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Isolation of a fraction from cauliflower mosaic virus-infected protoplasts which is active in the synthesis of (+) and (−) strand viral DNA and reverse transcription of primed RNA templates

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ABSTRACT

Sub-cellular fractions, isolated from cauliflower mosaic virus (CaMV)-infected turnip protoplasts, are capable of synthesising CaMV DNA *in vitro* on an endogenous template and of reverse transcribing oligo dT-primed cowpea mosaic virus RNA. The activity was not detected in mock-inoculated protoplasts. *In vitro*-labelled DNA hybridized to single-stranded M13 clones complementary to the putative origins of (−) and (+) strand CaMV DNA synthesis and to restriction endonuclease fragments encompassing more than 90% of the CaMV genome. The synthesis of (−) and (+) strand DNA appeared asymmetric. The template(s) for *in vitro* CaMV DNA synthesis are in a partially nuclease-resistant form. Fractions capable of *in vitro* CaMV DNA synthesis contained CaMV RNA both heterogeneous and as discrete species; they also contained a range of different sizes of CaMV DNA. Several lines of evidence indicate that this range of *in vitro*-labelled CaMV DNA, extending from 0.6kb to 8.0kb in length, represents elongating (−) strand DNA. These are discussed in relation to their role as possible replicative intermediates.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the type member of the caulimovirus group of plant viruses which have double-stranded DNA genomes (see 1-3). DNA sequencing has shown that the virus has the potential to code for 6-8 polypeptides in excess of 10 kda (4-6). The encapsidated DNA of most isolates of CaMV has three single-stranded discontinuities, one (G1) in the transcribed α (−) strand (7) and two (G2 and G3) in the complementary (+) strand delimiting the β and γ strand components (see Fig. 1).

Much circumstantial evidence has led several authors to suggest that CaMV may replicate by a process involving reverse transcription (8-11). The mechanism of reverse transcription explains the structure of encapsidated CaMV DNA and also that of

unencapsidated forms of viral DNA (12-14).

Comparisons of amino acid sequence have located regions of homology between the putative CaMV gene V product and polymerases from retroviruses and hepatitis B virus (15-17). DNA polymerases with some properties of reverse transcriptases have been detected in CaMV-infected plants (16,18), and a DNA polymerase activity of the predicted molecular weight for CaMV gene V product has recently been detected in partially purified CaMV replication complexes (19) and encapsidated in CaMV virions (20).

Recent research on CaMV replication has focussed on isolating subcellular fractions from CaMV-infected plants which synthesise CaMV DNA on an endogenous template (11,18,19,21). These fractions consist of nuclei and virus-induced inclusion bodies, the two main candidates for the site of CaMV DNA synthesis (18, 21-24). CaMV replication complexes have been isolated from these subcellular fractions by hypotonic leaching or nuclear lysis followed by sucrose gradient centrifugation (11,18,19). Our experience has been that the detection of CaMV DNA in fractions containing nuclei and inclusion bodies has proved difficult because: (a) the significant proportion of endogenous nuclear DNA synthesis prevented the detection of CaMV DNA synthesis by techniques other than hybrid selection and (b) the efficiency of hybrid selection of *in vitro*-labelled CaMV DNA is severely affected by the large amounts of unlabelled CaMV DNA released during nucleic acid extraction.

We have chosen an alternative system for studying CaMV DNA replication. Techniques have been developed for the efficient infection of turnip protoplasts with CaMV (25-27) which give near synchronous replication of the virus. Coupled with this is the observation that preparations of polysomes from CaMV infected leaves contain relatively large amounts of CaMV DNA in nucleoprotein complexes (Hull & Covey, unpublished observation); these show *in vitro* DNA synthesising activity. In this paper we describe the preparation and characterization of a subcellular fraction from CaMV-infected protoplasts active in (-) and (+) strand CaMV DNA synthesis and capable of reverse transcribing a primed exogenous RNA template.

MATERIALS AND METHODS

Virus.

CaMV isolates Cabb B-JI and NZ1 (28) were propagated in turnip (*Brassica rapa* L. cv. "Just Right") and purified as described by Hull *et al* (29). DNA was extracted from virus particles using the method of Hull & Howell (30).

Turnip protoplasts.

B. rapa protoplasts were prepared, inoculated, and cultured essentially as described by Maule (27).

Preparation of fractions active in CaMV DNA synthesis.

$30\text{-}60 \times 10^6$ protoplasts 60 hr post inoculation were pooled and pelleted at 100xg for 5 min. The pellet was washed twice in 0.4M mannitol and was resuspended at 4°C in 30ml of lysis buffer (100mM Tris-HCl, 25mM EGTA (pH 9.0) containing 35mM $MgCl_2$, 1M NaCl, 1% v/v Triton X-100 and 0.1% β-mercaptoethanol). The lysed protoplasts were filtered through two layers of miracloth and the filtrate was stored on ice for 30-45 min before centrifuging at 10,000xg for 10 min at 4°C. The low speed pellet fraction (LSP) was resuspended in 0.5-1.0ml 100mM Tris-HCl (pH 8.0), 24mM $MgCl_2$, 60mM KCl, 10mM DTT (2x concentrated assay buffer). The supernatant fraction was layered over a 10ml 60% w/v sucrose cushion in 40mM Tris-HCl, 10mM EGTA (pH 9.0), containing 30mM $MgCl_2$ and 200mM KCl, and centrifuged at 45K rpm for 5.5 hr in a Beckman Ti60 rotor at 4°C. The high speed pellet fraction (HSP) was resuspended in 0.5-1.0ml of 2x assay buffer. Both the LSP and HSP fractions were assayed immediately or glycerol was added to 25% v/v and the fractions stored at -20°C.

