The 80K Polypeptide Associated with the Replication Complexes of Cauliflower Mosaic Virus Is Recognized by Antibodies to Gene V Translation Product

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SUMMARY

The enzyme responsible for cauliflower mosaic virus (CaMV) DNA replication has been studied by isolating virus replication complexes and assaying the associated polymerase activity using activated calf thymus DNA or poly(rCm)-oligo(dG)12-18 as template–primer. The activity of the enzyme was specifically inhibited by immune serum raised against a synthetic peptide corresponding to a portion of the viral gene V protein. This result provides further evidence that this protein is the reverse transcriptase involved in CaMV replication.

A model for cauliflower mosaic virus (CaMV) replication has been proposed (Guilley et al., 1983; Hull & Covey, 1983; Pfeiffer & Hohn, 1983) which involves reverse transcription of a viral transcript. In this model, a major CaMV DNA transcript, 35S RNA, is transcribed in the first step of the replication process into cDNA starting from a tRNA primer binding site. Indeed, Volovitch et al. (1984) have demonstrated the presence in infected plants of a reverse transcriptase activity not detected in healthy plants. Toh et al. (1983) and Volovitch et al. (1984) have shown that the open reading frame (ORF) V polypeptide shares significant homology with reverse transcriptase from hepatitis B virus and avian retrovirus. From the CaMV nucleotide sequence, the ORF V protein is predicted to have a molecular weight of 79 000 (79K) (Franck et al., 1980). Recently, Ziegler et al. (1985) have used an antiserum raised against a synthetic peptide corresponding to a portion of the ORF V sequence to detect specifically an 80K protein in extracts of infected plants using immunoblotting techniques. There is no direct evidence, however, that the ORF V protein (the putative reverse transcriptase) is in fact the replicase activity detected in the CaMV replication complexes (CaMV-RC) (see Pfeiffer et al., 1984). We have shown previously (Pfeiffer et al., 1984) that the 80K polymerase responsible for CaMV DNA synthesis has some properties similar to those of the host γ-like DNA polymerase. Furthermore, a DNA polymerase activity of similar electrophoretic mobility can be detected in extracts of healthy plants although the amount of activity is variable and much lower than in infected plants (Pfeiffer et al., 1984). In view of these results we have used serological inhibition tests to provide further evidence that the CaMV-RC-associated enzyme is indeed the viral ORF V protein and that this protein has reverse transcriptase activity.

The replicase activity associated with CaMV-RC was isolated by hypotonic leaching of a heavy particulate fraction, enriched in nuclei and viral inclusion bodies, from CaMV-infected turnip leaves (Pfeiffer et al., 1984). Freshly washed leaves from plants that were healthy or had been inoculated 21 days previously were ground in 25 mM-MES pH 6, 5 mM-MgCl2, 2 mM-dithiothreitol (DTT), 250 mM-sucrose, 50% glycerol. After filtration through eight sheets of Miracloth, the crude extract was incubated in 5% Triton X-100 for 30 min at 0 °C. Nuclei (and viral inclusion bodies in the case of infected plants) were then sedimented through a sucrose cushion and taken up in 50 mM-Tris–HCl pH 7.8, 1 mM-MgCl2, 1 mM-DTT and incubated for 1 h at 0 °C. After centrifugation for 15 min at 15000 r.p.m. the supernatant fluid, which contained...
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Fig. 1. Comparison of the solubilization effects of (a) 1\% or (b) 5\% Triton X-100. Twenty \( \mu l \) aliquots of hypotonic extracts (500 \( \mu l \)/10 mg fresh leaves) were fractionated by electrophoresis on a 10\% SDS-polyacrylamide gel and immunoblotted. Lanes 2 and 3, immune serum; lanes 1 and 4, non-immune serum; lanes 1 and 2, infected extract; lanes 3 and 4, healthy extract.

Fig. 2. Immunodetection of virus-related 80K protein with immune serum with or without pre-incubation with the immunogenic oligopeptide. Blots of (a) infected or (b) healthy tissue were treated with immune serum at the same dilution as in Fig. 1 (500-fold) used without pretreatment or after incubation undiluted with synthetic polypeptide (1 \( \mu g/\mu l \)) in 0.5\% Tween 20 in PBS, 3\% bovine serum albumin for 6 h at room temperature and centrifugation for 5 min at 5000\( g \). Lanes 1, immune serum not pre-incubated with synthetic oligopeptide; lanes 2, immune serum pre-incubated with synthetic oligopeptide.

Small nucleoprotein complexes (Pfeiffer & Hohn, 1983) was removed with a Pasteur pipette and stored at 4 \( ^\circ C \). As shown in Fig. 1, treatment of the crude extract with 5\% Triton X-100 rather than 1\% Triton-100 as in our earlier work (Pfeiffer et al., 1984) improved the solubilization of the 80K polypeptide without significantly increasing the solubilization of the 110K \( \alpha \)-like DNA polymerase of the host plant (data not shown).

