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The Tobacco Mosaic Virus RNA Polymerase Complex Contains a Plant Protein Related to the RNA-Binding Subunit of Yeast eIF-3

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A sucrose density gradient-purified, membrane-bound tobacco mosaic virus (tomato strain L) (TMV-L) RNA polymerase containing endogenous RNA template was efficiently solubilized with sodium taurodeoxycholate. Solubilization resulted in an increase in the synthesis of positive-strand, 6.4-kb genome-length single-stranded RNA (ssRNA) and a decrease in the production of 6.4-kbp double-stranded RNA (dsRNA) to levels close to the limits of detection. The solubilized TMV-L RNA polymerase was purified by chromatography on columns of DEAE–Bio-Gel and High Q. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining showed that purified RNA polymerase preparations consistently contained proteins with molecular masses of 183, 126, 56, 54, and 50 kDa, which were not found in equivalent material from healthy plants. Western blotting showed that the two largest of these proteins are the TMV-L-encoded 183- and 126-kDa replication proteins and that the 56-kDa protein is related to the 54.6-kDa GCD10 protein, the RNA-binding subunit of yeast eIF-3. The 126-, 183-, and 56-kDa proteins were coimmunoaffinity selected by antibodies against the TMV-L 126-kDa protein and by antibodies against the GCD10 protein. Antibody-linked polymerase assays showed that active TMV-L RNA polymerase bound to antibodies against the TMV-L 126-kDa protein and to antibodies against the GCD10 protein. Synthesis of genome-length ssRNA and dsRNA by a template-dependent, membrane-bound RNA polymerase was inhibited by antibodies against the GCD10 protein, and this inhibition was reversed by prior addition of GCD10 protein.

The replication of positive-strand virus RNA requires at least one virus-encoded protein, an RNA-dependent RNA polymerase (5). Many positive-strand RNA viruses also encode a helicase-like protein which has an essential role in RNA replication (5). Replication of bacteriophage Qβ RNA requires, in addition to the phage-encoded subunit, four host-encoded proteins, ribosomal protein S1, protein synthesis elongation factors EF-Ts and EF-Tu, and a ribosome-associated protein, which is termed host factor (1, 4, 44). The involvement of host proteins in the replication of eukaryotic positive-strand RNA viruses has been implied from the copurification of host proteins with isolated virus RNA polymerase complexes (5). For example, a protein related to the 41-kDa subunit of wheat germ eIF-3 copurified with a detergent-solubilized brome mosaic virus RNA-dependent RNA polymerase, and this protein was shown to interact specifically with the virus-encoded 2a polymerase-like protein (37). Similarly, the human protein Sam68 was found in membrane-bound replication complexes of poliovirus and bound specifically to the $3D^{pol}$ RNA polymerase (28). The ability of host proteins to bind in vitro to terminal or internal regions of positive or negative viral RNA strands also suggests possible roles in RNA replication (5). In some cases, these host proteins have been identified, e.g., binding of calreticulin to 3′-terminal plus- and minus-strand sequences of rubella virus (41) and of the La protein to a 3′-terminal minus-strand sequence of Sindbis virus (33), but direct evidence for their role in virus RNA replication has yet to be obtained.

Tobacco mosaic virus (TMV) is a positive-strand RNA plant virus which encodes at least four proteins with molecular masses of 126, 183, 30, and 17.5 kDa, respectively (10, 11, 32). The 126-kDa protein has amino acid sequence motifs characteristic of RNA helicases and methyltransferases (25), whereas the 183-kDa protein, which is synthesized by translational readthrough of a UAG stop codon at the end of the open reading frame (ORF) for the 126-kDa protein, contains additionally a polymerase-like domain (25). Expression of both of these proteins is required for efficient RNA replication (23, 24). The 30-kDa cell-to-cell movement protein and the 17.5-kDa capsid protein, both translated from subgenomic mRNAs, are not essential for RNA replication (10, 11, 32). An additional subgenomic RNA, isolated from TMV-infected tobacco leaves (42), contains an ORF for a putative protein of 54 kDa, corresponding to the C-terminal region of the 183-kDa protein; however, this protein has not been detected in vivo (7). An early stage in the multiplication cycle of TMV has been shown to be sensitive to inhibition by actinomycin D, indicating a requirement for host transcription (9). However, no direct evidence for a role of host proteins in TMV RNA replication has been reported. We have recently purified a membrane-bound RNA polymerase from tomato plants infected with a tomato strain of TMV, TMV-L (30), and shown that, after removal of endogenous RNA with micrococcal nuclease, it is capable of complete replication of added TMV RNA and, therefore, must contain essential virus and host proteins (31). Here, we report the solubilization of the membrane-bound TMV-L RNA polymerase and its purification. Furthermore, we show that one of the host proteins which copurifies with the polymerase is related to the RNA-binding subunit of yeast eIF-3 and present evidence that this protein is an integral part of the TMV RNA polymerase complex.

MATERIALS AND METHODS

Propagation of virus and virus RNA. TMV-L (30) was propagated in tomato plants (*Lycopersicon esculentum* Craigella GCR 26), and virus particles and virus RNA were isolated as described previously (7).

