JOURNAL OF VIROLOGY, Sept. 1996, p. 6227–6234 Vol. 70, No. 9
0022-538X/96/$04.00+0

Complete Replication In Vitro of Tobacco Mosaic Virus RNA by a Template-Dependent, Membrane-Bound RNA Polymerase

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Received 29 February 1996/Accepted 30 May 1996

A crude membrane-bound RNA polymerase, obtained by differential centrifugation of extracts of tomato leaves infected with tobacco mosaic tobamovirus (tomato strain L) (TMV-L), was purified by sucrose density gradient centrifugation. Removal of the endogenous RNA template with micrococcal nuclease rendered the polymerase template dependent and template specific. The polymerase was primer independent and able to initiate RNA synthesis on templates containing the 3′-terminal sequences of the TMV-L positive or negative strands. TMV-*vulgare* RNA was a less efficient template, while RNAs of cucumber mosaic cucumovirus and red clover necrotic mosaic dianthovirus, or 5′-terminal sequences of TMV-L positive or negative strands, did not act as templates for the polymerase. A main product of the reaction with TMV-L genomic RNA as a template, carried out in the presence of [α-^{32}P]UTP, was genomic-length single-stranded RNA. This was shown to be the positive strand and uniformly labelled along its length, demonstrating complete replication of TMV-L RNA. Genomic-length double-stranded RNA, labelled in both strands, and small amounts of RNAs corresponding to the single- and double-stranded forms of the coat protein subgenomic mRNA were also formed. Antibodies to N-terminal and C-terminal portions of the 126-kDa protein detected the 126-kDa protein and the 183-kDa readthrough protein in purified RNA polymerase preparations, whereas antibodies to the readthrough portion of the 183-kDa protein detected only the 183-kDa protein. All three antibodies inhibited the template-dependent RNA polymerase, but none of them had any effect on the template-bound enzyme.

The replication of positive-strand virus RNA in eukaryotic cells takes place on membrane-bound complexes containing viral and host proteins and viral RNA. Membrane-bound complexes, capable of catalyzing the synthesis of viral RNA in vitro, can be isolated by differential centrifugation of homogenates of infected cells. Generally, RNA synthesis by such complexes consists of the completion of strands already initiated in vivo. However, removal of endogenous RNA from the complexes, before or after solubilization with a detergent, has produced primer-independent RNA polymerases from a number of positive-strand RNA viruses, which are able to initiate de novo RNA synthesis programmed by an added RNA template (2, 10, 33). In most of these systems, only negative-strand RNA was synthesized with the positive-strand RNA as a template. However, complete replication of positive-strand RNA in vitro has been reported for a few viruses. This was achieved for cucumber mosaic cucumovirus (strain Q) (CMV-Q) RNA with a purified, solubilized RNA polymerase (15), for flockhouse nodavirus by a membrane-bound RNA polymerase in the presence of added glycerophospholipids (36, 37), and for polio enterovirus with a combined in vitro translation and replication system (1, 24).

Tobacco mosaic tobamovirus (TMV) is one of the most widely studied positive-strand RNA plant viruses (8, 9). Membrane-bound replication complexes containing endogenous RNA templates and capable of elongating RNA chains initiated in vivo have been isolated from TMV-infected protoplasts or plants (34, 39, 40). However, there are no reports of the isolation of a template-dependent TMV RNA polymerase able to initiate RNA synthesis in vitro. Here we report the isolation of a template-dependent, membrane-bound RNA polymerase isolated from tomato plants infected with tomato strain L of TMV (TMV-L) and demonstrate its ability to catalyze the complete replication of the virus RNA in vitro.

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MATERIALS AND METHODS

Preparation of virus and virus RNA. TMV-L (25), TMV-*vulgare* (14), CMV-Q (7, 29, 30), and red clover necrotic mosaic dianthovirus (Australian isolate) (RCNMV-Aus) (36) were propagated, and virus particles and virus RNA were isolated as described previously (5, 16, 27).

DNA manipulations. The production of cDNA and amplification by reverse transcription-PCR, DNA cloning, analysis by agarose gel electrophoresis, and nucleotide sequencing for verification of constructs were as described previously (31).

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as described previously (26).

Isolation and purification of the membrane-bound TMV-L RNA polymerase. Leaves of young tomato plants (*Lycopersicon esculentum* Craigella GCR 26) were inoculated with TMV-L, and 4 days later the infected leaves (200 g) were homogenized in a blender at 4°C in 400 ml of buffer A (50 mM Tris-HCl [pH 7.4], 15 mM $MgCl_2$, 120 mM KCl, 0.1% β-mercaptoethanol, 1 μM pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 20% [vol/vol] glycerol). The homogenate was filtered through muslin, and the filtrate was centrifuged at 500 × *g* for 10 min at 4°C. The supernatant was then centrifuged at 30,000 × *g* for 30 min at 4°C to give a pellet (P30) and a supernatant (S30). The S30 supernatant was centrifuged at 160,000 × *g* for 3.5 h to give a pellet (P160) and a supernatant. The P30 and P160 pellets were resuspended separately in 40 ml each of buffer B (50 mM Tris-HCl [pH 8.2], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM leupeptin, 1 μM-pepstatin) and stored in 1-ml aliquots at −70°C. For purification, the resuspended crude P30 polymerase (0.2 ml) was layered on top of a 4.5-ml linear density gradient of 20 to 60% (wt/wt) sucrose in TED buffer (50 mM Tris-HCl [pH 8.0], 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% [vol/vol] glycerol) and centrifuged at 189,000 × *g* for 3 h in a Beckman SW50.1 rotor. Fractions (450 μl) were collected, assayed for RNA polymerase activity, and then stored at −70°C without removal of the sucrose. To make the RNA polymerase template dependent, active sucrose density gradient fractions 2 and 3 (numbered from the top) were dialyzed against buffer B, calcium acetate was added to a final concentration of 2 mM, and then micrococcal nuclease (MN) (Boehringer S7 nuclease; 1 U/μl) was added. The solution was incubated at 30°C for 30 min, and then EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] was added to a final concentration of 5 mM.

