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A DNA polymerase activity is associated with Cauliflower Mosaic Virus

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ABSTRACT

A DNA polymerase activity is found within the Cauliflower Mosaic Virus (CaMV) particle. Analysis of the reaction product reveals that the linear form of the virion DNA is preferentially labelled. The molecular weight of the DNA polymerase as determined on an "activity gel" is 76 kDa.

INTRODUCTION

Cauliflower Mosaic Virus (CaMV) is the best characterized member of the only group of plant viruses, the caulimoviruses, known to have a double-stranded DNA genome (for review see ref. 1 and 2). In the virus particle the major form of the DNA (8 kb) is circular, relaxed and contains three single-stranded discontinuities at specific sites, one in the minus strand and two in the plus strand (3,4,5). At the discontinuities the DNA has a triple-stranded structure with the 5'- and 3'- extremities of the interrupted strand overlapping for 8 to 20 nucleotides (5,6). Discontinuous molecules of this type co-exist in infected cells with supercoiled viral DNA (7,8,9) which is present as minichromosomes in the nuclei (8,10).

In the past year a model for CaMV replication has been put forward independently by several authors (11-15). It has been suggested that, as for the retroviruses (for review see ref. 16) or the Hepatitis-B type viruses (HBVs) (17), a reverse transcription step takes place during the CaMV infectivity cycle. In all three cases at some stage of the cycle an RNA species is produced that contains the complete viral genome. This RNA is then transcribed into cDNA. DNA-dependent DNA-polymerase activity has been described in mature HBV(s)(18). In the case of the retroviruses the encapsidated enzyme has a reverse transcriptase activity (19,20) but this has not yet been demonstrated in HBV(s). Recently Volovitch et al. (21) and Pfeiffer et al. (22) have described a reverse transcriptase activity in CaMV infected leaves.

In this paper, we report the presence in the CaMV particle of a template-dependent DNA polymerase activity. This virus-associated enzyme has a molecular weight of 76 kDa.

MATERIALS AND METHODS

Virus preparation

CaMV (Cabb-S and D/H strains) were propagated in turnip leaves (Brassica rapa L. c.v. Just Right) and isolated as described by Hull et al. (23). It was sometimes necessary to treat the preparation of virus with DNase I (0.1 µg/µl) to eliminate residual cellular DNA prior to the polymerase reaction. We have verified that such DNase I treatment does not digest encapsidated DNA. Some preparations were further purified by buoyant density centrifugation as described by Al Ani et al. (24) to eliminate residual contaminants. Before enzymatic assays, CaMV samples were extensively dialysed against 10 mM Tris-HCl pH 7.8. Purified CaMV preparations were stored at +4°C or at -20°C in 50% glycerol at a concentration of 4.5 µg/µl.

DNA polymerase assay

30 µg of purified CaMV was tested for DNA polymerase activity in 60 µl of polymerization buffer [50 mM Tris-HCl (pH 7.8) ; 5 mM $MgCl_2$; 20 mM KCl ; 1 mM DTT], containing 50 µg/ml BSA, 0.2 mM each of dATP, dGTP and dTTP and 3 µM [α-^{32}P]dCTP (Amersham, 3000 Ci/mMole).

Incorporation of radioactivity was assayed by TCA precipitation of aliquots spotted on Whatman 3 MM filter paper squares. For DNA electrophoretic analysis of the DNA, 30 µl of the DNA polymerase mixture were made up to 100 µl with water. The proteins were digested with proteinase K (1 µg/µl) for 16 h at 37°C in the presence of 1 % SDS, 50 mM EDTA and 500 mM NaCl. Proteins were extracted by 1 volume of phenol saturated with 50 mM Tris-HCl pH 8. DNA was purified by two successive ethanol precipitations in the presence of 200 mM ammonium acetate and 0.1% SDS.

Gel electrophoresis and autoradiography

DNA samples were analysed on a 1% agarose gel in a buffer containing 40 mM Tris-HCl pH 8.1 ; 20 mM Na acetate ; 2 mM EDTA. Electrophoresis was performed at 30 volts for 16 h. The gels were then stained for 15 min with 0.5 µg/ml of ethidium bromide and the DNA was visualised under U.V. illumination. Radioactive DNA was detected by autoradiography of the dried agarose gel.

Determination of the molecular weight of the enzyme by activity gel analysis

Electrophoresis was carried out essentially as described by Spanos et al.