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Solubilization and purification of the TMV-L RNA polymerase. A membrane-bound RNA polymerase was isolated from TMV-L-infected tomato leaves by differential centrifugation to give a 30,000 × *g* pellet (P30), which was resuspended in buffer B (50 mM Tris-HCl [pH 8.2], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin, 5% [vol/vol] glycerol) and purified by centrifugation through sucrose density gradients in TED buffer (50 mM Tris-HCl [pH 8.0], 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin, 5% [vol/vol] glycerol) as described previously (31). Fractions containing RNA polymerase activity from two sucrose gradients were diluted 10-fold with TED buffer and centrifuged at 40,000 × *g* for 1 h. The pellet containing the membrane-bound polymerase was resuspended in 1 ml of buffer B. A variety of ionic and nonionic detergents (obtained from Calbiochem) was tested for efficiency in solubilizing the RNA polymerase as described by Hayes and Buck (19). Sodium taurodeoxycholate was found to be the most efficient and was used in all subsequent steps in the purification. Sodium taurodeoxycholate (0.1 ml of a 10% [wt/vol] solution) was added to 0.9 ml of resuspended TMV-L polymerase and the mixture was incubated on ice for 1 h with occasional gentle agitation. After centrifugation at 100,000 × *g* for 1 h, the supernatant was applied to a column of DEAE–Bio-Gel A (10 by 1 cm; Bio-Rad), a weak anion exchanger which had previously been equilibrated in buffer B containing 1% sodium taurodeoxycholate (buffer C). The column was washed with 30 ml of buffer C at a flow rate of 1 ml min^{-1}, and 1-ml fractions were collected. Fractions containing RNA polymerase activity were applied to a 5-ml Econo-Pac cartridge of High Q (Bio-Rad), a quaternary ammonium strong anion exchanger which had previously been equilibrated in buffer B containing 0.1% sodium taurodeoxycholate (buffer D). A linear gradient of 0 to 1 M KCl in buffer D was then passed through the column at a flow rate of 1 ml min^{-1}. Fractions (1 ml) were collected, dialyzed against buffer D (three changes of 2 liters for 1 h each), and tested for RNA polymerase activity. Fractions with RNA polymerase activity (generally one or two fractions) were stored in 50-μl aliquots at −70°C.

DNA and RNA manipulations. DNA amplification by PCR, DNA cloning, S1 nuclease digestions, analysis by agarose gel electrophoresis, Northern blotting, and nucleotide sequencing for verification of constructs were as described elsewhere (40).

RNA polymerase reactions. RNA polymerase reactions were carried out by mixing 100 μl of RNA polymerase preparation with 25 μl of buffer B containing 4 mM ATP, 4 mM GTP, 4 mM CTP, 1 mM UTP, 20 μg of bentonite, and 10 μCi of [α-^{32}P]UTP (800 Ci/mmol). Reaction mixtures were incubated at 30°C for 1 h. The RNA product was isolated from reaction mixtures by phenol extraction and ethanol precipitation (19) and resuspended in 10 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. For monitoring RNA polymerase activity during the course of purification, 1 μl of the resuspended RNA product was spotted onto Whatman DE81 discs. The discs were dried, washed four times with 0.4 M Na_2HPO_4 and twice with water, and again dried. Radioactivity on the discs was then determined by scintillation counting. In all other cases, the resuspended RNA product was analyzed by polyacrylamide gel electrophoresis (PAGE) alongside size markers, as described previously (31), with quantification of bands by scanning of autoradiographs using a DeskTop scanning densitometer and analysis with Quantity One software.

Protein analysis. Sodium dodecyl sulfate (SDS)-PAGE of proteins was carried out in 8% gels by the method of Laemmli (26). Proteins were detected with a Bio-Rad Silver Stain Plus kit. For Western blotting, proteins were transferred electrophoretically to Immobilon P membranes (Millipore), and the membranes were blocked, incubated with the primary antibodies, and washed as described previously (2). Bound primary antibodies were located with a secondary antibody, donkey anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase, and chemiluminescence detection with a DuPont Renaissance kit according to the supplier's instructions. Wheat germ extract was from Promega. Protein concentrations were determined with the Bio-Rad Protein Assay kit as described by the supplier, with bovine serum albumin as a standard.

Production of a GCD10-MBP fusion. *Saccharomyces cerevisiae* genomic DNA was a generous gift from David O'Reilly. DNA corresponding to the GCD10 ORF (15) was PCR amplified with VENT DNA polymerase (New England Biolabs) and oligonucleotides TCGCGGATCCATGAATGCTTTGACAACCA TAG (corresponding to the first 22 nucleotides [nt] of the GCD10 ORF with an added *Bam*HI site) and TGCTCTAGAGTCGACTTATATTTTTTGTTTCTTA GCTCCATG (complementary to the last 27 nt of the GCD10 ORF including the stop codon with an added *Sal*I site). The 1.4-kbp PCR product was gel purified, cleaved with *Bam*HI and *Sal*I, and cloned into the corresponding sites of the vector pMAL-c2 (New England Biolabs), a derivative of pMAL-c (27), in *Escherichia coli* DH5αF′ cells. After growing the cells to an optical density at 600 nm of 0.5, expression of the protein was induced by addition of isopropyl-β-thiogalactopyranoside to a final concentration of 0.1 mM. After disruption of the cells by sonication, the GCD10-maltose binding protein (MBP) fusion was purified by binding to an amylose resin, followed by elution with maltose (27).

Antibodies. Antisera against wheat germ eIF-3 (37) and a GCD10-TrpE fusion protein (15) were generous gifts from Paul Ahlquist and from Minerva Garcia-Barrio and Alan Hinnebusch, respectively. Antisera against MBP fusions of N-terminal and C-terminal regions of the TMV-L 126-kDa protein and against an MBP fusion of the putative TMV-L 54-kDa protein (readthrough region of the 183-kDa protein) have been described previously (31). To raise an antiserum against the GCD10-MBP fusion protein, a rabbit was injected subcutaneously with 500 μg of the protein emulsified with an equal volume of Freund's complete adjuvant. Two further subcutaneous injections of 500 μg of the protein emulsified with an equal volume of Freund's incomplete adjuvant were given after 2 and 6 weeks, respectively. Blood was collected, and antiserum was prepared 12 days after the final injection. An antiserum against MBP was obtained from New England Biolabs. Igs were prepared from the antisera as described previously (2).

Immunoaffinity chromatography. Antibodies against the TMV-L 126-kDa protein (N-terminal region)-MBP fusion, the GCD10-MBP fusion, and the MBP (1 mg) were (separately) covalently linked to AminoLink gel in 2 ml of Amino Link AffinityPak columns, according to the directions of the supplier (Pierce), and the columns were equilibrated in buffer C. DEAE–Bio-Gel-purified TMV-L RNA polymerase (1 ml) in buffer C was applied to each column and allowed to enter the gel bed. A 0.2-ml volume of buffer C was applied to each column and allowed to enter the gel bed. The columns were then incubated at room temperature for 1 h to allow antibody-antigen complexes to form. Each column was then washed with 16 ml of buffer C to remove unbound protein. Bound proteins were eluted from the columns with an acidic buffer (Pierce ImmunoPure elution buffer). Fractions (1 ml) were collected and neutralized by the addition of 50 μl of 1 M Tris-HCl (pH 9.5). The bound proteins eluted in one or two fractions.