RNA polymerase reactions. The RNA polymerase preparation was added to buffer B containing 1 mM ATP, 1 mM CTP, 1 mM GTP, 10 μM UTP, 5 μCi of [α-^{32}P]UTP, bentonite (4.8 mg/ml) (prepared as described in reference 17), and, if required, an RNA template (25 μg/ml) in a total volume of 200 μl. Reaction mixtures for demonstrating initiation of synthesis contained either (i) 1 mM ATP, 1 mM CTP, 1 mM UTP, 10 μM GTP, and 20 μCi of [γ-^{32}P]GTP or (ii) 1 mM CTP, 1 mM GTP, 1 mM UTP, 10 μM ATP, and 20 μCi of [γ-^{32}P]ATP. Reaction mixtures were incubated at 30°C for 1 h. RNA products were extracted from reaction mixtures and analyzed by PAGE as described in protocols 7 and 10 of reference 17. Double-stranded RNAs (dsRNAs) of *Aspergillus foetidus* and *Penicillium stoloniferum* (4) and dsRNA reaction products of RNA polymerase reactions with CMV-Q (15) and RCNMV-Aus (2) were used as markers to determine the sizes of dsRNA reaction products. ^{32}P-labelled single-stranded RNA (ssRNA) transcripts synthesized in vitro by using T7 RNA polymerase and cDNA clones of RCNMV (38) and CMV (16), cleaved with various restriction endonucleases, were used as markers to determine the sizes of ssRNA reaction products. To produce full-length TMV-L transcripts for use as templates or markers, a 0.4-kbp fragment of a full-length cDNA clone of TMV-L RNA (pLFW30) (22) was amplified by PCR with VENT DNA polymerase and primers AGCGACCCGGGTAATACGACTCACTATAGTATTTTTACAACAATTACC (containing an *Xma*I site, a T7 promoter, and 20 nucleotides [nt] corresponding to the 5′ end of TMV-L) and TACCTCCGATATCATATGTC (complementary to nt 402 to 383 of TMV-L RNA, which includes an *Eco*RV site), gel purified, and cloned into the *Xma*I and *Eco*RV sites of plasmid pSL1180 (Pharmacia) to give pTMV4. The 6.0-kbp *Eco*RV-*Mlu*I fragment of pLFW3 was then cloned into the *Eco*RV and *Mlu*I sites of pTMV4 to give pTMV5, a full-length cDNA clone of TMV-L linked to the T7 promoter. Transcription of pTMV5 with T7 polymerase gave a 6.4-kb ssRNA which was infectious to *Nicotiana tabacum* Xanthi nc.

Treatment with S1 nuclease. RNA polymerase reaction products were treated with S1 nuclease as described previously (2).

Fusion proteins and antisera. Fusion proteins of regions of the TMV-L 126- and 183-kDa (54-kDa) proteins with a maltose-binding protein (MBP) were produced in *Escherichia coli*. The coding regions were amplified from pLFW3 (22) by using VENT DNA polymerase and the following pairs of primers: (i) for the N-terminal 653 amino acids of the 126-kDa protein, AGCTGAGGATCCA CAATGGCATACACACAAACAGCCACATCGTCCGC (contains a *Bam*HI site and TMV-L nt 72 to 94) and CATGACGTCGACGTAACTCACCACGG GCCATCG (contains an *Sal*I site and sequence complementary to nt 2031 to 2011 of TMV-L); (ii) for the C-terminal 470 amino acids of the 126-kDa protein, GGAGTCGAATTCACGATGGCCCGTGGTGAGTTAC (contains an *Eco*RI site and TMV-L nt 2013 to 2031) and TAGCTCGGTACCTGCAGCGGCCGC TATTGAGTACCTGCATCTAC (contains a *Pst*I site and sequence complementary to TMV-L nt 3423 to 3402); and (iii) for the 54-kDa protein (open reading frame within the readthrough part of the 183-kDa protein), CCCGGG GATCCTGAATTCGGAGATATATCTGATATGCAATTTTAC (contains an *Eco*RI site and TMV-L nt 3484 to 3509) and CTTGATGGCTCTAGTTGTTA AGGATCCTGCAGAAGCTTCGTCGA (contains a *Pst*I site and sequence complementary to TMV-L nt 4922 to 4902). The PCR products were gel purified, cleaved with *Bam*HI-*Sal*I (for primer pair i) or *Eco*RI-*Pst*I (for primer pairs ii and iii), and cloned into the corresponding sites of the vector pMAL-cRI (New England Biolabs), a derivative of pMAL-c (21), in *E. coli* DH5αF′ cells. The MBP fusions were expressed and purified, and antisera were raised in rabbits, as described previously (2).

Antibody-mediated inhibition of RNA-dependent RNA polymerase activity. Immunoglobulins were prepared from the antisera, and inhibition of the template-dependent TMV-L RNA polymerase by the antibodies was carried out, as described previously (2). In some experiments, the RNA polymerase was incubated with purified fusion protein (1 μg) at 0°C for 30 min prior to addition of the cognate antibody.

In vitro transcription. In vitro transcription of cDNA clones or PCR products with T7 or T3 RNA polymerase was carried out by using Megascript kits according to the directions of the supplier (Ambion). When labelled transcripts were required for use as size markers, 10 μCi of [α-^{32}P]UTP was added to the reaction mixture. Templates for producing transcripts corresponding to the 5′ and 3′ termini of the positive and negative strands of TMV-L, CMV-Q, or RCNMV-Aus were produced by PCR or reverse transcription-PCR from cDNA clones or viral RNA, respectively, by using *Pfu* DNA polymerase and pairs of primers, one of which contained a T7 promoter. A template for producing positive- and negative-strand transcripts corresponding to an internal sequence (nt 3335 to 4390) of TMV-L was obtained by cloning the 1.06-kbp *Eco*RI-*Kpn*I fragment of pLFW3 (22) into pBluescript SK(+).