(25) and Hubscher et al (26). Fifteen µg of CaMV particles and 5 U of E. Coli DNA polymerase were incubated at 37°C for 3 min in a buffer containing 50 mM Tris-HCl pH 6.8, 1% SDS, 5 mM DTT and 20% glycerol, and then loaded on a 0.1% SDS, 10% polyacrylamide gel containing 50 µg/ml of activated calf thymus DNA (prepared as described by Spanos et al. (25)). After electrophoresis SDS was removed from the gel by soaking for 2 hours in several changes of renaturation buffer (50 mM Tris-HCl, pH 7.8 ; 5 mM ß-mercaptoethanol ; 0.1 mM EDTA).

The gel was then sealed in a plastic bag containing 10 ml of the DNA polymerase reaction mixture and kept at 37°C for 16 hours. The gel was next washed for 4 hours in several changes of 5% TCA, dried and autoradiographed.

Analysis of the products of the DNA-polymerase reaction

Fifteen µg of CaMV and 5 U of E. Coli DNA polymerase were electrophoresed on an activity gel as described in the preceding paragraph. The proteins were renatured and the gel was incubated in the DNA-polymerisation medium. Bands were excised in the region of the gel where E. Coli DNA polymerase I (109 kDa) and CaMV DNA-polymerase (76 kDa) were expected to have migrated, and the DNA was electroeluated for 3 hours at 5 mA in 3 mM Tris-HCl pH 7.5, 3 mM NaH_2PO_4, 0.1 mM EDTA and 0.1% SDS. The recovered material was loaded on a sepharose 6B column equilibrated in 20 mM Tris-HCl pH 7.8, 0.5 mM EDTA to remove free deoxynucleotides. The DNA in the eluate is ethanol-precipitated. The pellets were resuspended in 20 µl sterilised water and divided in two aliquots. One was heated at 100°C for 3 min to denature the DNA. The fifty µl of S1 nuclease mixture contained 10 µg of the single-stranded (ss) or double-stranded (ds) labelled DNA, 10 µg of denatured carrier calf thymus DNA, 500 U of S1 nuclease (Sigma), 100 mM KCl, 1 mM Mg acetate, 5 mM Na acetate and 0.2 mM $ZnCl_2$, pH 4.5. Hydrolysis of aliquots spotted on Whatman 3 MM filter paper squares.

RESULTS

DNA polymerase activity in purified CaMV preparation

Preparations of purified CaMV virions were found to catalyze the incorporation of [α-^{32}P]dCTP into a trichloracetic acid insoluble product when incubated with the four deoxynucleotides as described in Materials and Methods. Fig. 1 shows the time course of a typical reaction. Two viral isolates (Cabb-S and D/H strains) were tested and gave similar results. No incorporation was detected when the virus was replaced by 1 µg of activated calf thymus DNA in the reaction mixture. No incorporation was observed in the

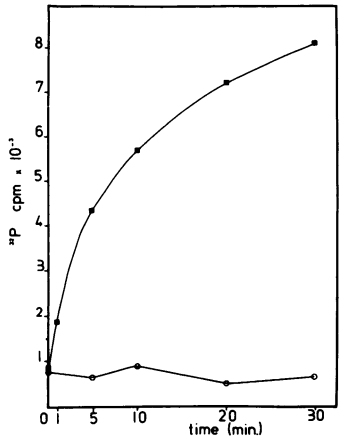

Figure 1. Time course of the DNA polymerase reaction.
(■) 30 µg of CaMV particles in 60 µl of DNA polymerase reaction mixture were incubated at 37°C with 10 µCi of [α-^{32}P]dCTP.
(○) Virus is replaced by 1 µg of activated calf thymus DNA.
After the indicated times of incubation, 9 µl of the reaction mixture was removed for counting as described in Materials and Methods.

absence of $MgCl_2$ and the optimal $MgCl_2$ concentration for the reaction was found to be 5 mM. The reaction was optimally stimulated by KCl at a concentration of 20 mM. No apparent structural changes were detected by electron microscopic observations of virions exposed to these conditions.

To help eliminate the possibility that the DNA polymerase activity would result from contamination of the virus preparation by cosedimenting plant DNA polymerases, we have tested the polymerase activity in a preparation of turnip yellow mosaic virus which was propagated in the same host plant. No incorporation was found (data not shown).