Antibody-mediated inhibition of RNA-dependent RNA polymerase activity. A membrane-bound, template-dependent TMV-L RNA polymerase (100 μl) was mixed with Ig (1 to 10 μg), and the mixture was incubated at 0°C for 30 min. TMV-L RNA (10 μg) and 25 μl of buffer B containing 4 mM ATP, 4 mM GTP, 4 mM CTP, 1 mM UTP, 20 μg of bentonite, and 10 μCi of [α-^{32}P]UTP (800 Ci/mmol) were then added, and the reaction mixture was incubated at 30°C for 1 h. Inhibition of single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) synthesis was monitored separately by analysis of products by PAGE and by scanning the autoradiographs. In some experiments, the RNA polymerase was incubated with purified MBP-fusion protein or MBP (10 μg) at 0°C for 30 min prior to the addition of the antibodies. Control experiments to check that the antibodies were free from ribonuclease activity were carried out as described previously (2, 20).

Antibody-linked polymerase assays. Antibody-linked RNA polymerase assays (43) were carried out as described previously (18, 19). Briefly, DEAE–Bio-Gel-purified TMV-L RNA polymerase or control proteins were subjected to SDS-PAGE and blotted onto Immobilon P membranes. The membranes were incubated with antibodies, washed, and then incubated with DEAE–Bio-Gel-purified TMV-L RNA polymerase to allow the polymerase to bind to the second antigen-binding site on the IgG antibody molecules. After further incubation with an RNA polymerase reaction mixture containing [α-^{32}P]UTP, the labeled products were detected by autoradiography.

RESULTS

Solubilization of the TMV-L membrane-bound RNA polymerase and purification of the solubilized polymerase. A membrane-bound RNA polymerase was isolated from tomato leaves infected with TMV-L by differential centrifugation as a resuspended 30,000 × *g* pellet fraction (P30) and purified by sucrose density gradient centrifugation. A range of nonionic and ionic detergents at concentrations of between 0.1 to 5.0% was tested for efficacy in solubilizing the membrane-bound polymerase. After incubation of the membrane-bound RNA polymerase with the detergent, samples were centrifuged at 100,000 × *g* for 1 h to give supernatant and pellet fractions. The products of RNA polymerase reactions with the supernatant and pellet fractions were analyzed by PAGE. Solubilization was assayed by the amount of genomic-length ssRNA and dsRNA produced by the supernatant fraction compared with that produced by the original membrane-bound RNA polymerase (31). Less than 5% solubilization was obtained with the detergents polyoxyethylene *p*-isooctylphenyl ether (Nonidet P-40, Triton X-100, and Triton X-114), polyoxyethylene dodecyl ether (Genapol X-80), polyoxyethylene tridecyl ether, *n*-dodecyl-β-D-maltoside, *n*-heptyl-β-D-glucopyranoside, *n*-octyl-β-D-glucopyranoside, octanoyl-*N*-methylglucamide (MEGA-8), decanoyl-*N*-methylglucamide (MEGA-10), *n*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14), 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)-dimethyl-ammonio]-2-hydroxy-1-propane sulfonate (CHAPSO), and sodium *N*-laurylsarcosinate (Sarcosyl) (not shown). This low level of solubilization was found to be due largely to inactivation of the polymerase, since only low levels of activity were recovered in

TABLE 1. Purification of the TMV-L RNA polymerase complex from infected tomato plants

Step	Protein concn (mg/ml)	RNA polymerase activity (cpm/ml)[a]	Sp act (cpm/mg)	Relative sp act
P30	11.2	2.7×10^4	2.4×10^3	1
Sucrose gradient	1.97	1.5×10^5	7.3×10^4	30
Taurodeoxycholate	0.318	6.4×10^5	2.0×10^6	821
DEAE–Bio-Gel A	0.039	1.2×10^6	3.2×10^7	13,100
High Q	0.002	1.6×10^6	9.0×10^8	368,000

[a] Incorporation of [α-^{32}P]UMP into RNA products (see Materials and Methods).

the pellet fractions. After incubation with the ionic detergent sodium deoxycholate at a 1% concentration, about 20% of the original activity was recovered in the supernatant. However, incubation with sodium taurodeoxycholate at a 1% concentration resulted in an increase in overall RNA polymerase activity, of which 70% was in the soluble (supernatant) fraction and 30% was in the pellet fraction. The sodium taurodeoxycholate-solubilized polymerase was used for further purification. No attempt was made to remove the endogenous bound RNA template at this stage, since viral RNA polymerases bound to their templates have been found to be more stable through subsequent purification stages (2, 20).

The solubilized RNA polymerase was purified by passage through a column of DEAE–Bio-Gel. The RNA polymerase did not bind to the column, since the activity was recovered in the flowthrough fractions, and no further activity was recovered by application of a 0 to 1.0 M KCl gradient through the column. The active fractions from the DEAE–Bio-Gel column were applied to a column of High Q. The RNA polymerase bound to this column, and passage of a 0 to 1.0 M KCl gradient through the column resulted in elution of the polymerase at about 0.65 M KCl.

The course of purification for a typical TMV-L RNA polymerase preparation is shown in Table 1. The specific activity of the High Q-purified RNA polymerase was increased by 368,000-fold compared to the membrane-bound P30 fraction.