LSP and HSP fractions were prepared in a similar manner from CaMV-infected plant tissue. 5-10g of systemically infected young leaves were homogenized in lysis buffer without Triton X-100 and NaCl. The homogenate was passed through two layers of miracloth (Calbiochem), and Triton X-100 (10% v/v) and 5M NaCl were added to 1% and 1M respectively. Subsequent steps in the preparation of LSP and HSP fractions were as described above.

Assay of CaMV DNA synthesis on endogenous template.

Assays were performed in a final volume of 100µl, comprising 50µl of LSP or HSP fractions and 50µl of a solution of 200µM dATP, dGTP, dTTP and 5-20µCi α^{32}P dCTP (3.2KCi/mmole) (New Eng-

land Nuclear). RNase A (5µg/ml), DNase 1 (5µg/ml) or Actinomycin D (100µg/ml) were added to LSP or HSP fractions and preincubated at 37°C for 15 min. The reaction was then started by the addition of the dNTP mix. Assays were at 37°C for 2-3 hr. Reactions were terminated by the addition of proteinase K to 1mg/ml, NaCl to 200mM, SDS to 1% (w/v), and EDTA to 10mM. After further incubation for 1 hr at 37°C the mixture was extracted with phenol and then with phenol:chloroform:isoamylalcohol (25:24:1) and the nucleic acids were precipitated from ethanol at -70°C.

Hybrid selection of in vitro-labelled CaMV DNA.

Labelled CaMV DNA was used to probe 'dot blots' of virion DNA or M13 clones using the methods described by Maule et al (31). For quantification, hybrid-selected labelled DNA on nitrocellulose discs was counted in a toluene-based scintillant.

Alternatively, labelled nucleic acid was used to probe gel-fractionated CaMV DNA transferred to nitrocellulose sheets as described by Southern (32).

Reverse transcriptase assay.

50µl of LSP or HSP fractions which had been stored at -20°C were added to 50µl of a solution containing 100µM dATP, dGTP, dCTP, dTTP, 1µg cowpea mosaic virus (CPMV) RNA primed with 0.1µg oligo dT_{12-18}. Assays were at 37°C for 2-3 hr and were terminated by the addition of proteinase K, NaCl, SDS and EDTA as described above.

Detection of cDNA products.

Nucleic acid, extracted as described above was incubated at 70°C for 15 min in 100mM NaOH containing 1mM EDTA. 0.01 volumes of glacial acetic acid was added to neutralize the reaction mixture and the alkali-resistant nucleic acid precipitated with ethanol. DNA was denatured in 10mM sodium phosphate buffer (pH 7.0) containing 70% v/v deionized formamide and 1M deionized glyoxal at 55°C for 15 min. The nucleic acid was electrophoresed in vertical 1% agarose gels as described by Hull & Howell (30). Fractionated nucleic acid was transferred to a nitrocellulose membrane (32) and probed with ^{32}P-labelled CPMV cDNA clones (see Fig. 8a). This assay was much more sensitive than one involving incorporation of label into cDNA products subsequently quantified by hybrid selection.

Molecular cloning.

CaMV NZ1 DNA (28) was cloned at its unique SalGI site in pAT153 (33). The orientation of CaMV DNA in this plasmid (pCNZ) with respect to the unique EcoRI site in pAT153 is shown in Fig. 1.

To prepare single-strand clones complementary to the 5' regions of the α, β and γ strands of CaMV DNA, pCNZ DNA was digested with BglII, electrophoresed through 1% LGT agarose(Sea plaque) in 89mM Tris, 89mM boric acid, 2.5mM EDTA, pH 8.0, and BglII fragments a and c extracted from the gel as described by Maniatis et al (34). Clone pNMα, complementary to the 5' region of the α strand, was the 1150bp BglII c fragment cloned in BamHI cut M13mp8 RF DNA as described by Messing (35). Clone pNMβ, complementary to the 5' region of the β strand, was a 300bp fragment from the HindIII cutting of the BglII a fragment cloned into BamHI/HindIII cut M13mp8 RF DNA. Clone pNMγ, complementary to the 5' region of the γ strand, was a 770bp fragment cut with XhoI and EcoRI from the BglII a fragment and cloned into SalGI/EcoRI cut M13mp8 RF DNA. The orientation of each clone was confirmed by dideoxy sequencing on M13 template as described by Sanger et al (36). The location and polarity of the three clones are shown in Fig. 1.

^{32}P-labelling of probe DNA.

Double-stranded DNA probes were labelled to high specific activity with α-^{32}P dCTP (3.2KCi/mmole)(NEN) by nick translation as described by Rigby et al (37). Southern blots were probed with ^{32}P-labelled DNA as described by Southern (32).

Single-stranded M13 clones were labelled to high specific activity by the method of Brown et al (38).

