Further Evidence that Viroplasms Are the Site of Cauliflower Mosaic Virus Genome Replication by Reverse Transcription during Viral Infection

By L. MAZZOLINI, P. DABOS, S. CONSTANTIN AND P. YOT*

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, Centre National de la Recherche Scientifique-Institut National de la Recherche Agronomique, BP 27, 31326 Castanet-Tolosan Cedex, France

(Accepted 10 August 1989)

SUMMARY

Cauliflower mosaic virus (CaMV) is a DNA plant virus that replicates its genome through an RNA intermediate. The cytoplasmic step of CaMV DNA replication was studied using a fraction consisting of purified viroplasms, which are virus-specific inclusion bodies accumulating in the infected plant cells. The isolated viroplasms retain a DNA polymerase activity able to synthesize CaMV DNA from endogenous templates. A further characterization of the viral DNA sequences produced in the isolated inclusion bodies indicates that newly synthesized DNA, mostly of polarity opposite to that of viral RNA, is single-stranded and partly associated with RNA by base-pairing. In addition to an RNA-dependent DNA polymerase activity, RNA molecules, which presumably originate from the viral RNA template used for reverse transcription, are found to accumulate in the purified inclusion bodies. Furthermore, a small DNA molecule strongly labelled in the purified fraction has been characterized and corresponds to the CaMV reverse transcription intermediate sa-DNA. These results provide further evidence that the reverse transcription of CaMV RNA occurs in the viroplasms. Additional data are presented which suggest that CaMV replication could occur in virion-related particles.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the most studied member of the caulimoviruses, the only group of plant viruses characterized so far to have a dsDNA genome, (for reviews see Shepherd, 1979; Guilfoyle, 1987; Gronenborn, 1987). The encapsidated form of CaMV DNA consists of a ds circular molecule, 8 kb in length, which contains three site-specific single-stranded interruptions (Volovitch et al., 1977). Viral DNA is also found in the nucleus of infected cells as a supercoiled molecule associated with histones (Olszewski et al., 1982; Menissier et al., 1983). This CaMV minichromosome is actively transcribed by the host RNA polymerase II during viral infection. One of the characterized viral RNA transcripts, the major 35S RNA, is of genome length and possesses an additional terminal redundancy of 180 nucleotides (Covey & Hull, 1981). Although this transcript is considered to be a possible polycistronic messenger (Dixon & Hohn, 1984), it could also represent an essential intermediate in the multiplication of the viral genome. Indeed, a replication model involving RNA-dependent synthesis of the CaMV DNA has been proposed (Guilley et al., 1983; Pfeiffer & Hohn, 1983; Hull & Covey, 1983). Such a model is now supported by numerous data obtained mainly from the characterization of intracellular CaMV DNA replication intermediates and from studies performed with isolated subcellular fractions active in viral DNA synthesis (for a review, see Hull et al., 1987). Furthermore, CaMV has been shown to share some coding sequence homologies with the retroid elements characterized in animals and yeasts (for a review, see Bonneville et al., 1987) and specifically codes for an RNA-dependent DNA polymerase...
(Takatsuji et al., 1986) essential to the reverse transcription step, an enzyme absent from uninfected plant cells (Volovitch et al., 1984).

Although evidence is accumulating that CaMV replicates by reverse transcription, only a few data on the intracellular localization of this process are presently available. Preliminary in vivo studies by Kamei et al. (1969) and Favali et al. (1973) suggested that the viral DNA synthesis could be cytoplasmic, occurring in viroplasms. These virus-specific inclusion bodies were also described as the site of assembly and storage of virus particles inside the infected cell (Conti et al., 1972). In contrast, further studies on the ability of isolated heavy organelles to perform viral DNA synthesis indicated that CaMV replication could take place in the nucleus (Ansa et al., 1982). However, additional experiments carried out with a heavy organelle fraction obtained from infected cells and active in DNA synthesis revealed that the newly synthesized viral DNA sequences accumulate preferentially in the viroplasms (Modjtahedi et al., 1984). More recently, a cell fractionation technique which allows an extensive purification of viroplasms has been developed (Bonneville et al., 1984). The purified organelles retain a DNA polymerase activity which acts predominantly on endogenous viral templates. A preliminary analysis of the DNA molecules synthesized in vitro in the viroplasms (Mazzolini et al., 1985) revealed that the viral DNA synthesis occurs throughout the whole CaMV genome and is asymmetric, producing DNA sequences mostly of polarity opposite to that of the CaMV RNA transcripts. In addition, electrophoretic analysis of the DNA synthesized in the isolated organelles indicated that most of the radioactive precursor is incorporated into small DNA molecules which are associated with RNA of heterogeneous size. Furthermore, an RNA-dependent DNA polymerase activity was found to co-purify with the viroplasms. Altogether these results suggested that some essential steps of the reverse transcription could occur in these organelles.