Analysis of the reaction products of the solubilized TMV-L RNA polymerase. The reaction products of the sodium taurodeoxycholate-solubilized RNA polymerase were compared with those of the membrane-bound RNA polymerase by PAGE. As found previously (31), the products of the sucrose gradient-purified RNA polymerase consisted of a genome-length 6.4-kb ssRNA and a 6.4-kbp dsRNA (Fig. 1, lane 1). After solubilization of the RNA polymerase with sodium taurodeoxycholate, the main reaction product was the 6.4-kb ssRNA, the amount of which was increased severalfold compared with that of the ssRNA produced from the membrane-bound polymerase (Fig. 1; compare lanes 1 and 3). Only a trace of the 6.4-kb dsRNA was detected. Some of the reaction product remained at the top of the gel after electrophoresis. This probably consisted of aggregates of ssRNA, since after digestion of the total RNA product with S1 nuclease, analysis by PAGE showed nothing at the top of the gel, no band of 6.4-kb ssRNA, and no increase in the amount of the 6.4-kb dsRNA (not shown). Hence, the total product of the solubilized polymerase was almost entirely single stranded. Likewise, the 6.4-kb genomic ssRNA was the predominant product detected after purification of the solubilized polymerase on columns of DEAE–Bio-Gel (Fig. 1, lane 4) and High Q (Fig. 1, lane 5). As a control, no product was detected from an equivalent membrane fraction from healthy tomato leaves after solubilization with sodium taurodeoxycholate (Fig. 1, lane 2).

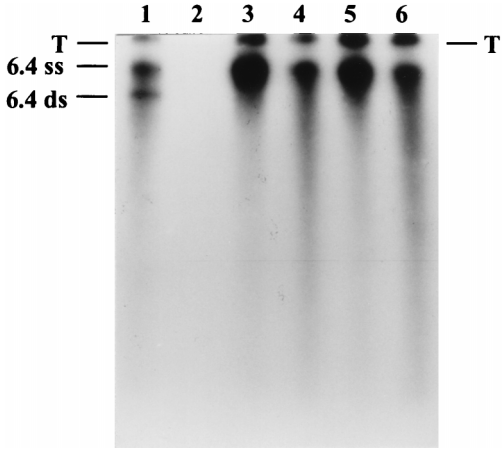

FIG. 1. Analysis of the products of the solubilized TMV-L RNA polymerase reactions by PAGE (4% gel). RNA polymerase reactions were carried out in the presence of [α-^{32}P]UTP and the following polymerase preparations: sucrose density gradient-purified, membrane-bound TMV-L RNA polymerase (lane 1); preparation from healthy tomato leaves equivalent to lane 1 (lane 2); TMV-L RNA polymerase (as in lane 1) after solubilization with sodium taurodeoxycholate (lane 3); solubilized TMV-L RNA polymerase after purification on DEAE–Bio-Gel (lane 4); solubilized TMV-L RNA polymerase after further purification on High Q (lane 5); 6.4-kb ssRNA transcripts synthesized with T7 RNA polymerase from a full-length cDNA clone of TMV-L RNA (lane 6) (31). The positions of the 6.4-kb ssRNA and 6.4-kbp dsRNA are indicated on the side of the autoradiograph. T, position of the top of the gel. Lanes 1, 3, 4, and 5 correspond to products from 100, 100, 20, and 50 μl of RNA polymerase, respectively.

Northern dot blots were used to determine the polarities of the reaction products of the sodium taurodeoxycholate-solubilized polymerase. Both the 6.4-kb ssRNA reaction product, which was extracted from a gel after PAGE, and the total reaction product (after denaturation) hybridized with a TMV-L negative-strand RNA, but not detectably with a TMV-L positive-strand RNA (Fig. 2, columns 1 and 2, rows A and B). Controls established that the TMV-L positive-strand RNA and negative-strand RNA hybridized only with TMV-L negative-strand RNA and positive-strand RNA, respectively (Fig. 2, columns 1 and 2, rows C and D). The results, therefore, indicate that the RNA synthesized by the solubilized polymerase was the positive strand.

Protein composition of the purified TMV RNA polymerase. SDS-PAGE analysis of preparations of the TMV-L RNA polymerase, after sodium taurodeoxycholate solubilization and DEAE–Bio-Gel and High Q purification, consistently showed the presence of proteins with molecular masses of 183, 126, 56, 54, 50, and 32 kDa. Four to five other proteins in the size range of 16 to 35 kDa were detected, but these were not consistent in size or amount between different preparations. SDS-PAGE profiles of representative preparations of purified TMV-L RNA polymerase from two different batches of infected plants are shown in Fig. 3, lanes 1 and 3. A 32-kDa protein was also detected in equivalent solubilized and purified fractions from leaves of healthy tomato plants (Fig. 3, lanes 2 and 4), and the protein of this size in the RNA polymerase preparations is therefore probably a host protein which fortuitously copurified with the polymerase. The 126- and 183-kDa proteins were considered likely to be virus encoded, since these two proteins were detected previously in the sucrose gradient-purified, membrane-bound TMV-L RNA polymerase (31). This was confirmed by Western blotting with antibodies to the N-termi-

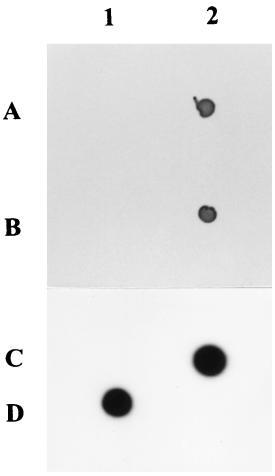

FIG. 2. Determination of the polarity of the reaction products of the sodium taurodeoxycholate-solubilized TMV-L RNA polymerase. Unlabelled positive-strand (column 1) and negative-strand (column 2) RNAs, corresponding to TMV-L RNA nt 3335 to 4390, were synthesized by using T7 and T3 RNA polymerases, respectively, from a TMV-L cDNA clone in pBluescript SK(+) (31), blotted onto filters, and hybridized with the ^{32}P-labelled 6.4-kb ssRNA product of reaction with the TDC-solubilized TMV-L RNA polymerase (extracted from a gel after PAGE [see Fig. 1, lane 3]) (row A), the total ^{32}P-labelled RNA product of reaction (after denaturation by heating to 100°C for 5 min, followed by rapid cooling) (row B), and ^{32}P-labelled positive-strand RNA and negative-strand RNA, corresponding to TMV-L RNA nt 3335 to 4390 (rows C and D, respectively). Hybridized RNAs were detected by autoradiography.