Two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis of CaMV DNA was as described by Favoloro et al (39) on a horizontal gel apparatus. For the non-denaturing dimension electrophoresis was in 40mM Tris-acetate, 1mM EDTA (pH 7.9) containing 5mM sodium acetate. For the denaturing dimension electrophoresis was in 30mM NaOH, 1mM EDTA. The gel was either blotted onto nitrocellulose (32) or neutralized in 1M Tris-HCl (pH 7.5), 1.5M NaCl before transfer under hybridizing conditions by a modification of the crossed

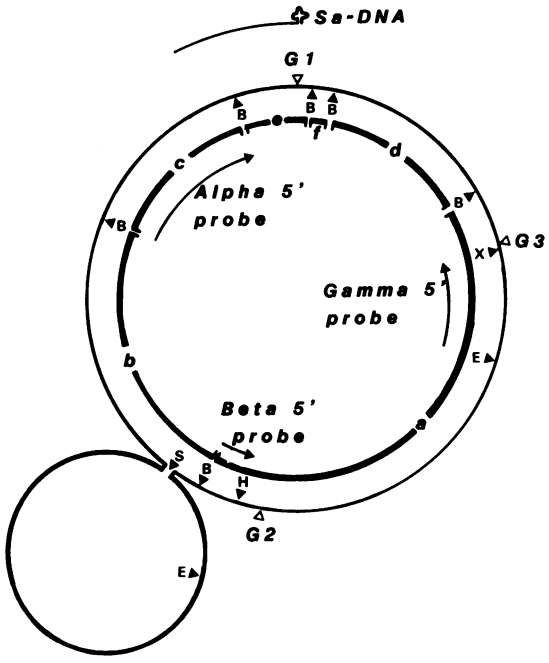

Figure 1. pCNZ DNA showing the location of the three single-strand discontinuities (G1, G2 and G3) on the CaMV genome and that of (-) "strong stop" DNA (sa-DNA); the location and 5'→3' polarity of α, β and γ strand specific probes are also indicated. BglII (B) fragments a-f of CaMV (NZ1) DNA, the unique XhoI (X) and SalGI (S) sites, and EcoRI (E) and HindIII (H) sites relevant to the construction of strand-specific probes are also shown.

contact procedure of Rozek & Timberlake (40). For this 300µg of pCNZ (100µg/ml) DNA, which had been treated with 10ng/ml DNase 1 for 10 min at 37°C and then heated to 100°C for 10 min, was cast in a 15cm x 15cm horizontal 1% agarose gel. The DNA was denatured by alkali, neutralized and transferred to a nitrocellulose sheet which was then baked at 80°C under vacuum for 2-3 hr. The gel containing the *in vitro*-labelled CaMV DNA, which had been fractionated by two-dimensional electrophoresis as described above, was soaked for 45 min in two changes of 60% v/v formamide in 4x SSC. The nitrocellulose sheet with bound pCNZ DNA was soaked in the same solution and layered over the gel. Labelled nucleic acid was transferred overnight at 25°C. The nitrocellu-

lose filter was washed at room temperature in two changes of 2x SSC containing 0.1% SDS and then at 65°C for 1h in 2x SSC, 0.1% SDS. The filter was autoradiographed to detect hybrid-selected CaMV DNA.

RESULTS

Isolation of a fraction from CaMV-infected turnip protoplasts active in CaMV DNA synthesis on an endogenous template.

Endogenous template assays were performed on the LSP and HSP fractions from mock-inoculated and CaMV-infected turnip protoplasts harvested at 60h post-inoculation. Results from hybrid selection of the *in vitro*-labelled DNA are shown in Table 1.

Both the LSP and HSP fractions from mock-inoculated turnip protoplasts synthesised DNA on an endogenous template; none of the labelled DNA hybridized to CaMV DNA (Table 1). The LSP and

TABLE 1
Detection of CaMV DNA synthesis on endogenous templates

	Total TCA precipitable ^{32}P cpm	Control filters (no bound CaMV)	CaMV DNA filters (1µg CaMV DNA/ filter)
(1) Mock-inoculated turnip protoplasts			
LSP	46,720	64	60
HSP	68,580	67	62
(2) CaMV-inoculated turnip protoplasts			
LSP	68,580	73	80
HSP	77,580	86	2793
(3) CaMV infected turnip plants			
LSP	94,536	162	166
HSP	207,520	140	3700

LSP and HSP fractions from turnip plants and protoplasts were assayed for their ability to synthesise CaMV DNA *in vitro* as described in materials and methods. Nucleic acids were extracted from each fraction by protease and phenol treatment, 5% of total nucleic acid was TCA precipitated and scintillation counted; the values indicated in the table are corrected to 100%. Residual nucleic acid was used to probe nitrocellulose filters with and without bound CaMV DNA. Hybridization, washing and quantification were as described in the text. All values in the table are the means of two determinations.

HSP fractions from CaMV-infected protoplasts also synthesised DNA on an endogenous template but only the HSP fraction synthesised a labelled product which hybridized to filter-bound CaMV DNA. Table 1 also shows that, as with protoplasts, only the HSP fractions prepared from CaMV-infected plants synthesised CaMV DNA.

Synthesis of labelled CaMV DNA was dependent upon the presence of the four deoxyribonucleoside triphosphates and Mg^{2+} (12mM). The optimal concentration of KCl was 30mM; KCl concentrations in excess of 50mM reduced the amount of labelled CaMV DNA to the control level (results not shown).

