended in buffer E.

Polyacrylamide gel electrophoresis. Acrylamide-bisacrylamide (10- and 0.25%, respectively) gels were prepared in Plexiglass tubes (12 by 1 cm) as described by Pelcher et al. (19). Three centimeters of gel (10%) was overlayed with 6 cm of 2.4% acrylamide. The gels were allowed to polymerize for 20 min, were transferred to buffer E, and were allowed to stand at 4°C for 72 h before use. The gels were then placed in Plexiglass tubes, one end of which was covered with a dialysis membrane, and prerun for 30 min. Approximately 20 μg of nucleic acid (including

markers) was applied, and electrophoresis was carried out for 180 or 210 min at 10 V/cm, 5 mA/gel. Gels were scanned at 260 nm before sectioning. Two 1-mm slices were added to each vial containing NCS solubilizer-water (9:1, vol/vol), sealed, and then heated at 50°C overnight. Radioactivity was determined by a Beckman (LS-133) liquid scintillation counter.

SDS-sucrose gradient centrifugation. The perchloric acid-extracted product of the reaction mixture, using DEAE-Sephadex-purified replicase as the enzyme source, was layered onto a linear 15 to 30% (wt/vol) sucrose density gradient in buffer D and centrifuged at 22,000 rpm in an SW 25.1 rotor for 18 h. Two equal samples were taken from each fraction. One was treated with RNase (10 μg/ml) in 2.0 ml of 2X SSC for 60 min at 25°C, and the other was incubated without RNase. Both samples were then made 5% in trichloroacetic acid after the addition of 0.5 mg of bovine serum albumin. The precipitates were collected on a Whatman GF/C filter and washed with 10 ml of 5% trichloroacetic acid, 10 ml of cold 95% ethanol, and 5 ml of ethanol-ether (1:1, vol/vol). After drying, the precipitates were solubilized with a solution of 20% NCS solubilizer, 3.75% water, and 76.25% toluene for 4 h at 37°C. Radioactivity was determined as previously described (19).

RESULTS

When homogenates of TMV-infected tobacco callus were centrifuged at 31,000 × *g*, the sedimenting material contained an RNA polymerase activity not present in the corresponding fraction from healthy material. This fraction was capable of incorporating the [^{3}H]UMP into trichloroacetic acid-insoluble product linearly for 30 min (Fig. 1).

Characteristics of the replicase reaction in vitro. The omission of each of the unlabeled nucleotriphosphates (ATP, CTP, GTP) considerably reduced the enzyme activity (Table 1). If all three nucleotide triphosphates were removed, almost no activity was detected. If the 31,000 × *g* pellet was not resuspended and recentrifuged several times, the incorporation of [^{3}H]UMP was high even in the absence of unlabeled nucleotide triphosphates, indicating the presence of large amounts of unlabeled nucleotides in the preparation. Replicase activity was strongly dependent on the presence of Mg^{2+}. The optimal Mg^{2+} concentration occurred in a broad range from 8 to 20 mM (not shown). The broad optimal Mg^{2+} ion concentration for stimulation of enzymatic activity may reflect chelation of the ion by nucleotides. A broad Mg^{2+} optimum has also been demonstrated for cowpea mosaic virus replicase (Zabel et al., 1974). The optimal Mn^{2+} concentration of 1.5 mM could replace Mg^{2+} but only gave 18% of the reaction rate of the assay containing Mg^{2+}. Replicase assay mixtures routinely contained

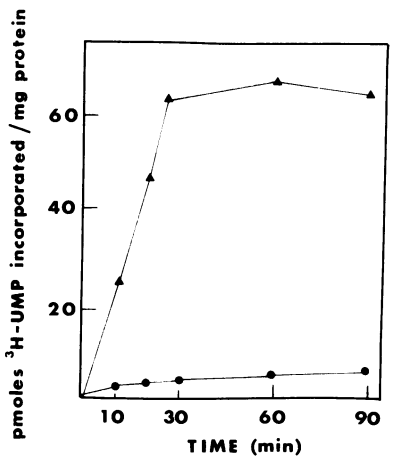

FIG. 1. *Time course of RNA synthesis by bound TMV replicase. Enzyme reaction mixture was incubated at standard conditions. At times indicated, two 50-μl samples were removed, immediately placed on Whatmann 3MM filter disc, washed with 5% trichloroacetic acid containing 1% $Na_4P_2O_7$ and 0.02% uracil, and further processed as described for the activity from TMV-infected tobacco callus (▲) and the corresponding fraction from healthy callus (●).*

TABLE 1. *Requirements of bound replicase*

Reaction conditions	Percent of control
Complete[a]	100
− ATP	21
− CTP	19
− GTP	23
− (ATP, CTP, GTP)	6
− Mg^{2+}	8
− Mg^{2+} + Mn^{2+}	18
− Actinomycin D	112
− Actinomycin D + DNase (30 μg/ml)	96
+ RNase (20 μg/ml)	78
+ TMV RNA	105
+ BMV RNA	103

[a] The complete reaction mixture was as described in Materials and Methods.