nal region or C-terminal region of the TMV-L 126-kDa protein, which specifically detected the 126- and 183-kDa proteins in the solubilized and purified TMV-L RNA polymerase but not in equivalent material from healthy plant leaves (Fig. 4A and not shown). Antibodies against the putative TMV-L 54-kDa protein (readthrough region of the TMV-L 183-kDa protein) detected the 183-kDa protein, but not the 126-kDa protein, in the solubilized and purified TMV-L RNA polymerase (not shown), as shown previously for sucrose gradient-purified, membrane-bound TMV-L RNA polymerase (31). The three proteins with molecular masses of 56, 54, and 50 kDa, which were not found in the equivalent fraction from healthy leaves (Fig. 3, lanes 2 and 4), did not bind antibodies against the N-terminal or C-terminal region of the TMV-L 126-kDa protein or antibodies against the putative TMV-L 54-kDa protein (readthrough region of the TMV-L 183-kDa protein) in Western blots of solubilized and purified TMV-L polymerase (Fig. 4A and not shown) or the membrane-bound TMV-L polymerase (31). These three proteins are therefore candidates for host-encoded components of the RNA polymerase holoenzyme.

Identification of a protein related to the RNA-binding subunit of yeast eIF-3 (GCD10 protein) in the purified TMV RNA polymerase. A Western blot of purified TMV-L RNA polymerase preparations was probed with antibodies to total wheat germ eIF-3. A 56-kDa protein was specifically detected in the TMV-L RNA polymerase preparations but not in an equivalent preparation from healthy tomato leaves (Fig. 4B). For further identification of this protein, the 54.6-kDa subunit (GCD10) of yeast eIF-3 (15) was expressed in *E. coli* as an MBP fusion. Antibodies raised against this protein were then used to probe Western blots of purified TMV-L RNA polymerase preparations and a wheat germ extract. A single 56-kDa protein was detected in both the polymerase preparations and the wheat germ extract (Fig. 4C). In similar Western blots, a protein of the same size was detected in the polymerase preparations and the wheat germ extract with antibodies against an *E. coli*-expressed GCD10-TrpE fusion protein (15), but no protein was detected with antibodies against the MBP or with preimmune sera (not shown). Hence, the 56-kDa protein in purified TMV-L RNA polymerase preparations and a protein of similar size in a wheat germ extract are related to the 54.6-kDa subunit (GCD10) of yeast eIF-3.

Evidence that the tomato GCD10-related protein is an integral part of the TMV-L RNA polymerase holoenzyme. To provide evidence that the tomato GCD10-like protein is a subunit of the TMV-L RNA polymerase holoenzyme, we asked whether it could be coimmunoaffinity selected with the virus-encoded 126- and 183-kDa proteins. DEAE–Bio-Gel-purified TMV-L RNA polymerase was passed into columns containing antibodies against the 126-kDa protein (N-terminal region)-MBP fusion, the yeast GCD10 protein-MBP fusion, or

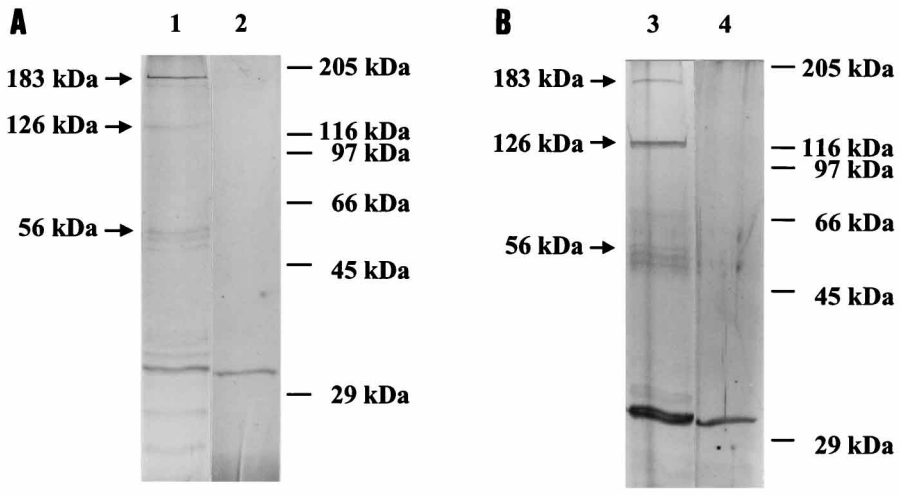

FIG. 3. Analysis of the protein compositions of solubilized, purified TMV-L RNA polymerase by SDS-PAGE in an 8% gel. Lanes 1 and 3, 35 μl of TMV-L RNA polymerase preparations from two different batches of TMV-L-infected tomato leaves, after solubilization and purification on DEAE–Bio-Gel and High Q; lanes 2 and 4, 35 μl of preparations from healthy tomato leaves equivalent to lanes 1 and 3, respectively. Two different gels (A and B) were run. The gels were silver stained. The positions and sizes of protein markers are shown on the right, and the positions of the 183-, 126-, and 56-kDa proteins are shown by arrows on the left of each gel.

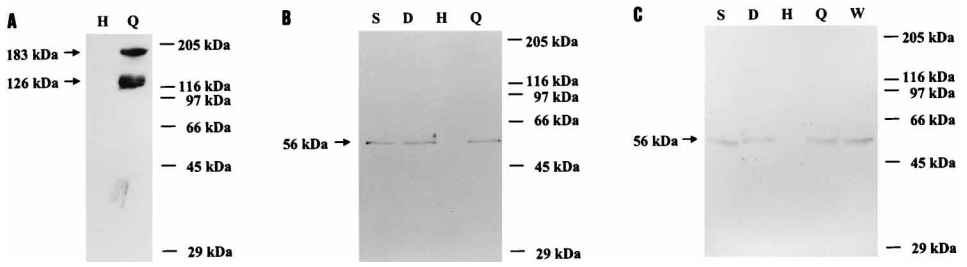

FIG. 4. Analysis of solubilized and purified TMV-L RNA polymerase preparations by Western blotting. Lanes: S, solubilized TMV-L RNA polymerase (35 μl); D, solubilized TMV-L RNA polymerase after DEAE–Bio-Gel purification (35 μl); H, preparation from healthy tomato leaves equivalent to S (35 μl); Q, solubilized TMV-L RNA polymerase after DEAE–Bio-Gel and High Q purification (35 μl); and W, wheat germ extract (1 μl). Proteins were separated by SDS-PAGE in 8% gels. Three separate blots were then prepared and probed with 1:5,000 dilutions of antisera against the following proteins: A, TMV-L 126-kDa protein (N-terminal region)-MBP fusion; B, wheat germ eIF-3; C, GCD10-MBP fusion. The positions and sizes of protein markers are shown to the right of the blots. The positions of the 183-, 126-, and 56-kDa proteins are indicated by arrows to the left of the blots.