The concentration of CaMV DNA in these assays was determined by a quantitative "dot hybridization" technique (31). A calibration curve was also constructed to determine the efficiency of hybridization of increasing concentrations of CaMV DNA. Using the calibration data it was calculated that CaMV DNA synthesis in the HSP fraction represented 40-50% of the total activity.

The HSP fraction from CaMV-infected protoplasts is capable of (-) and (+) strand CaMV DNA synthesis.

In vitro-labelled CaMV DNA from assays using the HSP fraction from infected protoplasts hybridized to all three single-stranded clones, pNMα, pNMβ and pNMγ, (Fig. 2A) thus showing that CaMV DNA of (-) and (+) strand polarity is synthesised on the CaMV endogenous template. Fig. 2A also shows that much more labelled DNA hybridizes to the (-) strand probe than to the (+) strand probes.

Labelled nucleic acid from assays in which the HSP fraction had been preincubated with RNase or Actinomycin D was used to probe Southern blots of the pNMα, pNMβ and pNMγ clones (Fig. 2A). Incubation with either RNase or Actinomycin D inhibited the synthesis of both (+) and (-) strand CaMV DNA by about 50%, as measured by areas of peaks from densitometer scans of autoradiographs such as Fig. 2A. The presence of DNase 1 in the assay did not significantly reduce CaMV (-) and (+) DNA strand synthesis (Fig. 2B).

CaMV nucleic acids present in the HSP fraction of CaMV-infected protoplasts.

CaMV DNA was prepared from the HSP fraction in either of

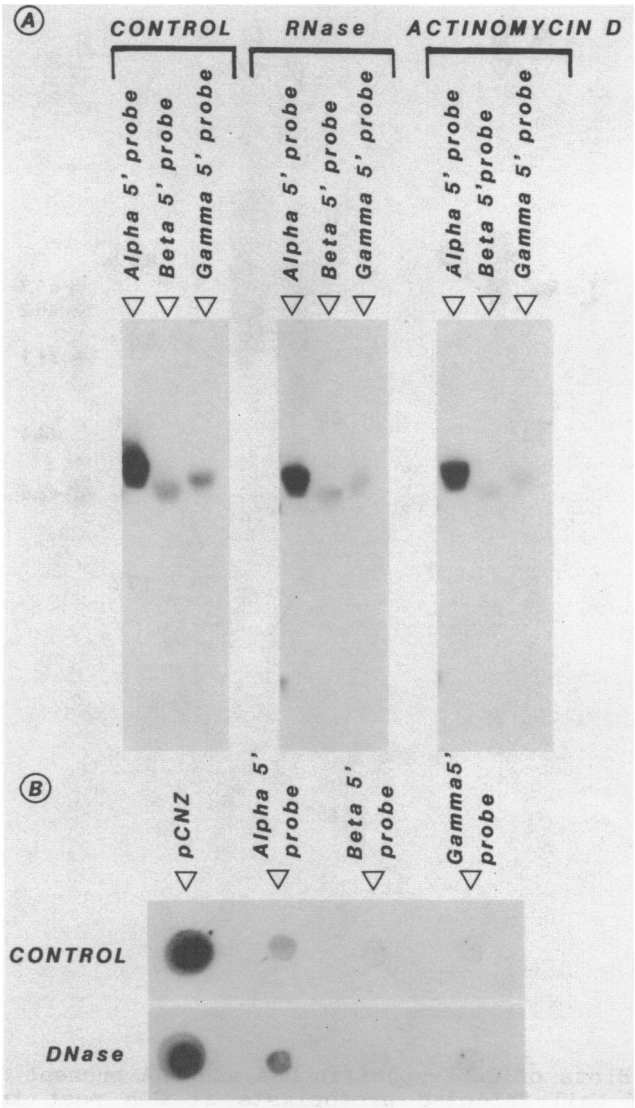

Figure 2. (A) In vitro DNA synthesis assays were performed as described in materials and methods. Labelled DNA from the HSP fraction of CaMV-infected turnip protoplasts synthesised in the absence of inhibitor (control), or in the presence of actinomycin D (100µg/ml) or RNase A (5µg/ml) was used to probe Southern blots of pNMα, pNmβ and pNMγ DNA (1.0µg per track), as described in materials and methods.

(B) In vitro-labelled DNA from the HSP fraction of CaMV infected protoplasts synthesised in the presence or absence of DNase 1 (5µg/ml) was used to probe "dot blots" of pCNZ, pNMα, pNMβ and pNMγ DNA.