AMD. RNA synthesis was only slightly higher in the absence of AMD. Most AMD-sensitive RNA polymerase activity was detected in the nuclear pellet (1,000 × *g*). Slight contamination of the 31,000 × *g* pellet with DNA-dependent RNA polymerase was indicated by higher incorporation of [^{3}H]UMP in assay mixtures in the absence of AMD. Treatment of the product with RNase A reduced the amount of radioactivity approximately 22%. Addition of TMV RNA or brome mosaic virus (BMV) RNA did not stimulate the amount of [^{3}H]UMP incorporated into product (Table 1).

Products of the bound replicase. To determine the nature of the products synthesized by replicase, samples were treated for 30 min in either high salt (2X SSC) or low salt (0.1X SSC) with RNase. Approximately 70% of the RNA synthesized was resistant to RNase treatment in high salt at all time periods tested. For the first 10 min, the amount of RNase-resistant product was greater than at later times. In low salt, nearly all the product was RNase sensitive. There was no loss of trichloroacetic acid-precipitable radioactivity of product incubated in the absence of RNase. It appears that most of the in vitro product is a partially double-stranded structure (Fig. 2).

The products were further analyzed by polyacrylamide gel electrophoresis. When the products were phenol extracted, two major regions of radioactivity were detected (Fig. 3). Using callus rRNA's of known molecular weight and 18S (0.75×10^6 daltons), 25S (1.2×10^6 daltons), and TMV RNA (2.05×10^6 daltons), the estimated molecular weights of the products were determined. Fraction number 10 had an estimated molecular weight of 4.0×10^6, was resistant to RNase degradation, and co-migrated with replicative form isolated from TMV-infected tobacco leaves. This peak appeared to be replicative form, similar to that described by Bradley and Zaitlin (4). Another higher-molecular-weight peak (approximately 5.0×10^6) (fraction no. 4), partially susceptible to RNase degradation, appeared to be replicative intermediate. Approximately 70% of the radioactive label was incorporated into replicative form; the remaining incorporated label was detected in replicative intermediate. If the products of the replicase assay were perchloric acid extracted, approximately 60% of the radioactive label was recovered in replicative intermediate, and the remaining counts were detected in replicative form. No significant amount of TMV RNA was detected. To determine whether single-stranded RNA was destroyed by endogenous RNase activity, [^{3}H]-labeled TMV RNA was added to the reaction mixture and, after 30 min of incubation, the amount of trichloroacetic acid-precipitable activity remaining was determined. In 30 min approximately 25% of the radioactivity was rendered trichloroacetic acid soluble. It appeared that the reaction mixture contained endogenous RNase activity; however, some single-stranded RNA could have been detected if synthesized.

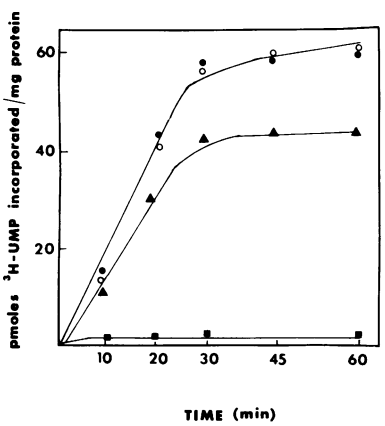

FIG. 2. *Time course of total RNA synthesis and of the fraction resistant to RNase. A 2-ml reaction mixture was incubated under standard assay conditions. At the indicated times, four 50-μl samples were removed. One sample was immediately assayed for total radioactivity (●); the second was incubated in 2 ml of 2X SSC containing RNase (▲); the third was heated with RNase in 0.1X SSC (■); and the last sample was incubated in 2 ml of 2X SSC without RNase (○). The reactions were terminated by the addition of trichloroacetic acid. Carrier protein was added, and the acid-insoluble precipitates were processed.*