RESULTS

Isolation and purification of a membrane-bound TMV RNA polymerase complex containing endogenous RNA template. Tomato plants were inoculated with TMV-L (25), and after 4 days, the infected, inoculated leaves were homogenized. The filtered homogenate was subjected to differential centrifugation, and the resuspended 30,000 × *g* (P30) and 160,000 × *g* (P160) pellets were assayed for RNA polymerase activity in the presence of [α-^{32}P]UTP. Most of the activity was found in the resuspended P30 pellet (designated the crude membrane-bound P30 polymerase) and did not require the addition of TMV-L RNA. The product of the reaction was an RNA species which migrated ahead of the genomic ssRNA in PAGE (Fig. 1, lane 2). The ds nature of this RNA species was shown by its resistance to S1 nuclease (Fig. 1, lane 3), and its size, estimated by comparison of its migration rate with those of dsRNA markers, was 6.4 kbp. It therefore corresponds in size to the ds form of the virus genomic RNA (28).

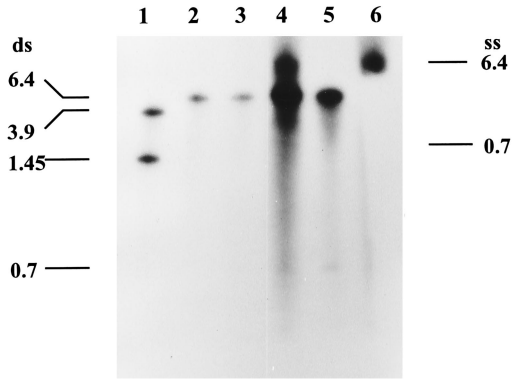

FIG. 1. Characterization of the products of reaction of membrane-bound TMV-L RNA polymerase preparations containing endogenous RNA template. RNA polymerase reactions were carried out with the crude membrane-bound P30 polymerase (lanes 2 and 3) and with sucrose density gradient-purified combined fractions 2 and 3 of the P30 polymerase (lanes 4 and 5) in the presence of [α-^{32}P]UTP. The reaction products were isolated and analyzed by PAGE followed by autoradiography. The reaction products in lanes 3 and 5 were treated with S1 nuclease prior to electrophoresis. The sizes of ssRNA and dsRNA bands (in kilobases and kilobase pairs, respectively) are shown on the right and left, respectively. Lane 1, dsRNA products of RCNMV RNA polymerase reaction (3.9 and 1.45 kbp) (2); lane 6, ssRNA transcripts of a full-length cDNA clone of TMV-L RNA (6.4 kb). Other ssRNA and dsRNA markers (not shown) are given in Materials and Methods.

The infection of plants with RNA viruses or viroids results in an increase in the activity of a host RNA-dependent RNA polymerase and a terminal nucleotidyl transferase (13, 32). In tomato plants, most of these activities are found in the 30,000 × *g* supernatant, but small amounts are found in the 30,000 × *g* pellet and hence could contaminate a crude, membrane-bound viral RNA-dependent RNA polymerase. In order to produce a template-dependent, membrane-bound TMV-L RNA polymerase, it was desirable first to remove any such contaminants. This was done by sedimenting the crude, membrane-bound P30 polymerase through a sucrose density gradient in a buffer lacking Mg^{2+} ions and containing EDTA, since it is known that the host RNA-dependent RNA polymerase and terminal nucleotidyl transferase activities are released from membranes by extraction with an Mg^{2+}-deficient buffer (11, 32, 41). After centrifugation, the gradient was separated into 11 fractions, numbered from the top. Most of the RNA polymerase activity was associated with a dark-green band, corresponding to fractions 5 to 8, which probably contained, inter alia, thylakoid membranes; however, a substantial amount of polymerase activity was found in colorless fractions (fractions 2 to 4) above the green band. The products of reaction obtained with the sucrose density gradient-purified polymerase fractions contained the dsRNA of 6.4 kbp, as found in reactions with the crude polymerase, together with three additional RNA species (Fig. 1, lane 4). The fastest migrating of these was shown by its

resistance to S1 nuclease to be dsRNA (Fig. 1, lane 5), and its size of 0.7 kbp corresponds to the ds form of the TMV coat protein subgenomic RNA (28). The other two RNAs were shown to be ss, because they were degraded by S1 nuclease (Fig. 1, lane 5), and their sizes, estimated by comparison of their migration rates with those of ssRNA markers, were 6.4 and 0.7 kb, corresponding to the sizes of the genomic and coat protein subgenomic ssRNAs, respectively (28). The 0.7-kb ssRNA band was difficult to photograph, because it was present in small amounts and sometimes was partially obscured by a smear extending down from the 6.4-kbp dsRNA band. However, it was reproducibly produced. The formation of all four RNA species did not require the addition of added TMV RNA; therefore, sedimentation of the polymerase complex through the sucrose gradient did not remove the endogenous RNA template. Purification of the crude P30 polymerase by sucrose density gradient centrifugation resulted in about a 10-fold increase in the amounts of reaction products.

Production of a membrane-bound, template-dependent and template-specific TMV RNA polymerase. To make the polymerase template dependent, endogenous RNA was removed by incubation with MN followed by inactivation of the nuclease with EGTA. For this purpose, fractions 2 and 3 of the sucrose density gradient-purified polymerase were selected, since these produced the most ssRNA. After MN treatment, no polymerase activity could be detected in the absence of added RNA template (Fig. 2, lane 1). The major products of reaction produced on addition of TMV-L virion RNA or RNA transcripts from a full-length TMV-L cDNA clone, characterized by their electrophoretic mobilities and sensitivities or resistances to S1 nuclease, corresponded to the 6.4-kb genomic ssRNA and its dsRNA form. A smaller amount of an RNA corresponding in size to the dsRNA form of the 0.7-kb coat protein mRNA was also detected (Fig. 2, lanes 2 and 3); in some reactions the 0.7-kb ssRNA could also be seen (see Fig. 6), although the amount produced was variable. A small amount of 6.4-kbp dsRNA was detected with TMV-*vulgare* virion RNA as the template (Fig. 2, lane 4). However, no products were detected when RNAs of viruses from different genera, i.e., CMV-Q RNA and RCNMV-Aus RNA, were added as putative templates to polymerase reaction mixtures (Fig. 2, lanes 5 and 6), indicating the template specificity of the polymerase and serving as additional controls for the absence of endogenous activity. The polymerase was also able to utilize 3′-terminal fragments of the TMV-L positive-strand and negative-strand RNAs as templates to produce dsRNA products (Fig. 2, lanes 8 to 10), but no products were produced with 5′-terminal fragments of the TMV-L positive-strand or negative-strand RNA (Fig. 2, lanes 11 and 12) or with 3′-terminal fragments of the CMV-Q positive-strand or negative-strand RNA 1 (Fig. 2, lanes 13 and 14). Attempts to prepare a template-dependent RNA polymerase by MN treatment of the crude, membrane-bound P30 polymerase were unsuccessful.