To prove that the enzymatic activity was located within the virus particles, a standard CaMV preparation was treated for 30 min at 37°C with a high concentration of DNase I (0.15 mg/ml) before testing its polymerase activity. In a control experiment, this amount of DNase completely digested "nick translated" calf thymus DNA, indicating that the DNase I was highly active in these conditions (Table I, line 3 and 4). Such treatment, however, had no effect on the ability of the CaMV preparation to incorporate [α-^{32}P]dCTP (line 1 and 2). These results suggest that the enzyme-template complex is in a protected state, presumably encapsidated within the virus particle, and so is not available as a substrate for DNase I.

Table I

Samples	Treatment by 0.15 µg/µl of DNase I	cpm ^{32}P Cerenkov
1. CaMV particles	none	4557
2. CaMV particles	DNase I	4550
3. Calf thymus ^{32}P DNA	none	27725
4. Calf thymus ^{32}P DNA	DNase I	630

Analysis of the reaction product

After an endogenous DNA polymerase reaction, the DNA of CaMV particles was purified as described in Materials and Methods and analysed on a 1% agarose gel. Encapsidated CaMV DNA preparations are composed of open circular DNA (Fig. 2,a), a faster migrating population consisting of knotted open circular DNA (10) and linear DNA molecules. Radioactive CaMV DNA, labelled by the endogenous polymerase activity was shown by autoradiography to comigrate exclusively with the linear CaMV DNA (Fig. 2,b). Purified CaMV DNA was tested in vitro for its ability to incorporate radioactive deoxynucleoside triphosphate as follows : 1 µg purified viral DNA was labelled by 5 U of purified avian myoblastosis virus reverse transcriptase in the DNA polymerase reaction mixture.The labelled DNA was analysed on a 1% agarose gel and autoradiographed. This experiment revealed that the linear form of purified virion DNA was preferentially labelled (data not shown).

Molecular weight of the CaMV-DNA polymerase

The molecular weight of the virus-associated DNA polymerase was determined in polyacrylamide gels containing activated DNA, as described in Materials and Methods. In this procedure virus is dissociated in 1% SDS for 3 min at 37°C (instead of the usual 100°C). Polyacrylamide gel electrophoresis of virus treated in this fashion showed that dissociation of the capsid was complete. When SDS is removed after electrophoresis, the enzyme renatures as it interacts with activated DNA, and can then incorporate [α-^{32}P]dCTP in the presence of the 3 other deoxynucleotides. Analysis of a CaMV virus preparation detected a DNA polymerase activity corresponding to a single protein (Fig. 3, slot b). This protein comigrates with the large

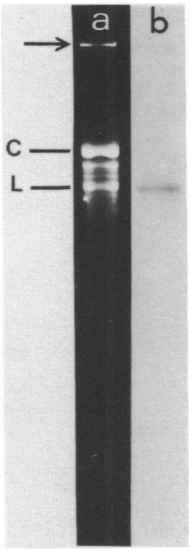

Figure 2. Electrophoresis of the endogenous DNA polymerase product.
After 30 min. of the DNA polymerase reaction, DNA was purified from CaMV particles and analysed on a 1% agarose gel. Slot a : the gel stained with ethidium bromide and photographed under U.V. illumination ; circular DNA is referred to as C and linear DNA as L. Intermediate bands have been characterized as knotted circular double-stranded DNA (10). The arrow indicates the top of the gel. The autoradiogram of (a) is shown in (b). Only the linear band of DNA is labelled (5 days exposure).

Klenow fragment of E. Coli DNA polymerase I (Fig. 3, slot a) and has a molecular weight of about 76 kDa. The pattern obtained from a crude extract of healthy plant polymerases showed two major active peptides migrating very close to the E. Coli DNA polymerase I (109 kDa) and to the large Klenow fragment (76 kDa) (Fig. 3, slot c).

In order to determine the nature of the reaction catalysed by the CaMV-associated enzyme the DNA labelled on an activity gel was purified and submitted to the action of the S1 nuclease (Fig. 4B). The E. Coli DNA-polymerase reaction product was used as a control (Fig. 4A). This experiment shows that the reaction product of our virion-associated enzyme (as does the E. Coli polymerase) synthesized S1-resistant, i.e. double-stranded DNA. As expected, the reaction product becomes sensitive to S1 nuclease after heat treatment. Therefore we are not dealing with a nucleotidyl transferase, but rather with a bone fide template-dependent polymerase.