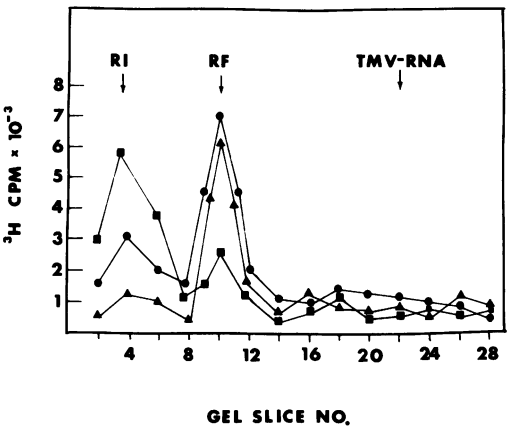

FIG. 3. *Polyacrylamide gel electrophoresis of labeled bound TMV replicase product. A 2-ml reaction mixture was incubated at 33°C for 30 min and subsequently deproteinized by phenol (●) or perchloric acid (■) and analyzed by polyacrylamide gel electrophoresis (210 min at 5 mA/gel). A portion of the reaction mixture extracted with perchloric acid was treated with RNase mixture before gel analysis (▲).*

Partial purification of replicase. The use of divalent cation-deficient buffers to solubilize ATPase from membranes is well established (1, 27). Cowpea mosaic virus replicase has been solubilized by the use of Mg^{2+}-deficient buffers (33). TMV replicase was released from the membranes by the addition of divalent cation-deficient buffer and stirring the mixture. The resulting supernatant contained 70 to 80% of

the replicase activity now template-free. The enzyme was nearly completely dependent upon the addition of exogenous RNA for activity. After further purification on a glycerol gradient, all tested RNAs (TMV RNA, BMV RNA, or callus rRNA) stimulated incorporation of [^{3}H]UMP into product (Table 2). Fractions from the glycerol gradient containing enzyme activity were pooled, applied to a DEAE-Sephadex column, and eluted by a KCl gradient. Using TMV RNA as the template in the assay mixtures, a single peak of activity was detected (Fig. 4). Approximately 55 to 70% of the replicase activity applied to the column was recovered. The specific activity of the purified replicase was 25 to 40 pmol of [^{3}H]UTP incorporated per mg of protein per h. The variability in specific activity was due to membrane-bound replicase preparations. At this stage of purification, template-free replicase still contained RNase activity and other contaminating polypeptides.

TABLE 2. *Requirements of partially purified replicase*

Reaction conditions	Gradient-purified replicase	DEAE-Sephadex-purified replicase
Complete[a]	100	100
− TMV RNA	4	2
− TMV RNA + BMV RNA	113	21
− TMV RNA + TYMV RNA	87	9
− TMV RNA + callus rRNA	65	12
− (ATP, CTP, GTP)	3	2
− Mg^{2+}	NT[b]	8
− Mg^{2+} + Mn^{2+}	NT	14
+ RNase	94	83
+ DNase	99	96

[a] The complete reaction mixture was as described in Materials and Methods.
[b] NT, Not tested.

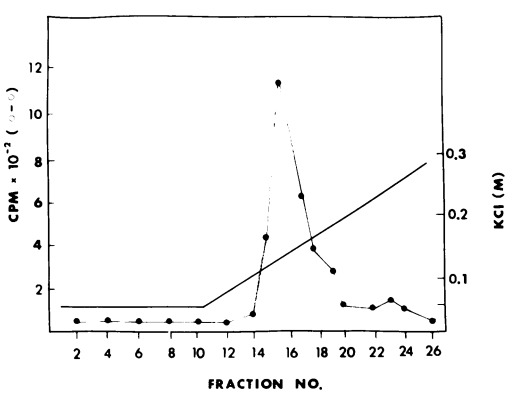

FIG. 4. *DEAE-Sephadex column chromatography of solubilized TMV replicase. Solubilized replicase was prepared by releasing mixing buffer C with bound TMV replicase. The DEAE-Sephadex column was equilibrated in buffer C. After the flow-through material had emerged, the column was treated with a linear 0.05 to 0.3 M KCl gradient (——) in buffer C. Fractions (0.5 ml) were collected at flow rate of 10 ml/h; 200-μl portions of each fraction were assayed for replicase activity for 90 min* (●).