De novo initiation of RNA synthesis and complete replication of TMV RNA by the TMV membrane-bound, template-dependent RNA polymerase. The production of genomic-length ssRNA by the template-dependent TMV-L RNA polymerase (Fig. 2, lanes 2 and 3) suggests that the polymerase has catalyzed the complete replication of the virus genomic RNA. To prove this unequivocally, three experiments were carried out. First, to prove that the genomic RNA was not labelled by any traces of TNTase activity which may have remained in the sucrose density gradient-purified RNA polymerase, RNA polymerase reactions were carried out in the absence of GTP, CTP, or ATP. When any one of the nucleoside triphosphates (NTPs) was left out of the polymerase reaction mixture, no products could be detected (not shown).

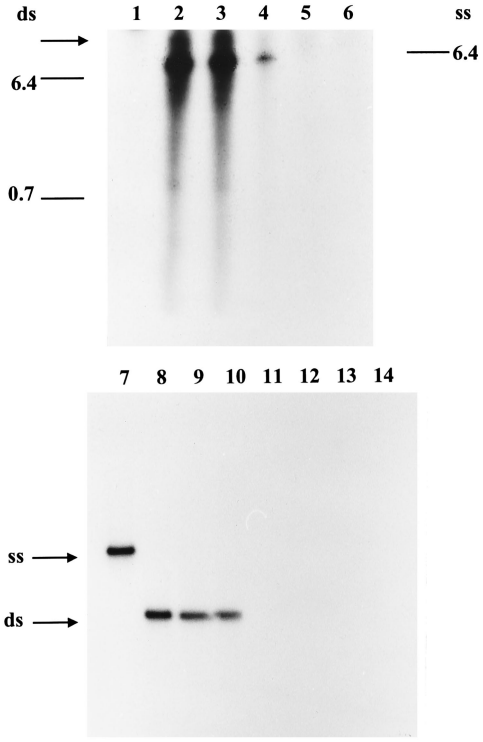

FIG. 2. Characterization of the reaction products and template specificity of a template-dependent, membrane-bound TMV-L RNA polymerase. RNA polymerase reactions were carried out, in the presence of [α-^{32}P]UTP and the templates indicated below, with sucrose density gradient-purified, membrane-bound TMV RNA polymerase combined fractions 2 and 3, from which endogenous RNA had been removed by treatment with MN. The reaction products were analyzed as described in the legend to Fig. 1. Lane 1, no added template; lane 2, TMV-L virion RNA; lane 3, TMV-L RNA transcribed from a full-length cDNA clone with T7 RNA polymerase; lane 4, TMV-*vulgare* virion RNA; lane 5, CMV-Q RNA; lane 6, RCNMV-Aus RNA; lane 8, 278-nt 3′-terminal fragment of the TMV-L positive-strand RNA; lane 9, 271-nt 3′-terminal fragment of the TMV-L negative-strand RNA; lane 10, 271-nt 3′-terminal fragment of the TMV-L negative-strand RNA, containing an extra G residue at the 3′ terminus; lane 11, 392-nt 5′-terminal fragment of the TMV-L positive-strand RNA; lane 12, 277-nt 5′-terminal fragment of the TMV-L negative-strand RNA; lane 13, 312-nt 3′-terminal fragment of the CMV-Q positive-strand RNA; lane 14, 245-nt 3′-terminal fragment of the CMV-Q negative-strand RNA fragment, containing an extra 3′-terminal G residue. Lane 7, ^{32}P-labelled ssRNA marker corresponding to the 278-nt 3′-terminal fragment of the TMV-L positive-strand RNA. For lanes 1 to 6, the sizes of ssRNA and dsRNA bands (in kilobases and kilobase pairs, respectively) are shown on the right and left, respectively, and an arrow marks the position of the top of the gel. For lanes 7 to 14, the arrows indicate the positions of the 278-nt ssRNA band (ss) or the 271- or 278-nt dsRNA band (ds).

Second, to prove that the TMV template-dependent RNA polymerase was capable of initiating the synthesis of new RNA strands, reactions were carried out with the TMV-L template-dependent RNA polymerase in the presence of TMV-L RNA template and [γ-^{32}P]GTP as the only labelled NTP. Analysis of the reaction products by gel electrophoresis revealed a band corresponding to the 6.4-kb genomic-length dsRNA species (Fig. 3, lane 1). The band is faint compared with that produced in reactions with [α-^{32}P]UTP (Fig. 3, lane 2). For quantitation, the products of the reaction carried out with [α-^{32}P]UTP were serially diluted and electrophoresed alongside the product of the reaction carried out with [γ-^{32}P]GTP (not shown). From the dilution at which the intensities of the 6.4-kb dsRNA bands with each labelled substrate were approximately equal, it was calculated that only one nucleotide of the 6.4-kb species was

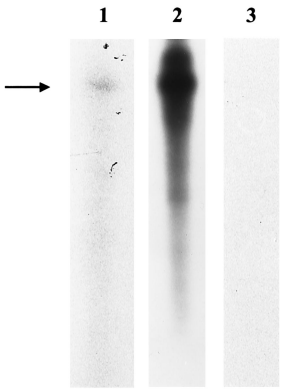

FIG. 3. Initiation of RNA synthesis by the TMV-L template-dependent RNA polymerase. RNA polymerase reactions were carried out with the TMV-L template-dependent RNA polymerase, TMV-L RNA as a template, unlabelled NTPs, and the following labelled NTPs: [γ-^{32}P]GTP (lane 1), [α-^{32}P]UTP (lane 2), and [γ-^{32}P]ATP (lane 3). The reaction products were analyzed as described in the legend to Fig. 1. Lanes 1 and 3 contained 10 times as much reaction product as lane 2. The arrow marks the position of the 6.4-kb dsRNA product.