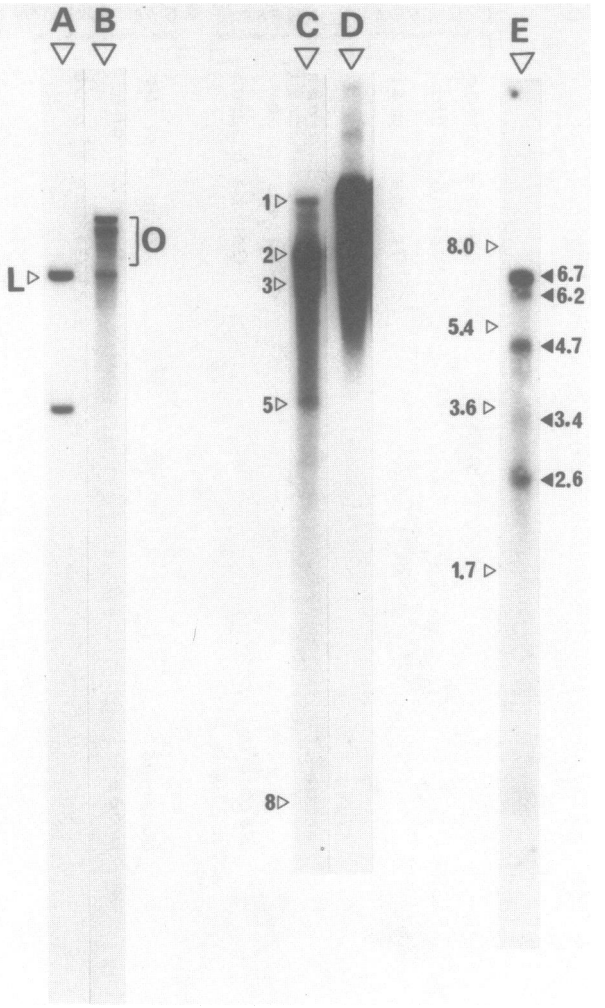

Figure 3. Blots of CaMV-specific DNA and RNA present in the HSP fraction of CaMV-infected protoplasts at 60h post inoculation electrophoresed without denaturation on 1.1% agarose gels and probed with pCNZ. Lane A - pCNZ DNA linearised by SalGI; "L" indicates the location of 8.0kb linear CaMV DNA; the faster band is the cloning vector pAT153 (3.565kb). Lane B - Virion nucleic acid was electrophoresed after protease digestion and phenol extraction. "O" indicates the region of the gel corresponding to the open circular conformers of CaMV DNA. Lane C - Nucleic acids were extracted with phenol alone. The numbering of CaMV forms corresponds to that in ref (14). Lane D - An equivalent amount of the HSP fraction to that used in Lane C was digested with protease prior to phenol extraction. Lane E - DNase resistant nucleic acid (25µg/ml for 30 mins at 30°C) was denatured by

glyoxal treatment prior to electrophoresis and Southern blotting. Glyoxylated CaMV restriction fragments were co-electrophoresed as size markers and are indicated down the left-hand side of the track. The lengths of several CaMV specific RNAs are indicated down the right-hand side of the track.

two ways, by protease digestion followed by phenol extraction or with phenol alone. Protease digestion followed by phenol extraction was needed to release all of the in vitro-labelled CaMV DNA from the HSP fraction. A number of CaMV DNA forms characteristic of encapsidated DNA were present when nucleic acid was prepared with protease followed by phenol extraction (Fig. 3). These include full length linear CaMV DNA and the open circular and 'twisted' conformers (Fig. 3, lanes B and D) described by several authors (41-43). When nucleic acids were extracted from the HSP fraction with phenol alone, a treatment which does not release encapsidated CaMV DNA (12,44,45) a range of smaller CaMV molecules were detected in addition to open circular and full-length linear molecules (Fig. 3, lane C). The protease and phenol treatment releases relatively so much of the larger material that the sensitivity for detection of smaller CaMV molecules is reduced (Fig. 3, lane D); they are detected on longer exposures. The molecules have been partially characterized by Maule (45). A similar spectrum of phenol-extractable CaMV molecules are present in CaMV-infected plants. It has been suggested that they are replication intermediates which can be predicted from the reverse transcription model for CaMV replication (12,14).

CaMV-specific RNA was also found in the HSP fraction of CaMV-infected turnip protoplasts (Fig. 3, lane E). In addition to heterogeneous CaMV RNA ranging in size from 1.0 to 7.0kb, several discrete molecules of 6.7kb, 6.2kb, 4.7kb, 3.4kb and 2.6kb were detected.

Analysis of in vitro-labelled CaMV DNA.

When in vitro-labelled CaMV DNA was used to probe Southern blots of gel-fractionated BglII, EcoRI, HindIII and PstI fragments of the CaMV genome it hybridized to most, if not all, of the restriction fragments (results not shown). Restriction fragments on the left-hand side of the genome (see Fig. 1) tended to be more heavily labelled than those on the right-hand side.