Requirements of the solubilized replicase. Using the DEAE-Sephadex-purified replicase, other RNAs beside TMV RNA were tested for their ability to stimulate enzymatic activity (Table 2). BMV RNA, turnip yellow mosaic virus RNA, or callus rRNA failed to stimulate significant amounts of [^{3}H]UMP incorporation. Omission of the nucleotide triphosphates or Mg^{2+} reduced the enzyme activity considerably. The product was partially sensitive to RNase action but relatively insensitive to DNase action. Isolation of the product and analysis on sucrose gradients demonstrated a polydisperse product (Fig. 5). Most radioactivity was found in an RNase-resistant structure, which had a sedimentation coefficient of 16S and appeared to be replicative form. A significant portion of the radioactive counts were in structures smaller than replicative form and RNase sensitive. Replicative intermediate or TMV RNA was not detected. The DEAE-Sephadex-purified replicase in the presence of TMV-RNA incorporated [^{3}H]UMP into trichloroacetic acid-insoluble product linearly for 6 h (Fig. 6). Only a small amount of radioactivity was incorporated by purified enzyme in the absence of TMV RNA (Fig. 6).

Soluble RNA polymerase. The soluble fraction of healthy and TMV-infected callus contains an AMD-insensitive RNA polymerase. Table 3 shows the requirements of the enzyme isolated from TMV-infected cells. Although not shown, the requirements of the enzyme from healthy callus were essentially identical. Enzyme activity was greatly reduced if the nucleotide triphosphates were omitted. Mn^{2+} could only replace Mg^{2+} to the extent of 22% of the Mg^{2+}-stimulated reaction. AMD had little effect upon [^{3}H]UMP polymerization, and its increased rate in the absence of AMD was probably due to contaminant DNA-dependent RNA polymerase. The product was resistant to DNase action but only partially resistant to RNase. Addition of TMV RNA or BMV RNA failed to stimulate [^{3}H]UMP incorporation. Analysis of the enzyme on glycerol gradients revealed an estimated molecular weight of 150,000. Any of the RNAs tested (TMV RNA, BMV RNA, or callus rRNA) could be used to stimulate activity.

Kinetics of the synthesis of replicase, RNA

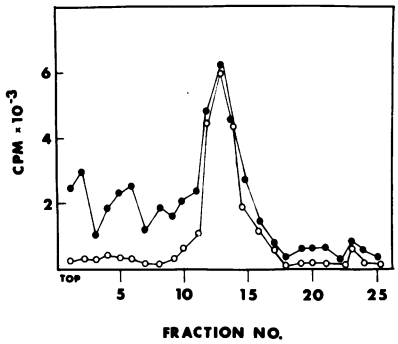

FIG. 5. *Sucrose-SDS density gradient centrifugation of DEAE-Sephadex-purified replicase reaction products. A 1-ml reaction mixture was incubated at 33°C and subsequently deproteinized by perchloric acid. A portion of extract was layered onto a 15 to 30% linear sucrose gradient in buffer D and centrifuged at 22,000 rpm for 18 h in an SW25.1 rotor. Fractions were collected from the top, and two equal portions were assayed for total RNA* (●) *and RNase-resistant RNA* (○).

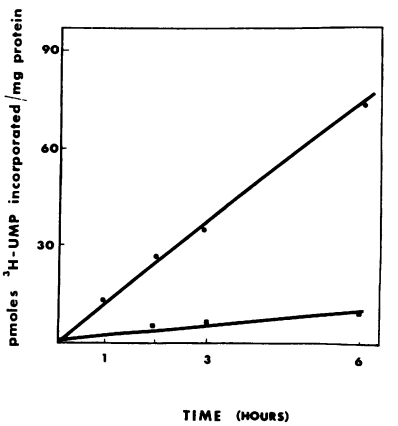

FIG. 6. *Time course of RNA synthesis by DEAE-Sephadex-purified replicase. Enzyme reaction mixture was incubated at standard conditions. At times indicated, two 50-μl samples were removed, immediately placed on separate Whatman 3MM filter disks, washed with 5% trichloroacetic acid containing 1% $Na_4P_2O_7$ and 0.02% uracil, and further processed as described in Materials and Methods. Enzymatic activity in the presence* (●) *or absence* (■) *of TMV RNA (50 μg/ml).*