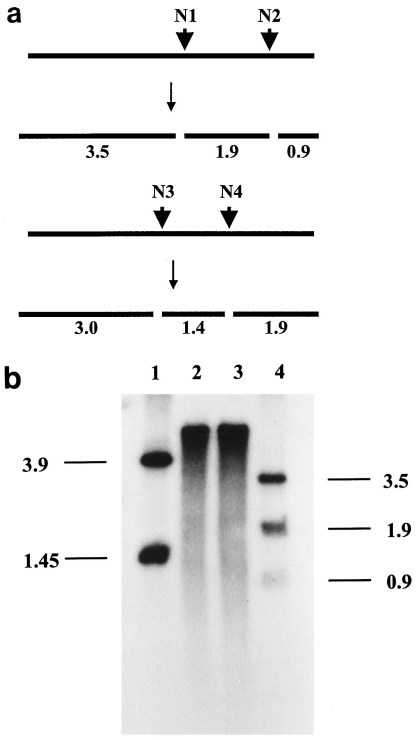

FIG. 4. Analysis of the genomic-length ssRNA synthesized by the template-dependent TMV-L RNA polymerase with TMV-L RNA template. (a) Diagram showing the expected products of RNase H digestion of TMV-L positive-strand RNA hybridized to oligodeoxyribonucleotides N1 and N2 or TMV-L negative-strand RNA hybridized to oligodeoxyribonucleotides N3 and N4. The sequences of the oligodeoxyribonucleotides are as follows: N1, 5′-GTGGCTGTTTGTGT GTATGCC-3′ (complementary to nt 3554 to 3535); N2, 5′-CCTCCATCGTTC ACACTCGT-3′ (complementary to nt 5459 to 5440); N3, 5′-CCTGTGCATTA CACGTGCAG-3′ (nt 3018 to 3037); and N4, 5′-GTCTAGCATCAATGCTCC CG-3′ (nt 4453 to 4472). (b) The ^{32}P-labelled genomic-length ssRNA polymerase reaction product was extracted after PAGE and annealed to oligodeoxyribonucleotides; the hybrids were then incubated with RNase H. The products were then electrophoresed through a formaldehyde-agarose gel and detected by autoradiography. Lane 1, 3.9- and 1.45-kb ssRNA markers synthesized by transcription from full-length cDNA clones of RCNMV-Aus RNA 1 (pRCI1G) and RNA 2 (pRCI2G) with T7 RNA polymerase; lane 2, genomic-length ssRNA reaction product after gel extraction; lane 3, genomic-length ssRNA reaction product plus N3, N4, and RNase H; lane 4, genomic-length ssRNA reaction product plus N1, N2, and RNase H. Sizes (in kilobases) of marker RNAs and product RNAs are shown on the left and right, respectively.

labelled with [γ-^{32}P]GTP. Since the β and γ phosphates of the NTP substrates are eliminated during RNA synthesis, only the 5′-terminal nucleotide will retain the γ phosphate in the newly synthesized RNA, proving unequivocally that the template-dependent RNA polymerase is able to catalyze the de novo initiation of negative-strand RNA synthesis, since the 5′-terminal nucleotide of the negative strand is G. When polymerase reactions were carried out with TMV-L RNA template and [γ-^{32}P]ATP as the only labelled nucleotide, no products were detected (Fig. 3, lane 3).

In the third experiment, the labelled genomic-length ssRNA reaction product was extracted from a gel and hybridized with two oligodeoxyribonucleotides, N1 and N2, with sequences complementary to internal sequences in TMV-L RNA (Fig. 4a). Digestion of the hybrid with RNase H, which is specific for RNA-DNA hybrids, hydrolyzed the RNA specifically and completely at the sites of hybridization with N1 and N2 to produce fragments of the predicted sizes (3.5, 1.9, and 0.9 kb) with nucleotide incorporation as expected from their lengths and nucleotide compositions (Fig. 4b; compare lanes 2 and 4), as estimated by scanning the autoradiograph with a DeskTop scanning densitometer and by analysis with Quantity One software. Hence, the genomic-length ssRNA reaction product consisted of positive-strand RNA uniformly labelled along its length. When the labelled genomic-length ssRNA reaction product was annealed with two oligodeoxynucleotides, N3 and N4, with sequences complementary to internal sequences of the TMV-L negative-strand RNA, followed by RNase H digestion, the RNA was not cleaved and the predicted digestion products of 3.0, 1.9, and 1.4 kb were not detected (Fig. 4b; compare lanes 2 and 3). Hence, the genomic-length ssRNA reaction product contained no negative-strand RNA detectable by the method used.

Ratios of genomic-length positive-strand and negative-strand RNAs synthesized by the TMV membrane-bound, template-dependent and template-bound RNA polymerases. To determine the ratios of labelled positive and negative strands in the genomic-length ssRNA and dsRNA species synthesized by the template-dependent and template-bound RNA polymerases, the ^{32}P-labelled ssRNA and dsRNA species from polymerase reactions were separated by gel electrophoresis, extracted from the gels, denatured, and used as probes to hybridize to Northern (RNA) blots of positive-strand and negative-strand RNAs corresponding to a 1-kb internal fragment of TMV-L RNA. The labelled genomic-length ssRNA synthesized by the template-dependent RNA polymerase hybridized to the negative-strand RNA (Fig. 5, lane 2) but not detectably to the positive-strand RNA (Fig. 5, lane 1), confirming the RNase H results (Fig. 4) that the synthesized RNA is the positive strand. In a similar way, the labelled genomic-length ssRNA reaction product of the sucrose-purified template-bound RNA polymerase was also shown to be the positive strand (Fig. 5, lanes 7 and 8). However, differences between the dsRNA products of the template-dependent and template-bound RNA polymerases were observed. The labelled genomic-length dsRNA reaction product of the template-dependent RNA polymerase hybridized to both positive- and negative-strand RNAs (Fig. 5, lanes 3 and 4), showing that it contained newly synthesized negative and positive strands, whereas the labelled genomic-length dsRNA reaction products of the crude and purified template-bound RNA polymerases hybridized