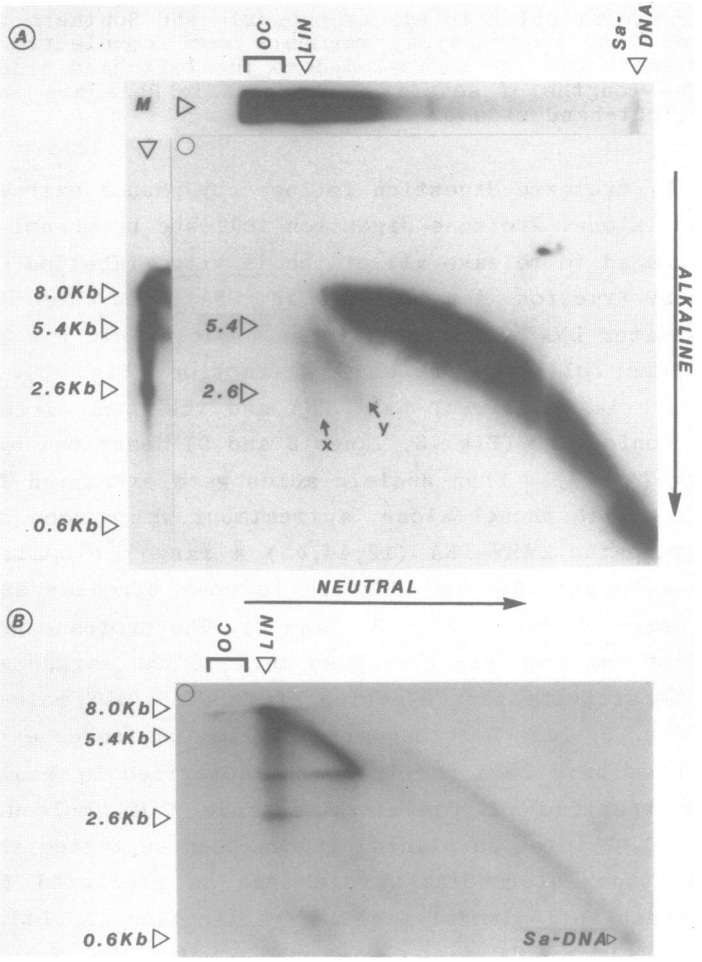

Figure 4. (A) Analysis of in vitro-labelled CaMV DNA by two-dimensional gel electrophoresis. In vitro-labelled CaMV DNA from the HSP fraction of CaMV-infected protoplasts was fractionated by two-dimensional gel electrophoresis and CaMV DNA was hybrid-selected as described in materials and methods. The marker tracks "M" were non-labelled CaMV DNA extracted by protease treatment and phenol extraction from the same HSP fraction as was used to assay CaMV DNA synthesis. "O" indicates the origin of gel electrophoresis, "OC", "Lin" and "sa-DNA" correspond to CaMV open circular, linear and (-) "strong stop" DNAs respectively. "x" and "y" correspond to unidentified elongating molecules.

(B) Total nucleic acid from CaMV-infected protoplasts at 60h post-inoculation was fractionated by two-dimensional gel electrophoresis, Southern blotted, and probed with ^{32}P-labelled CaMV DNA. "O" indicates the origin of gel electrophoresis. Labelled 2.6kb and 5.4kb spots originating from the open circular region were visible in the original autoradiograph.

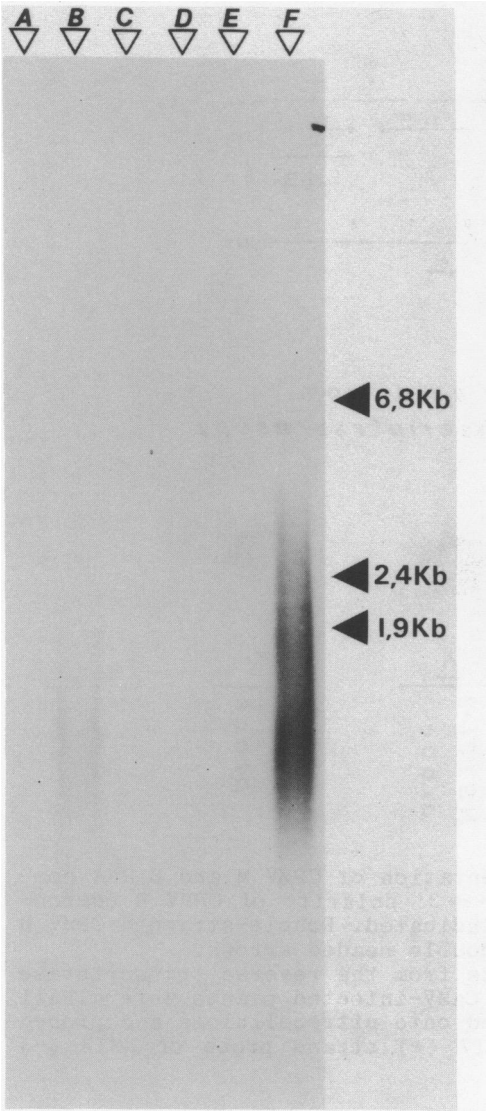

Figure 5. LSP and HSP fractions from turnip plants and protoplasts were assayed for reverse transcriptase activity as described in materials and methods. Reaction products were treated with alkali, denatured, electrophoresed and Southern blotted. The Southern blot was probed with nick-translated CPMV M and B specific cDNA clones and autoradiographed. Lane A - LSP fraction from CaMV-infected plants. Lane B - HSP fraction from CaMV-infected plants. Lane C - LSP fraction from mock-inoculated turnip protoplasts. Lane D - HSP fraction from mock-inoculated turnip protoplasts. Lane E - LSP fraction from CaMV-infected turnip protoplasts. Lane F - HSP fraction from CaMV-infected turnip protoplasts.