In the present report, a further characterization of the purified viroplasms, and of the viral DNA synthesis performed in such cytoplasmic inclusion bodies, is described. It is shown that the newly synthesized DNA consists mostly of single-stranded molecules which are found either free or associated with endogenous RNA by base-pairing. Indeed viral RNA sequences which may originate from the 35S RNA of genome length, are detected in the purified viroplasms. In addition, a DNA molecule, preferentially labelled upon in vitro DNA synthesis, is revealed to be the 'sa-DNA', a reverse transcription intermediate already identified in infected plant cells (Covey et al., 1983; Guilley et al., 1983; Turner & Covey, 1984). Other data indicate that viral DNA synthesis performed in the viroplasms could occur in virion-related nucleoprotein complexes.

**METHODS**

Viruses and nucleic acids. CaMV (Cabbage B, strain PV 147 from ATCC) was maintained in *Brassica campestris* ssp. *rapa* (cv. Just Right) and was purified from infected leaves according to Hull et al. (1976). Plasmid pLW414 (Howell et al., 1980) contains the complete CaMV genome of the strain CM4-184 (Gardner et al., 1981; Howarth et al., 1981) inserted into the SalI site of pBR322. Plasmid DNAs were prepared as described by Cieweil (1972). Cellular RNA was extracted from uninfected and infected leaves as follows. Twenty g of fresh leaves, without midribs, were frozen and ground to a fine powder in liquid nitrogen. The cells were lysed at 0 °C for 15 min by the addition of 20 ml of 200 mM-Tris-HCl pH 7.0, 200 mM-disodium EDTA and 1% SDS mixed with one equal volume of phenol : chloroform : isooamyl alcohol (24 : 1). The aqueous phase was re-extracted once with the same mixture and twice in succession with chloroform : isooamyl alcohol (24 : 1). Nucleic acids were precipitated from the aqueous phase with 2.5 volumes of ethanol and dissolved in 20 mM-Tris-HCl pH 7.4 and 10 mM-MgCl₂. The contaminating DNA was eliminated by treatment with DNase I (Worthington) at 50 μg/ml for 1 h at 0 °C. Residual RNase activity was removed from the DNase by affinity chromatography as described by Maxwell et al. (1977). Nucleic acids were extracted successively with an equal volume of phenol : chloroform : isooamyl alcohol (25 : 24 : 1) and chloroform : isooamyl alcohol (24 : 1), each of the two extractions having been performed twice. Sodium acetate pH 5.5 was added to the aqueous phase to give a final concentration of 0.3 M and RNA was precipitated with 2.5 volumes of ethanol overnight at −20 °C. The sa-DNA molecule labelled in vitro in the viroplasms was purified by electroelution from a preparative agarose gel according to Chouikl et al. (1979).

**Purification of viroplasms and extraction of nucleic acid.** Purification of the viroplasms from infected turnip leaves was performed according to a published technique (Mazzolini et al., 1985). Nucleic acids were obtained after proteinase K (Merck) treatment and subsequent extractions with organic solvents as already described (Mazzolini et al., 1985). For RNA extraction the isolated nucleic acids were digested with DNase devoid of RNase, as mentioned above.


**DNA synthesis in the purified viroplasms.** The conditions used for endogenous *in vitro* DNA synthesis were as previously described (Mazzolini *et al.*, 1985). Radioactivity of the acid-insoluble material was measured according to Bollum (1975).

**Labelling of nucleic acids.** Nick translation labelling of DNA was performed according to Rigby *et al.* (1977) using [α-32P]dCTP (3000 Ci/mmol, New England Nuclear). RNA labelling with [γ-32P]ATP (7000 Ci/mmol, New England Nuclear) and polynucleotide kinase (Boehringer Mannheim) was performed according to Goldbach *et al.* (1978).

**Enzymic and chemical treatments of nucleic acids.** Restriction endonucleases (Boehringer Mannheim and Bethesda Research Laboratories) were used as recommended by the manufacturers. RNase A (Sigma) treatment at low ionic strength and S1 nuclease (Boehringer Mannheim) digestion were performed as described by Mandoli *et al.* (1982). Site-specific chemical cleavage at 7-methyl guanosine residues by treatment with aniline was performed according to Turner & Covey (1984).