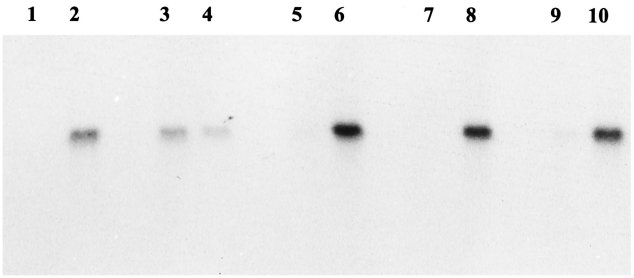

FIG. 5. Synthesis of positive-strand and negative-strand RNAs by TMV-L RNA polymerase preparations. Unlabelled positive-strand (lanes 1, 3, 5, 7, and 9) and negative-strand (lanes 2, 4, 6, 8, and 10) RNAs, corresponding to TMV-L RNA nt 3335 to 4390, were synthesized by using T7 and T3 RNA polymerases, respectively, from a TMV-L cDNA clone in pBluescript SK(+), electrophoresed in formaldehyde-agarose gels, and blotted onto membranes. The membranes were then probed with the ^{32}P-labelled genomic-length ssRNA (lanes 1, 2, 7, and 8) or (denatured) dsRNA (lanes 3, 4, 5, 6, 9, and 10) products of reactions carried out in the presence of [α-^{32}P]UTP with (i) the membrane-bound, template-dependent RNA polymerase with TMV-L RNA as template (lanes 1 to 4); (ii) the crude membrane-bound P30 RNA polymerase with no added template (lanes 5 and 6); or (iii) sucrose density gradient-purified, membrane-bound RNA polymerase combined fractions 2 and 3 with no added template (lanes 7 to 10). Hybridized bands were detected by autoradiography.

mainly to the negative strand (Fig. 5, lanes 6 and 10) and only very weakly to the positive strand (Fig. 5, lanes 5 and 9), showing that mainly the positive strand of the dsRNA was newly synthesized.

Characterization of the subgenomic RNAs produced by the TMV template-dependent RNA polymerase. The synthesis of small amounts of 0.7-kb ssRNA and dsRNA species by the TMV-L template-dependent RNA polymerase suggested that they may be the ss and ds forms of the coat protein subgenomic mRNA. To confirm this, the 0.7-kb ssRNA and dsRNA were mapped. Each ^{32}P-labelled RNA was separated by gel electrophoresis (Fig. 6), extracted from the gel, and then used as a probe in dot blot hybridization with unlabelled 5′-terminal and 3′-terminal fragments of the TMV-L positive-strand and negative-strand RNAs (Fig. 7). The 0.7-kb ssRNA hybridized with the 277-nt 5′-terminal negative-strand fragment but not with the 271-nt 3′-terminal negative-strand fragment or with the 392-nt 5′-terminal and 278-nt 3′-terminal positive-strand fragments. The 0.7-kb subgenomic ssRNA therefore maps to the 3′ end of the positive strand, consistent with its identity as the coat protein subgenomic mRNA. The 0.7-kb dsRNA hybridized with both the 5′-terminal negative-strand fragment and the 3′-terminal positive-strand fragment but did not hybridize with either the 5′-terminal positive-strand or 3′-terminal negative-strand fragment. It is clear, therefore, that the 0.7-kb dsRNA corresponds to the ds form of the coat protein subgenomic mRNA and is labelled in both strands.

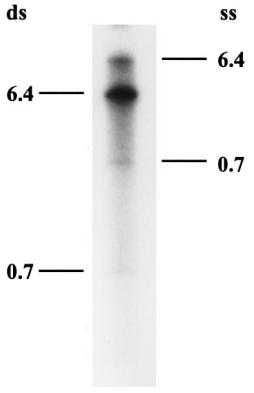

FIG. 6. Synthesis of subgenomic ssRNA and dsRNA by the membrane-bound, template-dependent TMV-L RNA polymerase programmed with TMV-L RNA. The sizes of ssRNA and dsRNA bands (in kilobases and kilobase pairs, respectively) are shown on the right and left, respectively.

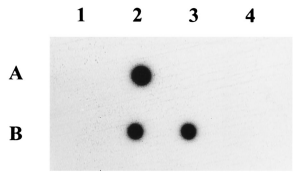

FIG. 7. Characterization of subgenomic RNAs synthesized by the membrane-bound, template-dependent TMV-L RNA polymerase. A 392-nt 5′-terminal fragment of the TMV-L positive-strand RNA (lane 1), a 277-nt 5′-terminal fragment of the TMV-L negative-strand RNA (lane 2), a 278-nt 3′-terminal fragment of the TMV-L positive-strand RNA (lane 3), and a 271-nt 3′-terminal fragment of the TMV-L negative-strand RNA (lane 4) were blotted onto filters and hybridized with the ^{32}P-labelled 0.7-kb subgenomic ssRNA (row A) or (denatured) 0.7-kb subgenomic dsRNA (row B) products of the reaction with the membrane-bound, template-dependent RNA polymerase carried out in the presence of added TMV-L RNA (T7 transcripts) as a template and [α-^{32}P]UTP. Since the amounts of subgenomic RNAs synthesized were small (Fig. 6), it was necessary to extract the bands from eight lanes to obtain enough material for the hybridizations. Autoradiographs were exposed for 10 days.