the MBP, each covalently linked to AminoLink agarose gel. After incubation to allow the formation of antigen-antibody complexes, the columns were washed to remove unbound proteins. Immunoaffinity-selected proteins were then eluted with an acidic buffer, neutralized, and separated by SDS-PAGE, followed by blotting onto membranes and probing separate blots with antibodies against the 126-kDa protein (N-terminal region)-MBP fusion, the yeast GCD10 protein-MBP fusion, or the MBP. The antibodies against the MBP did not immunoaffinity select detectable amounts of the 183-kDa protein or the 126-kDa protein (Fig. 5A, lane 3) or of the 56-kDa GCD10-related protein (Fig. 5B, lane 3). In contrast, antibodies against the 126-kDa protein immunoaffinity selected the 183-kDa protein, the 126-kDa protein, and the 56-kDa GCD10-related protein. Antibodies against the GCD10 protein also selected these three proteins in relative amounts similar to those selected by antibodies against the 126-kDa protein (Fig. 5A, lanes 1 and 2; Fig. 5B, lanes 1 and 2). As a control, no proteins were detected when the third blot was probed with antibodies against the MBP (not shown). These results indicate that the TMV-L-encoded 183- and 126-kDa proteins and the 56-kDa tomato GCD10-related protein are bound together in a complex.

Support for this conclusion was obtained by antibody-linked polymerase assays. The DEAE–Bio-Gel-purified TMV-L RNA polymerase, the 126-kDa protein (N-terminal region)-MBP fusion, the GCD10 protein-MBP fusion, and a fraction from healthy, mock-inoculated tomato leaves were electrophoresed in an SDS-polyacrylamide gel, blotted onto a membrane, and incubated with antibodies against the 126-kDa (N-terminal-region)-MBP fusion, the GCD10 protein-MBP fusion, or the MBP as a control. The membranes with antibodies bound to their cognate proteins were then incubated with the DEAE–Bio-Gel-purified TMV-L RNA polymerase to allow the polymerase to bind to the second antigen binding site on cognate antibody molecules. After further incubation with an RNA polymerase reaction mixture containing [α-^{32}P]UTP, labelled products were detected by autoradiography (Fig. 6). In the polymerase assays with antibodies against the 126-kDa (N-terminal region)-MBP fusion, bands in the positions of the 126- and 183-kDa proteins in the RNA polymerase preparation (Fig. 6A, lane 1) and the 126-kDa protein (N-terminal region)-MBP fusion (calculated size, 125 kDa, but migrates with an apparent molecular mass of 115 kDa) (Fig. 6A, lane 2)

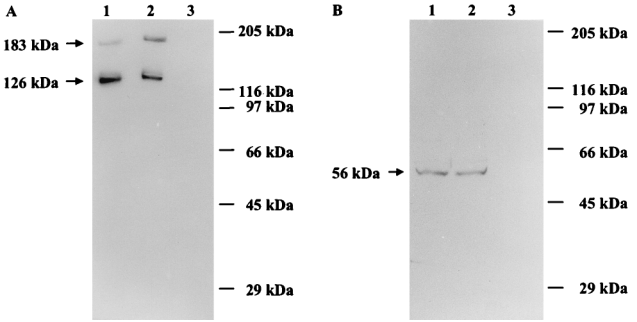

FIG. 5. Coimmunoaffinity selection of the TMV-L 183- and 126-kDa proteins with the 56-kDa GCD10-related protein. DEAE–Bio-Gel-purified TMV-L RNA polymerase was incubated with antibodies against the TMV-L 126-kDa protein (N-terminal region)-MBP fusion, the GCD10-MBP fusion, and the MBP. Immunoaffinity-selected proteins were analyzed by SDS-PAGE in 8% gels and by Western blotting. Lanes: 1, proteins immunoaffinity selected with antibodies against the TMV-L 126-kDa protein (N-terminal region)-MBP fusion; 2, proteins immunoaffinity selected with antibodies against the GCD10-MBP fusion; 3, proteins immunoaffinity selected with antibodies against the MBP. Separate blots were prepared and probed with antibodies against the TMV-L 126-kDa protein (N-terminal region)-MBP fusion (A) and the GCD10-MBP fusion (B). The positions and sizes of protein markers are shown to the right of the blots. The positions of the 183-, 126-, and 56-kDa proteins are indicated by arrows to the left of the blots.

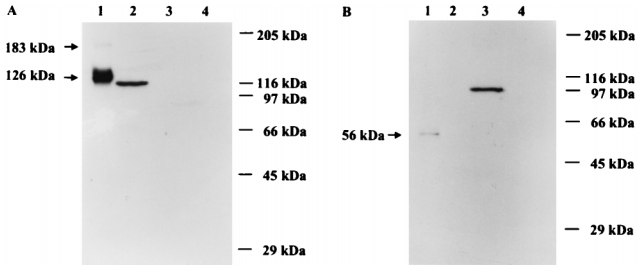

FIG. 6. Binding of active TMV-L RNA polymerase to antibodies against the GCD10 protein demonstrated by the antibody-linked polymerase assay. Solubilized TMV-L RNA polymerase after DEAE–Bio-Gel purification (lane 1), the TMV-L 126-kDa protein (N-terminal region)-MBP fusion (lane 2), the GCD10-MBP fusion (lane 3), and a fraction from healthy tomato leaves equivalent to lane 1 (lane 4) were electrophoresed in SDS-8% polyacrylamide gels and blotted onto separate membranes (membranes A and B). Membrane A (A) was then incubated with antibodies against the TMV-L 126-kDa protein (N-terminal region)-MBP fusion, and membrane B (B) was incubated with antibodies against the GCD10-MBP fusion. Both membranes were then incubated with DEAE–Bio-Gel-purified TMV-L RNA polymerase (containing endogenous RNA template), followed by an RNA polymerase reaction mixture containing [α-^{32}P]UTP. Bands were detected by autoradiography. The positions and sizes of protein markers are shown to the right of the membranes. The positions of the 183-, 126-, and 56-kDa proteins are indicated by arrows to the left of the membranes.