In order to analyse labelled replicative intermediates from the endogenous reaction, the DNA was fractionated by two-dimensional gel electrophoresis, and then hybrid-selected as described in Materials and Methods (Fig. 4A). Unlabelled CaMV DNA extracted from the HSP fraction was co-electrophoresed in each dimension, Southern blotted and probed with pCNZ to serve as size markers. A labelled molecule, of apparent denatured size

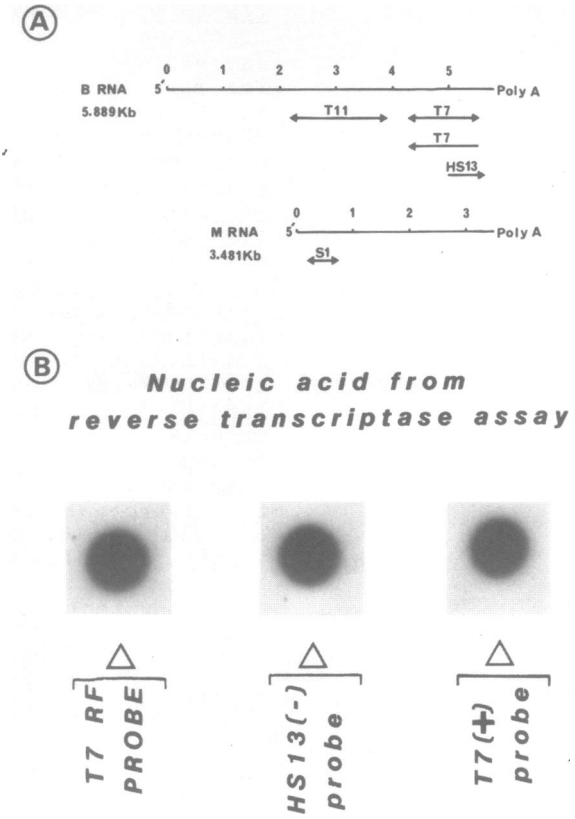

Figure 6. (A) Schematic representation of CPMV M and B RNA components. The location and 5' ---➔3' polarity of CPMV B component strand-specific probes are indicated. Double-stranded CPMV M and B cDNA clones are shown as double headed arrows.
(B) CPMV cDNA products from the reverse transcriptase assay using the HSP fraction of CaMV-infected plants were alkali treated, neutralized, dot blotted onto nitrocellulose and probed with T7 replicative form DNA, T7 (+) strand probe or HS13 (-) strand probe.

0.6kb, comigrated in the neutral dimension of the gel with the (-) strand "strong stop" DNA (sa-DNA) described by several authors (10,13,18,46). The heterogeneous range of labelled CaMV DNA extended from sa-DNA up to a maximum size of 8kb (Fig. 4A) and is similar to that found in total nucleic acid from CaMV-infected protoplasts (Fig. 4B). Two minor spots of hybrid-selected DNA with single-strand molecular weights of 2.6kb and

5.4kb were visible in the region of the gel corresponding to open circular CaMV DNA. These sizes corresponded to those of γ and β strands respectively and were not associated with labelled DNA of the length expected for the α strand. As predicted by the reverse transcription model (+) strand DNA synthesis is only completed after (-) strand DNA has circularized (8,9,11). Two additional elongating molecules (x and y in Fig. 4A) were also detected in the region of linear native DNA. The polarity of these molecules is unknown but they may represent elongating (+) strand molecules on (-) strand templates.

Fractions active in CaMV DNA synthesis are capable of reverse transcribing primed RNA templates.

Central to the proposed reverse transcription model for CaMV replication is the presence of an RNA-dependent DNA polymerase activity in CaMV-infected plants. To detect reverse transcriptase activity oligo dT-primed CPMV RNA was added to LSP and HSP fractions isolated from CaMV-infected protoplasts and plants and from mock-inoculated protoplasts. Reverse transcription assays were performed as described in Materials and Methods and alkali-resistant nucleic acid was then denatured, electrophoresed and Southern blotted. Hybridization of these blots (Fig. 5) with nick-translated CPMV probes S1 and T11 (47) (see Fig. 6A), revealed that only the HSP fractions from CaMV-infected plants and protoplasts were capable of synthesising a CPMV cDNA product. Controls in which CPMV RNA was omitted, did not produce any hybridizing nucleic acid. Hence there is a positive correlation between the ability to synthesise CaMV DNA on an endogenous template and the ability to synthesise a cDNA product from an exogenous primed RNA template.

Using strand specific probes for CPMV cDNA (see Fig. 6A) it was shown that the products of the reverse transcription reaction resulted from both first and second strand cDNA synthesis (Fig. 6B).

DISCUSSION

We have described a procedure for the isolation of a fraction active in the synthesis of CaMV DNA from virus-infected protoplasts. The procedure differs from those for isolating CaMV

replication complexes from infected plant tissue (11,18,19) both in the pH of extraction and in the use of protoplasts. Probably because of the difference in extraction procedure our replication complexes are in the HSP rather than the LSP as found by other authors. The use of protoplasts to study virus multiplication is advantageous since the infections are near synchronous and the active phase of virus growth well characterized (27,45). We have estimated that the CaMV-specific activity of our preparations was about 40-50% of the total activity.