polymerase, and viral RNA. Experiments were initiated to simultaneously determine the rate of appearance of replicase, TMV RNA, and soluble polymerase. At 12-h intervals after inoculation, a sample of the inoculated cells was transferred to liquid medium containing [³H]uridine (100 μCi/ml) and incubated for 4 h. At the end of the labeling period, total RNA was extracted and subjected to electrophoresis. The rate of incorporation was established by determining the counts per minute per gel slice corresponding in position to the TMV RNA optical density peak. From the remaining cell sample, viral replicase and soluble RNA polymerase were isolated and quantified. The amount of viral RNA increased in a linear manner from 24 to 60 h after inoculation. The rate of [³H]uridine incorporation into TMV RNA increased from 24 to 72 h after inoculation. The amount of [³H]uridine incorporated into TMV RNA at 60 h was approximately six times that incorporated at 24 h after inoculation. Replicase activity increased 10-fold from 24 to 60 h post inoculation (p.i.) and then declined. The soluble RNA polymerase activity increased about threefold from 24 to 72 h p.i. To determine whether the increase in soluble RNA polymerase was due to change in the cells present at inoculation or to new growth, mannitol was substituted for sucrose-glucose in the medium. Mannitol is an efficient osmoticum, but is not metabolized by tobacco cells (27). Inoculated cells were planted on medium containing either mannitol or glucose/sucrose. Replicase that was isolated from cells incubated on medium supplemented with mannitol showed a linear increase in activity from 24 to 60 h p.i. (Fig. 7B) similar to that of cells grown on sucrose/glucose medium (Fig. 7A). However, the RNA polymerase activity remained relatively constant throughout the duration of the experiment (Fig. 7B). The threefold increase in RNA polymerase in infected callus grown on medium containing glucose/sucrose may be a result of new cell growth and not stimulation of the enzyme in cells present at the time of inoculation.

DISCUSSION

A particulate cell-free preparation from TMV-infected callus, enriched in cytoplasmic membranes, contained an RNA-dependent RNA polymerase (replicase) that appeared to be viral specific. The activity of TMV replicase, like that of other viral replicases (4, 5, 8, 9, 20, 24, 31), was markedly stimulated by the presence of Mg^{2+}, whereas Mn^{2+} was a poor substitute ion (Table 1). Products of the bound replicase were partially sensitive to RNase in high salt (2.0X SSC) but completely sensitive in low salt (0.1X SSC) (Fig. 2).

Analysis of the phenol-extracted products revealed the synthesis of the replicative form and only small amounts of replicative intermediate (Fig. 3). These results are consistent with those reported by other workers (4). However, if the product was extracted with perchloric acid, larger amounts of radioactive label were detected in replicative-intermediate form than in

TABLE 3. *Requirements of the soluble RNA-dependent RNA polymerase from TMV-infected tobacco tissue culture cells*

Reaction conditions	Percent of control
Complete[a]	100
− ATP	15
− CTP	12
− GTP	14
− (ATP, CTP, GTP)	3
− Mg^{2+}	6
− Mg^{2+} + Mn^{2+}	22
− Actinomycin D	117
− Actinomycin D + DNase (30 μg/ml)	92
− RNase	84
+ TMV RNA	92
+ BMV RNA	89

[a] The complete reaction mixture was as described in Materials and Methods.

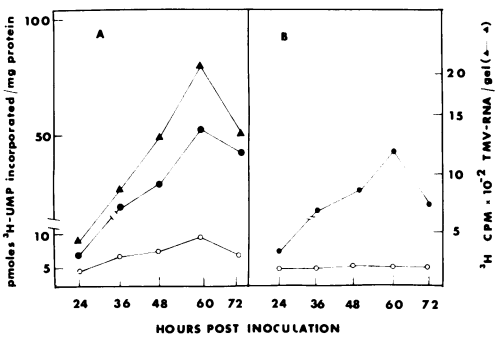

FIG. 7. *Rate of incorporation of [³H]uridine into viral RNA, accumulation of viral RNA, and the time course appearance of TMV replicase and soluble RNA polymerase. Callus was exposed to 100 μCi of [³H]uridine per ml for 4 h before nucleic acid extraction. Electrophoresis was carried out on 2.4% gels for 150 min at 5 mA/gel. Radioactivity determinations were confined to that portion of the gel known to contain TMV RNA. Rate calculations were made by determining counts per minute per gel corresponding in position to the TMV RNA optical density peak. All calculations were corrected for background radioactivity observed with nucleic acid from control callus. Total viral RNA determinations were made by converting the area under the optical density peak to micrograms of RNA. Time course appearance of bound TMV replicase (●), soluble RNA polymerase (○), and TMV RNA (▲) in callus incubated on medium containing glucose/sucrose as carbon sources (A). (B) Time course appearance of bound replicase (●) and soluble RNA polymerase (○) in callus incubated on the nonmetabolizable sugar, mannitol.*

replicative form. Perchloric acid has been reported to be a simple and fast method for isolating RNA from viruses, plants, and bacteria. The reason why it is superior to phenol in certain instances is not clearly understood, but since it deproteinizes solutions very rapidly it may be of great importance in deactivating RNases (28, 29).