A synthetic polypeptide containing the C-terminal 25 amino acids of the CaMV ORF V protein was synthesized and injected into rabbits (Ziegler et al., 1985). In immunobLOTS (Towbin et al., 1979) the immune serum so obtained could detect an 80K protein in infected plants. Other proteins of 56K, 33K, 23K and 20K were also detected by the immunoblotting technique (Fig. 1, 2). Certain of these polypeptides are probably degradation products of the 80K gene V product whereas others are recognized by non-specific antibodies present in the antisera. Thus, pre-incubation of immune serum with the synthetic polypeptide (1 \( \mu g \) polypeptide/1 \( \mu l \) undiluted immune serum for 6 h at room temperature) before reaction with the blotted proteins suppressed reaction with the 80K and 33K polypeptides and most of the reaction with the 56K polypeptide, indicating that these species are specifically recognized by antibodies raised against the synthetic polypeptide. The 23K and 20K proteins, on the other hand, are probably host proteins recognized by other (non-specific) antibodies present in the serum. The 56K and 33K proteins may be degradation products of the 80K protein which is the only one detected on activity gels.
Fig. 3. Serological inhibition test of DNA polymerase activities. Empty symbols, infected hypotonic extract; filled symbols, healthy hypotonic extract. \( \Diamond, \bullet \), Pretreatment with \( \text{H}_2\text{O} \) (control), no added calf thymus DNA; \( \circ, \bullet \), pretreatment with immune serum, no added calf thymus DNA; \( \blacksquare, \bigcirc \), pretreatment with \( \text{H}_2\text{O} \) (control), plus 20 \( \mu \)g/ml activated calf thymus DNA; \( \bigcirc, \bullet \), pretreatment with immune serum, plus 20 \( \mu \)g/ml activated calf thymus DNA.

Fig. 4. Serological inhibition test of reverse transcriptase activity. Empty symbols, infected extracts; filled symbols, healthy extracts. \( \blacksquare, \bigcirc \), Pre-incubation with sterile water; \( \bigtriangleup, \bigcirc \), pre-incubation with non-immune serum; \( \bigcirc, \bullet \), pre-incubation with immune serum; \( \bigcirc, \bullet \), minus poly(rCm)-oligo(dG)\(_{12-18}\), pre-incubation with sterile water 1 h at 4 °C as in control.

(Pfeiffer et al., 1984). It is noteworthy, however, that immune serum reacted with 56K, 23K and 20K bands in extracts from both infected and healthy plants whereas non-immune serum did not react significantly (Fig. 1). Thus, the 56K band must contain, in addition to the gene V-specific polypeptide, a second species of host origin which is recognized by non-specific antibodies in extracts from both healthy and infected plants. This explains why the detection of the 56K protein was not entirely suppressed by pre-incubation of immune serum with the synthetic polypeptide.

Hypotonic extracts of CaMV-infected tissue have been shown to contain DNA polymerase activities (Pfeiffer & Hohn, 1983; Pfeiffer et al., 1984). Tests for serological inhibition of this activity were performed as follows. Twenty \( \mu \)l aliquots of hypotonic extracts were pre-incubated for 1 h at 4 °C in the presence of either 5 \( \mu \)l sterile water, 5 \( \mu \)l non-immune serum or 5 \( \mu \)l immune serum plus 5 \( \mu \)l PBS (1-5 mm-KH\(_2\)PO\(_4\), 8 mm-Na\(_2\)HPO\(_4\), p\( \text{H} \) 7-4, 140 mm-NaCl, 2-7 mm-KCl, 3 mm-Na\(_3\)) in each case to favour the formation of antigen–antibody complexes. The reaction was started by addition of 60 \( \mu \)l of 1-5-fold concentrated mixture to give final concentrations of
50 mM-Tris-HCl pH 7.8, 50 mM-KCl, 5 mM-MgCl₂, 2 mM-DTT, 100 μM each of dATP, dCTP and dTTP, 1 μCi [α-³²P]dGTP (2000 Ci/mmole, Amersham), 0.05% Triton X-100 with or without 20 μg/ml activated calf thymus DNA (prepared as described by Spanos & Hübscher, 1982). At intervals during incubation at 37 °C, aliquots of 15 μl were spotted on Whatman 3MM filter papers for precipitation with TCA. After several washes, acid-insoluble radioactivity was determined by Cerenkov counting. As shown in Fig. 3, the activity detected in CaMV-RC without addition of template was inhibited by pre-incubation with immune serum when compared with untreated extract. Non-immune serum had no effect on the activity; the kinetics of incorporation obtained were identical to those obtained with an untreated extract (data not shown). It has been shown previously that the endogenous CaMV replicase activity is fully resistant to aphidicolin (Pfeiffer et al., 1984). The hypotonic extract prepared from healthy plants as a control had a rather low polymerase activity which was totally inhibited by aphidicolin (data not shown), suggesting that the enzyme is a co-purifying host θ-like DNA polymerase. This activity was unaffected by the addition of immune serum even when the ratio (v/v) was increased from 1:4 to 1:1.