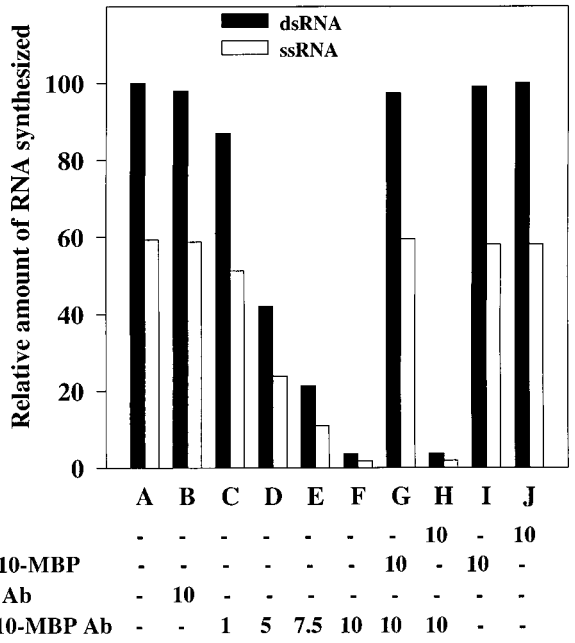

FIG. 7. Inhibition of a membrane-bound, template-dependent TMV-L RNA polymerase by polyclonal antibodies against the GCD10 protein. A membrane-bound, template-dependent TMV-L RNA polymerase preparation (31) was incubated with no protein (A to F), the GCD10-MBP fusion (10 μg) (G and I), or the MBP (10 μg) (H and J) and then with antibodies (Ab) against MBP (10 μg [B]), antibodies against the GCD10-MBP fusion (1 μg [C], 5 μg [D], 7.5 μg [E], and 10 μg [F to H]), or no antibody (A, I, and J). TMV-L RNA was then added, and RNA polymerase reactions were carried out in the presence of [α-^{32}P]UTP. The reaction products were analyzed by PAGE in 4% gels, and the relative proportions of genome-length, ssRNA and dsRNA were measured by scanning autoradiographs of the gels.

were detected. In the assays with antibodies against the GCD10 protein-MBP fusion, bands in the positions of the 56-kDa GCD10-related protein (Fig. 6B, lane 1) and the 97-kDa GCD10 protein-MBP fusion (Fig. 6B, lane 3) were detected. No bands were detected in assays with either of these antibodies in the lanes containing extracts from mock-inoculated uninfected plants (Fig. 6A, lane 4; Fig. 6B, lane 4) or in any of the lanes in assays with antibodies against the MBP (not shown). These results extend the coimmunoaffinity selection experiments by showing that an active TMV-L RNA polymerase complex binds to antibodies against both the virus-encoded 126- and 183-kDa proteins and the GCD10 protein. The 126- and 183-kDa virus-encoded proteins and the 56-kDa GCD10-related protein are, therefore, part of the complex. The variability in the ratio of the 183- and 126-kDa proteins in different RNA polymerase preparations and the fact that the intensity of silver staining varies widely between different proteins (45) have so far precluded determination of the stoichiometry of these three proteins in the complex.

Finally, we asked if antibodies to the GCD10 protein would inhibit the TMV-L RNA polymerase. Attempts to produce a primer-independent RNA polymerase, dependent on added TMV-L RNA template, by micrococcal nuclease treatment of the purified, solubilized polymerase were unsuccessful. Therefore, the TMV-L template-dependent, membrane-bound RNA polymerase described previously (31) was used for antibody inhibition experiments. Antibodies to the GCD10 protein-MBP fusion inhibited the synthesis of ssRNA and dsRNA by the template-dependent RNA polymerase on a TMV-L genomic RNA template approximately equally in a concentration-dependent fashion (Fig. 7). At the highest concentration of antibody used, inhibition was greater than 95% (Fig. 7F). The inhibition was reversed by prior addition of the GCD10 protein-MBP fusion (Fig. 7G) but not by prior addition of MBP (Fig. 7H). Addition of the GCD10 protein-MBP fusion or MBP in the absence of antibody had no effect on RNA polymerase activity (Fig. 7I and J). No significant inhibition was detected with antibodies to the MBP (Fig. 7B). Antibodies against the GCD10-MBP fusion did not inhibit membrane-bound or solubilized TMV-L RNA polymerase preparations containing endogenous RNA template (not shown).