Inhibition of the TMV template-dependent RNA polymerase by antibodies to the TMV-encoded 126- and 183-kDa proteins. TMV encodes two proteins believed to be involved in the virus RNA replication, a 126-kDa protein containing amino acid sequence motifs typical of RNA helicases, NTP-binding proteins, and methyltransferases (19) and a 183-kDa protein, synthesized by translational readthrough of the 126-kDa protein open reading frame, which additionally contains motifs characteristic of RNA-dependent RNA polymerases (19). Additionally, a subgenomic RNA containing an open reading frame encoding a putative 54-kDa protein, corresponding to the C-terminal portion of the 183-kDa protein, has been detected in TMV-infected plants, although the 54-kDa protein has not been detected (6, 8). The 126- and 183-kDa proteins have both been detected in crude membrane-bound and partially purified, solubilized preparations of TMV (U1 strain) RNA polymerase preparations (39, 40), but inhibition by antibodies has not been reported, probably because a template-dependent polymerase has not hitherto been available. We have prepared antisera to regions of the TMV-L 126- and 183-kDa proteins linked to MBP for use in Western blots (immunoblots) to detect virus-encoded proteins in sucrose density gradient-purified, membrane-bound TMV-L RNA polymerase preparations and to test them for inhibition of the template-dependent and template-bound polymerases.

Antisera to the C-terminal and N-terminal regions of the 126-kDa protein were able to detect both the 126- and 183-kDa proteins (shown for the C-terminal region antiserum in Fig. 8, lane 2); an antiserum to the putative 54-kDa protein (readthrough region of the 183-kDa protein) detected the 183-kDa protein but not the 54-kDa protein (Fig. 8, lane 3). No proteins were detected by the preimmune serum (not shown) or antibodies to MBP (Fig. 8, lane 1).

When the template-dependent TMV-L RNA polymerase was incubated with antibodies to the 54-kDa protein prior to addition of the TMV-L RNA template and NTP substrates, RNA polymerase activity was completely inhibited, as no products could be detected (Fig. 9, lane 9). Incubation of the template-dependent polymerase with the same amounts of antibodies to the C-terminal or N-terminal region of the 126-kDa

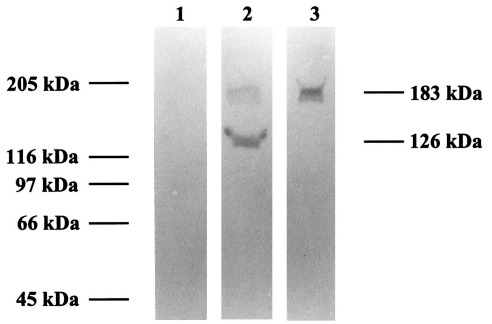

FIG. 8. Detection of the 126- and 183-kDa polypeptides in the sucrose density gradient-purified TMV-L RNA polymerase. Sucrose density gradient-purified, membrane-bound TMV-L RNA polymerase combined fractions 2 and 3 were electrophoresed in an SDS-polyacrylamide gel, and the separated proteins were blotted onto a membrane. The membrane was then probed with antibodies to MBP (lane 1), to the C-terminal portion of the 126-kDa protein (amino acids 648 to 1116)–MBP fusion (lane 2), or to the 54-kDa protein (amino acids 1143 to 1616 of the 183-kDa protein)–MBP fusion (lane 3). Antisera were used at a dilution of 1:2,000. The positions of protein markers are shown on the left, and the positions of the 126- and 183-kDa proteins are shown on the right.

protein led to partial inhibition of the polymerase (Fig. 9, lanes 3 and 6), although incubation with larger amounts of these antibodies gave complete inhibition (not shown). The inhibition was shown to be specific because prior incubation of the polymerase with the corresponding MBP fusion, prior to addition of the antibodies, reversed the inhibition (Fig. 9, lanes 2, 5, and 8). Incubation of the polymerase with any of the MBP fusions in the absence of antibodies (Fig. 9, lanes 1, 4, and 7), with antibodies to MBP alone, or with preimmune sera (not shown) had no effect on activity. When the sucrose density gradient-purified, template-bound TMV-L RNA polymerase (prior to MN treatment) was incubated with antibodies to the 54-kDa protein or with antibodies to the C-terminal or N-terminal region of the 126-kDa protein, no inhibition of the polymerase activity was observed (not shown).

DISCUSSION

Previous characterization of the reaction products of a crude, membrane-bound RNA polymerase obtained from tobacco plants infected with TMV-U1 identified the genomic-length dsRNA (replicative form) and a polydisperse partially ssRNA and dsRNA product similar to the replicative intermediates isolated from infected plants (39, 40). In our crude membrane-bound RNA polymerase preparations from tomato plants infected with TMV-L, we were able to detect only the genomic-length dsRNA. However, after purification by sucrose density gradient centrifugation, the RNA polymerase synthesized genomic-length ssRNA as well as the dsRNA. Replicative-intermediate forms were not detected, because the ssRNA, after extraction from a gel, was fully sensitive to S1 nuclease digestion. It is possible that the crude RNA polymerase preparations contain inhibitors, which may prevent the reaction from going to completion and may partially degrade the product. It is clear that the sucrose density gradient step was effective in removing such inhibitors, as well as any host RNA polymerase or terminal transferase which may have contaminated the crude enzyme. It is noteworthy that sedimentation of a crude membrane-bound flockhouse virus RNA polymerase through a glycerol cushion was required to remove a contaminating nuclease (37).

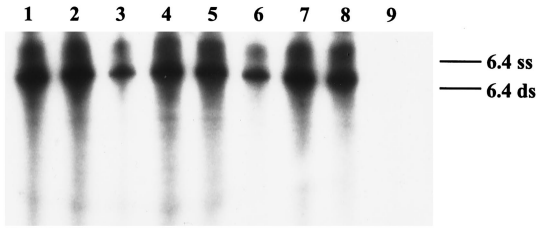

FIG. 9. Inhibition of the template-dependent TMV-L RNA polymerase by polyclonal antibodies to the 126- and 183-kDa (54-kDa) proteins. The membrane-bound, template-dependent TMV-L RNA polymerase was incubated with the C-terminal portion of the 126-kDa protein–MBP fusion (lanes 1 and 2), the N-terminal portion of the 126-kDa protein–MBP fusion (lanes 4 and 5), the 54-kDa protein–MBP fusion (lanes 7 and 8), or no protein (lanes 3, 6, and 9) and then with antibodies to the C-terminal portion of the 126-kDa protein–MBP fusion (lanes 2 and 3), the N-terminal portion of the 126-kDa protein–MBP fusion (lanes 5 and 6), or the 54-kDa protein–MBP fusion (lanes 8 and 9) or with no antibody (lanes 1, 4, and 7). 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 as described in the legend to Fig. 1. The positions of the 6.4-kb ssRNA and dsRNA reaction products are shown on the right.