DISCUSSION

Our experiments indicate that there is an enzyme activity associated with CaMV particles, which has the ability to catalyze the polymerisation of deoxynucleoside triphosphates in the absence of added template. Our results do not agree with those reported earlier by Hull (27), in which it was indicated that CaMV particles did not contain polymerase activity. This discrepancy can be explained in view of the variability of our own results

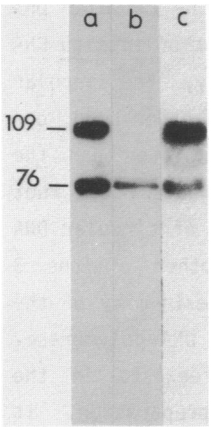

Figure 3. Autoradiogram of a 10% polyacrylamide "activity gel" of DNA polymerase of E. Coli and purified CaMV.
Slot a : 5 U of E. Coli DNA polymerase I (109 kDa). The large fragment of Klenow (76 kDa) is a proteolytic product of the 109 K enzyme (1 min. exposure).
Slot b : 15 µg of purified CaMV. One activity band is detected, it comigrates with the large fragment of Klenow (15 min. exposure).

with the virus preparation. Fresh virus preparations are poorly active in liquid medium but very efficient in activity gel. The contrary was observed for virus preparations stored at -20°C in 50 % glycerol during several weeks (data not shown). It should be pointed out that the electrophoretic pattern of the virion-associated enzyme(s) is different from that obtained with the host plant because the 109 kDa enzyme is not found. This tends to prove that virus preparations are not contaminated by plant polymerases. Analysis of the labelled CaMV-DNA reveals that, whereas circular relaxed DNA is predominant in native DNA preparation, only linear CaMV DNA becomes labelled. This is

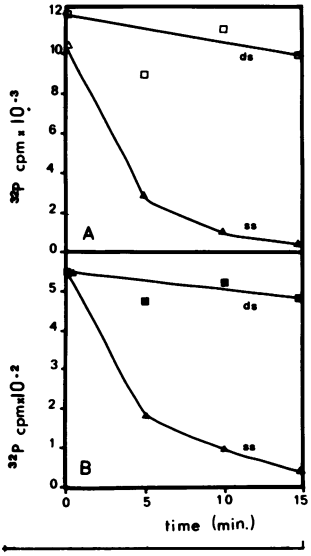

Figure 4. Analysis of the products of the DNA-polymerase reaction.
A. E. Coli DNA polymerase labelled DNA.
B. CaMV associated DNA polymerase labelled DNA.
The double-stranded (ds) DNA resists to the action of the S1 nuclease, but after heat treatment, the single-stranded (ss) DNA becomes sensitive to S1 nuclease.

different from what happens in the case of HBV(s) in which the circular DNA is partially double-stranded (28,29). In this case the result of in situ DNA polymerisation is to fill in the large gap and, consequently, the circular DNA is found to be labelled (30). In the case of CaMV, on the other hand, our results strongly suggest that linear DNA is a better template than the circular form. In earlier experiments we and others (31) have noted that linear encapsidated DNA does not result from random breakage of circular DNA but rather from preferential cleavage at one or the other of the 3 interruptions (unpublished results). In such a case the 3' extremity of the interrupted linear strand should be a good template for a DNA polymerase. Our results may suggest that the linear form of DNA preexists in the particles and is not produced as an artefact of the DNA preparation. It should be pointed out that the extent of incorporation of deoxynucleotides into DNA within the particles is rather low. This observation may be explained in two ways : one possibility is that the deoxynucleoside triphosphate substrate does not reach the enzyme-matrix complex. We feel this explanation is unlikely since the architecture of CaMV has been shown to be rather loose by neutron scattering studies (Kruse et al. in preparation). Alternatively, the viral DNA may be a poor template for the enzyme. Indeed, the genome of CaMV is entirely double-stranded except in the vicinity of the interruptions. On the other hand, once released from the viral DNA by SDS-gel electrophoresis, the enzyme can be renatured with a good template e.g. the activated DNA) and then becomes quite active. It is likely that, since the polymerase activity is restored after removing SDS, the enzyme is monomeric or composed of subunits which coelectrophorese in polyacrylamide gels.

We can only speculate as to what the role of this enzyme might be in the virion. It seems likely that one of the first steps in the infection cycle of CaMV is the elimination of the overlapping sequences present at each interruption of the virion DNA and repair of the discontinuities so as to generate the supercoiled form of the DNA. We cannot exclude the possibility that the virion-associated enzyme facilitates this process.