Non-ionic detergents have been shown to protect the putative active site of reverse transcriptase of avian myeloblastosis virus (Green & Gerard, 1974). We tested different concentrations of Triton X-100 (0.005% to 1%) with purified avian myeloblastosis virus DNA polymerase in the presence of either activated calf thymus DNA or poly(rCm)-oligo(dG)₁₂₋₁₈ in DNA polymerase assays. We found that the highest activity was obtained in the presence of 0.05% Triton X-100 (data not shown), and therefore have added 0.05% Triton X-100 to all reaction mixtures. In these conditions a significant activity was detected in extracts of infected plants when 20 μl/ml activated calf thymus DNA was added. The activity was detected in extracts of healthy plants but at a much lower level (Fig. 3). Pre-incubation of hypotonic extracts of infected plants with immune serum resulted in a marked inhibition of the enzyme activity. Non-immune serum never produced a similar result; incorporation of labelled precursors was unaffected. Pre-incubation of the hypotonic extract of healthy plants with immune serum before starting the reaction with activated calf thymus DNA did not change the level of incorporation as compared to that of an untreated extract or an extract pre-incubated with non-immune serum (not shown). Thus, the inhibition by immune serum of DNA polymerase activity in extracts of infected plants detected in the presence of activated calf thymus DNA reflects the specific inhibition of the virus-coded DNA polymerase associated with viral replication complexes. The residual activity, which was similar to that found in extracts of healthy plants in the presence of activated calf thymus DNA and immune serum, is assumed to be the background of host polymerases not inhibited by the immune serum.

The inhibition of the DNA polymerases that was associated with replication complexes and detected with endogenous or exogenous template by immune serum raised against the C terminus of the viral ORF V protein implicates the viral gene V protein in the CaMV-RCs, which is specifically detected in extracts of infected plants by immunoblotting techniques (Fig. 1, 2).

We also tested the activity of the CaMV-RC-associated enzyme using poly(rCm)-oligo(dG)₁₂₋₁₈ (molar ratio C:G = 20:1) which is an efficient primer-template for reverse transcriptases. This template is also resistant to RNase attack and, because of the large amounts of RNase in our extracts, such a template is required to detect reverse transcriptase activity. The assay was performed as for the DNA polymerase reaction but with 20 μg/ml poly(rCm)-oligo(dG)₁₂₋₁₈ in place of activated calf thymus DNA. The three unlabelled deoxyribonucleotides were not included in the reaction mix because only [α-³²P]dGTP is required to copy poly(rCm)-oligo(dG)₁₂₋₁₈ into poly(dG), and to eliminate the detection of DNA polymerase activities. No activity could be found in extracts of healthy plants with this template (Fig. 4), in agreement with the results described by Volovitch et al. (1984). In contrast, an activity was found in infected plants which was dependent upon the addition of poly(rCm)-oligo(dG)₁₂₋₁₈ (Fig. 4). Analysis of the DNA synthesized by electrophoresis in a sequencing gel showed that about 60 nucleotides on average were incorporated per template specifically in extracts of infected plants. After denaturation for 5 min at 80 °C and quenching on ice, unprimed poly(rCm) was not copied into poly(dG).
The reverse transcriptase activity specific for extracts of infected plants was strongly inhibited by immune serum but not by non-immune serum as compared with the untreated extract. The ability of the CaMV-RC-associated enzyme to copy poly(rCm)-oligo(dG)$_{12-18}$ and the specific inhibition of this activity by immune serum support the argument in favour of the involvement of the viral gene V protein in the replication of the CaMV genome. The model of CaMV replication involving reverse transcription implicates an enzyme capable of copying RNA. The ability of the replicase to copy poly(rCm)-oligo(dG)$_{12-18}$ into DNA and the inhibition of this activity by immune serum suggest that the viral gene V protein is the reverse transcriptase implicated in CaMV multiplication.

However, the viral replicase encoded by the CaMV ORF V is probably not the sole component of the enzyme involved in CaMV replication. As discussed by Hübscher (1984) DNA polymerases responsible for chromosomal DNA replication of prokaryotes and eukaryotes are part of multipolypeptide complexes. The functional forms of DNA polymerases that have so far been isolated have been designated as DNA polymerase holoenzymes which are required for efficient and accurate in vitro replication of naturally occurring genomes such as those of bacteriophages or DNA viruses of animals. Perhaps the same will apply to the enzyme responsible for CaMV DNA replication.

REFERENCES


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