Neither the crude nor sucrose-purified RNA polymerase required the addition of RNA template for its activity; they therefore contained endogenous TMV-L template, and the reaction probably consisted of the completion of strands initiated in vivo, as found for the crude TMV-U1 polymerase (39, 40). We also detected small amounts of ssRNA and dsRNA corresponding to the coat protein subgenomic mRNA with the sucrose-purified TMV-L polymerase. Subgenomic RNAs were not detected with the TMV-U1 polymerase (39, 40), but indirect evidence was obtained for synthesis of coat protein subgenomic mRNA with a subcellular fraction obtained from tobacco protoplasts infected with TMV-OM (34).

Removal of the endogenous RNA template from the sucrose-purified TMV-L RNA polymerase with MN rendered it template dependent. It was also template specific, being able to utilize RNAs of TMV-L and, less efficiently, TMV-*vulgare* as templates but not RNAs of viruses in other genera. The template-dependent RNA polymerase was able to catalyze the complete replication of TMV-L RNA, producing genomic-length, positive-strand ssRNA. The ability of the RNA polymerase to initiate negative-strand synthesis was shown by the incorporation of [γ-^{32}P]GTP into the negative strand of the dsRNA product; this eliminates the possibility that the dsRNA was formed by using the 3′-OH as a primer in a "copy-back" mechanism, as can occur in vitro with the poliovirus 3D^{pol} (6) and hepatitis virus C NS5B (3) polymerase proteins. The polymerase must also be able to initiate synthesis of positive strands, because uniformly labelled positive strands were synthesized by using [α-^{32}P]UTP. However, no incorporation of [γ-^{32}P]GTP was detected in the positive strand synthesized. This may be related to the fact that the 5′ terminus of TMV RNA has a 7-methylguanosine triphosphate cap structure. The capping and methylating activities are believed to reside in the 126-kDa protein (12), and formation of the cap will involve prior hydrolysis of the γ-position phosphate. Hence, it is likely that the template-dependent TMV-L RNA polymerase preparation contained a phosphatase capable of hydrolyzing the 5′ γ phosphate of the positive strand, but further work will be required to determine if it is competent for addition of the 7-methylguanosine cap. It is also noteworthy that the polymerase could use a 3′-terminal negative-strand RNA as a template, again indicative of the ability to initiate positive-strand RNA synthesis. However, unlike a CMV RNA polymerase preparation, which required an additional 3′-G residue to enable it to utilize a negative-strand satellite RNA as a template (35), the

TMV-L polymerase was able to use 3′-terminal negative-strand templates whether or not they contained an extra G residue.

The template-dependent TMV-L RNA polymerase was also able to synthesize small amounts of the ss and ds forms of the 0.7-kb coat protein subgenomic mRNA. As expected, only the positive strand of the 0.7-kb ssRNA was detected. However, the ds form was labelled in both strands. A similar observation was made for a subgenomic RNA synthesized in vitro by an isolated RNA polymerase of flockhouse nodavirus (37). It is likely that the 0.7-kb subgenomic RNA is synthesized from a subgenomic promoter on a genomic-length negative-strand RNA template, as shown for the coat protein subgenomic RNA of brome mosaic bromovirus (23). The subgenomic ssRNA could then serve as a template for the synthesis of the ds form, which would be a dead-end product. Neither the ss nor the ds form of the 1.6-kb movement protein subgenomic RNA was detected in the reaction products of the template-dependent or template-bound polymerase. The ss form would have been difficult to detect because of the smear below the genomic-length dsRNA band. However, the ds form should have been readily detectable, particularly after S1 nuclease treatment, which removes the background smear. This could indicate different requirements for recognition of the movement protein and coat protein subgenomic promoters. Indeed, it is known that the movement protein is expressed early and the coat protein is expressed late in the infection cycle. The RNA polymerase studied here was isolated 4 days after inoculation, when synthesis of the movement protein would have decreased to a low level (20).

Genetic studies have shown that both the 126- and 183-kDa proteins are required for normal levels of TMV replication (9), and crude TMV RNA polymerase preparations have been shown to contain proteins of these sizes (39, 40). Here we have shown by immunoblotting with specific antisera that the sucrose-purified polymerase contains both the 126- and 183-kDa proteins. The ratios of the signals for the two proteins varied for different preparations, although the relative strengths of Western blot signals may not be directly related to relative protein levels, since the common epitopes recognized by an antibody may not be equally accessible in these overlapping proteins. Inhibition of the template-dependent RNA polymerase with specific antibodies showed the importance of the 126-kDa protein N-terminal region (contains a methyltransferase-like domain) and C-terminal region (contains a helicase-like domain) and of the 183-kDa readthrough region (contains the polymerase domain) in the RNA polymerase activity. It is noteworthy that none of these antibodies inhibited the template-bound enzyme, suggesting that the inhibitory antibodies targeted regions of the polymerase involved in interaction with the template.

This is the first report of the production of a template-dependent TMV RNA polymerase and of the complete replication of a plant virus positive-strand RNA by a membrane-bound RNA polymerase. The key to obtaining template dependency and complete replication may lie in the removal of an inhibitor or nuclease by sucrose density gradient centrifugation, since attempts to produce a template-dependent polymerase from crude enzyme preparations were unsuccessful. Complete replication of flockhouse nodavirus RNA by a membrane-bound RNA polymerase has been reported; in that case, added glycerophospholipids were needed to obtain synthesis of ssRNA (36, 37). This may reflect differences in the membranes involved and the structural organization of the polymerase within the membrane. Although we have demonstrated complete replication of TMV RNA, the ratio of positive to negative strands in the reaction products was much lower than that observed in vivo (18). Further work will be required to determine whether this is due to the absence of the capsid protein or some other essential protein in the replication system.

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council.

We thank T. Meshi for plasmid pLFW30.

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