Another possibility is that, as assumed for HBV, this enzyme is left over from a late stage of the replication cycle. This could happen if encapsidation of the double-stranded virion DNA started before the second strand was fully synthesized ; the enzyme would thus become entrapped in the particle. In this hypothesis, one would expect the virion DNA-polymerase to be identical to the enzyme involved in replication ; numerous arguments suggest that the latter enzyme is a RNA-dependent DNA polymerase (reverse

transcriptase). Indeed, preliminary experiments carried out with the virion enzyme show that it is able to polymerize [α-^{32}P]dGTP when provided with the duplex poly(C)d(G)$_{12-18}$, which is a specific template for reverse transcriptase. More experiments are in progress to further clarify this point.

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REFERENCES

1. Shepherd, R.J. (1979) Annu. Rev. Plant Physiol. 30, 405-423.
2. Hohn, T., Richards, K. and Lebeurier, G. (1981) Curr. Topics Microbiol. Immunol. 96, 193-236.
3. Volovitch, M., Drugeon, G. and Yot, P. (1978) Nucleic Acids Res. 5, 2913-2925.
4. Hull, R., Covey, S.N., Stanley, J. and Davies, J.W. (1979) Nucleic Acids. Res. 7, 669-677.
5. Franck, A., Guilley, H., Jonard, G., Richards, K. and Hirth, L. (1980) Cell 21, 285-294.
6. Richards, K.E., Guilley, H. and Jonard, G. (1981) FEBS Lett. 134, 67-70.
7. Mênissier, J., Lebeurier, G. and Hirth, L. (1982) Virology 117, 322-328.
8. Olszewski, N., Hagen, G. and Guilfoyle, T.J. (1982) Cell 29, 395-402.
9. Hull, R. and Covey, S.N. (1983a) Nucleic Acids Res. 6, 1881-1895.
10. Mênissier, J., de Murcia, G., Lebeurier, G. and Hirth, L. (1983) The EMBO J. 2, 1067-1073.
11. Guilley, H., Richards, K.E. and Jonard, G. (1983) The EMBO J. 2, 277-282.
12. Guilley, H., Jonard, G. and Richards, K. (1983) in Current Communications in Molecular Biology - Plant Infectious Agents (Robertson, H.D., Howell, S.H., Zaitlin, M. and Malmberg, R.L.), 17-22. Cold Spring Harbor Laboratory, New York.
13. Pfeiffer, P. and Hohn, T. (1983) Cell 33, 781-789.
14. Hull, R. and Covey, S.N. (1983b) Trends Biochem. Sci. 119-121.
15. Marco, Y. and Howell, S.H. (1984) in press.
16. Teich, N. (1982) in RNA Tumor Viruses (Weiss, R., Teich, N., Varmus, H. and Coffin, J.), 25-207. Cold Spring Harbor Laboratory, New York.
17. Summers, J. and Mason, W.S. (1982) Cell 29, 403-415.
18. Kaplan, P.M., Greenman, R.L., Gerin, J.L., Purcell, G.R. and Robinson, W.S. (1973) J. Virol. 12, 995-1005.
19. Baltimore, D. (1970) Nature 226, 1209-1211.
20. Temin, H.M. and Mizutani, S. (1970) Nature 226, 1211-1213.
21. Volovitch, M., Modjtahedi, N., Yot, P. and Brun, G. (1984) The EMBO J. 3, 309-314.
22. Pfeiffer, P., Laquel, P. and Hohn, T. (1984) Plant Molec. Biol. 3, 261-270.
23. Hull, R., Shepherd, R.J. and Harvey, J.D. (1976) J. Gen. Virol. 31, 93-100.
24. Al Ani, R., Pfeiffer, P., Lebeurier, G. and Hirth, L. (1979) Virology 93, 175-187.

25. Spanos, A., Sedgwick, S.G., Yarranton, G.T., Hubscher, U. and Banks, G.R. (1981) Nucleic Acids Res. 9, 1825-1839.
26. Hubscher, U., Spanos, A., Albert, W., Grummt, F. and Banks, G.R. (1981) Proc. Natl. Acad. Sci. USA 78, 6771-6775.
27. Hull, R. (1980) in Plant Cell Cultures. Results and Perspectives (Sala, F., Parisi, B., Cella, R. and Ciferri, O.), 219-224.
28. Summers, J., O'Connell, A. and Millman, I. (1975) Proc. Natl. Acad. Sci. USA 72, 4597-4601.
29. Delius, H., Gough, N.M., Cameron, C.H. and Murray, K. (1983) J. Virol. 47, 337-343.
30. Landers, T.A., Greenberg, H.B. and Robinson, W.S. (1977) J. Virol. 23, 368-376.
31. Hull, R. and Howell, S.H. (1978) Virology 86, 482-493.