The HSP fraction from CaMV-infected protoplasts synthesised CaMV DNA of (-) and (+) strand polarity. However, in contrast to the observations of Pfeiffer et al. (19) our results suggested that there was asymmetry in the synthesis of the two strands. Fig. 2 shows that much more in vitro-synthesised CaMV DNA hybridized to the (-) strand (α) probe than to the (+) strand (β and γ) probes. This difference is much more than can be accounted for by differences in the sizes of the probes. The interpretation of the two-dimensional separation of in vitro-labelled CaMV DNA (Fig. 4) is also consistent with asymmetry of strand synthesis. Stand-specific probing has shown that the "overarc" from 8Kb to the (-) strand "strong stop" sa DNA (Fig. 4B) is predominantly (-) strand DNA (Thomas and Maule, unpublished observations). Asymmetry of strand synthesis is characteristic of retroviruses (48,49) and of hepatitis B viruses (50-52).

The reason for (-) strand DNA replicative intermediates resolving as heterogeneous material under non-denaturing gel electrophoresis is unclear. The presence of heterogeneous CaMV RNA in this fraction (Fig. 3) and in CaMV replication complexes isolated by Pfeiffer and Hohn (11) suggests this may be due to RNAse H activity as in retrovirus (48,49) and HBV (-) DNA synthesis (53). (-) CaMV DNA:35S RNA hybrids may be dissociated during extraction liberating a heterogeneous population of 5' coterminal (-) strand DNA. Because the synthesis of CaMV DNA on endogenous template is partly nuclease insensitive (Figs 2A,2B) it is unlikely that these labelled molecules are degradation products.

In addition to (-) strand DNA, two discrete molecules corresponding to the lengths expected for the (+) β and γ strands

were hybrid-selected from the region of the gel corresponding to the open circular DNA (Fig. 4A, spots 5.4 and 2.6kb). The apparently low levels of labelled circular DNA may be a consequence of inefficient hybrid selection due to the large amounts of unlabelled virion DNA released during nucleic acid extraction (Fig. 3). Labelled circular DNA was not detected in replication complexes isolated from infected plants (19) but was synthesised in nuclei isolated from infected plants (24). Furthermore two elongating molecules (x and y in Fig. 4A) were detected in the region of linear CaMV DNA. These may represent elongating (+) strands on (-) strand templates but the polarity of these molecules has yet to be determined.

As with the replication complexes of Pfeiffer & Hohn (11) our HSP fraction contained heterodisperse CaMV RNA; it also contained several discrete RNA species. The 4.7kb transcript may be similar to one of the CaMV-specific RNAs present in infected plants (54,55). However CaMV-specific RNAs of 6.7kb, 6.2kb and 2.6kb in length have not been reported previously. The 2.6kb RNA is the expected size for a transcript encompassing the putative CaMV gene V protein. A probe specific for CaMV gene V has detected a transcript of similar size in the poly(A)$^+$ RNA fraction of CaMV-infected cells (S.N. Covey, personal communication).

The replication model for CaMV proposes that (-) strand DNA is synthesised from the 35S RNA template (8-11). Thus one would expect (-) strand DNA synthesis to be abolished by pretreatment of the replication complex with RNase. Pfeiffer et al (19) reported that (-) strand DNA synthesis was reduced by 80% after RNase treatment whereas (+) strand synthesis was less affected (30% reduction). In our system, RNase reduced both (-) and (+) strand DNA synthesis by about 50%. This suggests that the (+) strand RNA template in the complexes is, in part, protected against nucleases and also that there is far more of this template than of the (-) strand DNA template in the complexes when they are isolated. Actinomycin D (100µg/ml) also inhibited the synthesis of both strands equally. A similar observation has been made previously (19) although the overall reduction in synthesis was much greater in their case. These results suggest that both the (+) strand RNA and the (-) strand DNA templates in

our replication complexes are inaccessible to RNase and Actinomycin D to a certain extent. Guilfoyle et al (18) noted that the incorporation of deoxyribonucleotides into CaMV nucleic acid in preparations of nuclei was largely resistant to RNase and DNase. CaMV replication complexes sedimented over a range of sizes on sucrose gradients and lighter fractions were slightly sensitive to RNase (18). These results, and those presented here suggest that encapsidation and replication may proceed concurrently in vivo. Circumstantial evidence that this may be so is that DNA polymerase activity can be detected in purified virions (20). The failure of RNase to inhibit (-) strand DNA synthesis completely may be due to only a small percentage of replicative intermediates remaining in a nuclease accessible form.

Analysis of the nucleic acids in replication complexes and of the effects of nucleases on the incorporation of label into CaMV DNA provides circumstantial evidence for an RNA dependent DNA polymerase. Direct evidence for a reverse transcribing enzyme was provided in the experiment illustrated in Fig. 5. Only fractions from CaMV-infected plants and protoplasts active in CaMV DNA synthesis, were capable of synthesising CPMV cDNA from a primed RNA template. The replication complexes isolated by Pfeiffer et al (19) did not transcribe exogenous heterodeoxyribopolymer or homoribopolymer templates. Volovitch et al (16) found enzyme activity in a fraction active in CaMV DNA synthesis (21) which incorporated labelled deoxyribonucleotides in the presence of primed polyA$^+$ mRNAs; in the latter case the products were not characterised. In our system this property was only apparent after freezing the HSP fraction at -20°C in 25% w/v glycerol, a treatment which may be sufficient to dissociate the enzyme from its template or to release it from subcellular particles. Probing of the cDNA products indicated that extensive tracts (in excess of 2.5kb) are reverse transcribed into DNA from the primed CPMV RNA template.

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