Traditional attempts to solubilize proteins, including TMV replicase (23, 32), involved the use of detergents to disrupt membranes and have met with limited success. However, TMV replicase released from membranes by the use of Mg^{2+}-deficient buffer was relatively stable. Based on its sedimentation in glycerol gradients, the solubilized replicase has a molecular weight of approximately 150,000. This molecular weight is in the same range as that reported for TMV replicase from tobacco leaves (32). A protein of this size has been detected as a virus-stimulated protein in TMV-infected tissues (22, 33).

Analysis of the DEAE-Sephadex-purified enzyme revealed that TMV RNA was greatly superior as a template to the other tested RNAs. The product was partially sensitive to RNase and resistant to DNase. Of the plant viral replicases that have been purified to the template-free stage, most have shown little template specificity. Recently, however, Sela and Hauschner (23) purified TMV replicase to an extent where it was completely dependent upon TMV RNA as a template. Hadidi and Frankel-Conrat (10) have purified BMV replicase to a similar degree. In our system, the product of DEAE-Sephadex-purified replicase was partially sensitive to RNase. Further analysis of the product on SDS-sucrose gradients revealed a heterogeneous product. The majority of the radioactive label was found in an RNase-resistant structure with a sedimentation value of approximately 16S that appeared to be replicative form. The remaining counts were found to have sedimentation values less than 16S and were RNase sensitive. In no instance was complete TMV RNA detected. DEAE-purified replicase appeared to synthesize the complement of the template provided (replicative form) but failed to reinitiate RNA synthesis on the minus strand to give rise to replicative intermediate. The ability of DEAE-Sephadex-purified replicase to incorporate [³H]UMP into trichloroacetic acid-insoluble product for longer periods of time (Fig. 6) was similar to that reported by Zabel et al. (30) for cowpea mosaic virus replicase.

Duda et al. (6) reported a soluble RNA-dependent RNA polymerase (molecular weight, 160,000) in healthy tobacco leaves that synthesizes a small double-stranded RNA. Its activity was markedly increased upon TMV infection. The soluble fraction of healthy and TMV-infected tobacco callus contained RNA-dependent RNA polymerase that had many properties

(Table 3) similar to those of the enzyme from tobacco leaves (6). In contrast, its activity was not stimulated in the in vitro assay upon the addition of exogenous RNA as was the enzyme from tobacco leaves. In our experiments, RNA polymerase enzymatic activity increased approximately threefold in healthy and TMV-infected tobacco callus 72 h after transfer to fresh agar medium. Duda et al. (6) reported a slightly greater than threefold increase in this activity 5 days after inoculation with TMV when compared with healthy tissue. When healthy or TMV-infected callus was incubated on mannitol-containing medium, a medium that substantially reduces cell division, the rate of enzymatic synthesis was different from that of comparable callus incubated on glucose/sucrose medium. RNA polymerase activity from healthy or TMV-infected callus increased approximately threefold from 24 to 72 h after transfer to fresh medium. If the callus was incubated on the mannitol medium, no significant change in levels of the enzyme occurred. The activity of replicase was not affected by incubation of infected callus on either glucose/sucrose- or mannitol-containing medium. The increase in RNA polymerase enzyme in infected callus grown on medium containing glucose/sucrose may be a result of new cell growth and not stimulation of the enzyme in cells present at the time of inoculation.

During the first 60 h after inoculation of callus cells, the rate of viral RNA synthesis increased and revealed a maximum during the 48- to 60-h period. This conclusion is based on the observation that the rate of [^{3}H]uridine incorporated into viral RNA increased during each successive 4-h labeling period up to 60 h p.i. The rate declined sharply after the first 60 h. These results are similar to those reported previously (19). TMV replicase activity increased 10-fold and RNA polymerase activity increased threefold from 24 to 60 h p.i. When callus was incubated on a mannitol-containing medium, no significant increase in soluble RNA polymerase was detected (Fig. 7); however, TMV replicase activity was not affected. Although the function of the soluble RNA polymerase is still uncertain, its stimulation of activity is not a prerequisite for TMV replicase activity. The ability to isolate relatively stable template-free TMV replicase may aid in the understanding of the mechanism of viral RNA synthesis.

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