DISCUSSION

The membrane-bound TMV-L RNA polymerase was found to be refractory to solubilization by a wide range of nonionic and ionic detergents, including Nonidet P-40, Triton X-100, and dodecyl-β-D-maltoside, which have been used successfully in the solubilization of polymerases of other positive-strand RNA plant viruses, such as alfalfa mosaic virus (36), brome mosaic virus (35, 37), cucumber mosaic virus (18), and red clover necrotic mosaic virus (2). The TMV-U1 RNA polymerase has also been reported to be poorly solubilized by Triton X-100 and dodecyl-β-D-maltoside, although 14% solubilization was achieved with CHAPS (48). The large variations in solubilization of different polymerases with specific detergents may be due to differences in the membrane to which the polymerase is attached and differences in the organization of the polymerase in relation to the membrane. Although the membrane site of replication has been determined rigorously for only a few positive-strand RNA plant viruses, evidence suggests that membranes derived from several different subcellular organelles are the sites of replication of different viruses, e.g., the endoplasmic reticulum (brome mosaic virus [38] and poliovirus [3]), chloroplast outer membrane (alfalfa mosaic virus [12] and turnip yellow mosaic virus [16]), tonoplast (cucumber mosaic virus [17]), modified endosomes and lysosomes (alphaviruses [14, 34]), peroxisome-derived membranes (cymbidium ringspot virus [6]), and mitochondrion-derived membranes (carnation Italian ringspot virus [6]). The TMV-U1 126- and/or 183-kDa replication protein accumulates in cytoplasmic granular inclusions or viroplasms, which may mature to form X bodies (13, 22, 39). X bodies are reported to contain endoplasmic reticulum, although this has not been proved to be the site of RNA replication (13). TMV-L has been reported not to form viroplasms or X bodies, and the TMV-L 126- and 183-kDa proteins were localized to virus bundles (8).

Efficient solubilization of the TMV-L RNA polymerase was achieved by using sodium taurodeoxycholate. Solubilization resulted in a severalfold increase in the synthesis of the 6.4-kb genome-length positive-strand ssRNA and a decrease in the production of the 6.4-kb dsRNA to levels close to the limits of detection. It is possible that interaction of sodium taurodeoxycholate with the membrane or the polymerase altered the configuration of the polymerase complex to allow more-efficient release of completed ssRNA strands from their templates. It is noteworthy that a membrane-bound flockhouse virus RNA polymerase synthesized only negative-strand RNA (isolated as dsRNA) with a genomic RNA template, but on addition of certain neutral or negatively charged phosphoglycerolipids (PGLs), both negative and positive strands were produced (46, 47). It was suggested that initiation of positive-strand synthesis may have resulted from a direct PGL-polymerase interaction or to a change in membrane configuration (47). Attempts to produce a solubilized TMV-L RNA polymerase, dependent on added TMV-L RNA, were not success-

ful, suggesting that the configuration of the polymerase within the membrane is important for initiation of negative-strand RNA synthesis or that an essential component of the complex was dissociated on solubilization.

After DEAE–Bio-Gel and High Q chromatography, the solubilized TMV-L RNA polymerase contained only 10 to 11 protein components detectable by silver staining. One of these proteins was also found in equivalent preparations from healthy tissue, and 4 to 5 others were not consistently found in all preparations. The remaining proteins with molecular masses of 183, 126, 56, 54, and 50 kDa are candidates for virus-encoded or host-encoded subunits of the polymerase complex. Western blotting showed that the two largest proteins were the virus-encoded 183- and 126-kDa proteins. The proportions of these two proteins varied considerably in different RNA polymerase preparations; two extremes in ratios are shown in Fig. 3. Differences in the ratios of these two proteins have been noted previously in Western blots of different preparations of the membrane-bound TMV-L RNA polymerase (31). Both the 183- and the 126-kDa proteins have been shown to be essential for efficient replication of TMV RNA in vivo (23, 24). The significance of their different ratios in different isolated polymerase preparations is not known; preparations with widely differing ratios had similar activities in vitro.

The 56-kDa protein component of the TMV-L RNA polymerase was shown by Western blotting to be related to the 54.6-kDa yeast GCD10 protein, the RNA-binding subunit of the translational initiation factor eIF-3 (15). Several lines of evidence indicate that this 56-kDa GCD10-related protein is a genuine component of the TMV-L RNA polymerase complex. (i) The protein was not found in the equivalent healthy purified fraction from healthy leaves. (ii) The 183-, 126-, and 56-kDa proteins were coimmunoaffinity selected by antibodies against either the N-terminal region of the 126-kDa protein or the GCD10 protein. (iii) In antibody-linked polymerase assays, active TMV-L RNA polymerase bound to antibodies to the N-terminal region of the 126-kDa protein and to antibodies to the GCD10 protein. (iv) Synthesis of ssRNA and dsRNA by the membrane-bound, template-dependent TMV-L RNA polymerase on a positive-strand RNA template were inhibited by antibodies to the GCD10 protein, and inhibition was reversed by the addition of the GCD10 protein. The anti-GCD10 antibodies probably inhibited the initiation of negative-strand RNA synthesis by the template-dependent RNA polymerase, since RNA polymerase preparations containing endogenous RNA template were not inhibited by this antibody. The inhibition of positive-strand RNA synthesis by the anti-GCD10 antibodies could, at least in part, be a secondary effect of the inhibition of negative-strand synthesis. Elucidation of the role of the tomato GCD10-related protein in the replication of TMV-L RNA will require further work; however, if, like the yeast GCD10 protein (15), it has RNA-binding activity, such a role could be related to this activity. It is noteworthy that binding of the phage Qβ RNA polymerase holoenzyme to its genomic RNA template requires two of its host subunits which have RNA-binding activity, namely, the ribosomal protein S1 (29) and the host factor (1).

A purified brome mosaic virus RNA polymerase has been shown to contain a barley protein related to the 41-kDa subunit of wheat germ eIF-3 (37). Wheat germ eIF-3 contains 10 subunits with molecular masses of 116, 107, 87, 83, 56, 45, 41, 36, 34, and 28 kDa (21). In Western blots, antibodies to the yeast GCD10 protein reacted with only a 56-kDa protein in a wheat germ extract, suggesting that the 56-kDa component of wheat germ eIF-3 is the RNA-binding subunit. Mouse monoclonal antibodies against the 41-kDa subunit of wheat germ eIF-3 reacted with the brome mosaic virus RNA polymerase, but antibodies against the 56-kDa subunit did not react (37). It is clear, therefore, that the eIF-3-related protein associated with the brome mosaic virus RNA polymerase is not an analog of the yeast 54.6-kDa GCD10 RNA-binding eIF-3 subunit or of the tomato 56-kDa GCD10-related protein in the TMV-L RNA polymerase. These host protein constituents of the TMV-L and brome mosaic virus RNA polymerases, therefore, correspond to different eIF-3 subunits and are likely to play different roles in the replication of their respective viruses.

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