**Agarose gel electrophoresis and hybridization.** DNA was subjected to electrophoresis in agarose gels containing 40 mM-Tris-acetate pH 8.0 and 2 mM-disodium EDTA, and then transferred to nitrocellulose sheets (Schleicher & Schüll) according to Southern (1975). Agarose gel electrophoresis of RNA was performed under denaturing conditions in the presence of methylmercuric hydroxide (Bailey & Davidson, 1975) and the RNA was directly blotted onto nitrocellulose. Hybridizations with a probe consisting of 32P-labelled DNA were performed as described by Wahl *et al.* (1979).

When using 32P-labelled DNA as a probe, nitrocellulose filters were incubated for 2 h at 65°C in prehybridization solution [900 mM-NaCl, 90 mM-sodium citrate pH 7.0, 0.1% w/v Ficoll 400 (Pharmacia), 0.1% w/v polyvinylpyrrolidone 40 (Sigma), 0.1% w/v bovine serum albumin (fraction V; Sigma), 5 mg/ml yeast tRNA (Boehringer Mannheim) and 0.1% w/v SDS]. Following heat denaturation the probe was hybridized for 14 h at 65°C in hybridization solution [i.e. prehybridization solution containing 10% w/v dextran sulphate (Pharmacia)]. The filters were then washed three times for 15 min at 65°C in 150 mM-NaCl, 15 mM-sodium citrate, pH 7.0 and 0.1% (w/v) SDS and then three times for 15 min at room temperature in 15 mM-NaCl, 1.5 mM-sodium citrate, pH 7.0 and 0.1% SDS. Nitrocellulose sheets were then exposed to Kodak X-Omat S, using DuPont Lightning Plus intensifying screens at −70°C.

**Caesium sulphate density gradient centrifugation.** This was performed according to Williams & Vinograd (1971). Briefly, 32P-labelled nucleic acids were dissolved in 10 mM-Tris-HCl pH 7.5, 1 mM-disodium EDTA and 10% (v/v) dimethylsulphoxide. Solid caesium sulphate was then added to give a final density of 1.55 g/ml. Two ml of the solution was centrifuged at 27000 r.p.m. at 20°C for 60 h in a Beckman SW60 rotor. The fractions were collected from the bottom of the tube with the use of a peristaltic pump and their buoyant density and radioactivity values were determined respectively by weighing, and counting using the Cerenkov effect.

**Disruption of viroplasms, and analysis of the replication complexes by centrifugation in a caesium chloride density gradient.** After [32P]DNA synthesis the viroplasms were recovered by centrifugation at 12000 g for 1 min at 4°C and resuspended in a lysis medium consisting of 10 mM-Tris-HCl pH 7.4, 1 mM-disodium EDTA, 5% (v/v) Triton X-100 and 1.5 mM-urea. The viroplasm suspension was then loaded at the top of a centrifuge tube filled with 10 mM-potassium phosphate buffer pH 7.15 containing caesium chloride dissolved to give a final density of 1.369 g/ml. The tubes were centrifuged at 25000 r.p.m. for 45 h at 20°C in a Beckman SW60 rotor. Gradient fractions were collected from the top and the TCA-insoluble radioactivity was determined.

**RESULTS**

The newly synthesized viral DNA molecules are partly associated with RNA by base-pairing.

In order to gain insight into the DNA synthesis performed in the purified viroplasms, the newly synthesized labelled nucleic acids were extracted and centrifuged to equilibrium in a caesium sulphate density gradient which allows the separation of DNA and RNA molecules. Analysis of the distribution of the radioactive material in the gradient (Fig. 1) revealed that although some of the labelled molecules were present at the position expected for DNA, a significant amount of the radioactive nucleic acids had a higher apparent density, close to that of ssRNA. However, all the radioactivity was detected at the position of DNA when the extracted nucleic acids were treated with pancreatic RNase prior to the centrifugation step. These results indicated that some of the DNA synthesized in the viroplasms is bound to long RNA sequences, the resulting molecules having a significantly higher buoyant density than that of pure DNA. The radioactivity peak corresponding to the RNA-associated DNA molecules was also displaced towards the position of DNA in the gradient when the purified nucleic acids were heat-denatured prior to centrifugation (data not shown). This observation suggests that the
newly synthesized DNA was associated with RNA by base-pairing rather than by covalent linkage.

In order to characterize further the native structure of the DNA molecules synthesized in the purified viroplasms as well as their mode of association with RNA, the nucleic acids $^{32}P$-labelled during endogenous DNA synthesis were isolated and their sensitivity to S1 nuclease was tested. This was done by measuring the remaining percentage of TCA-insoluble radioactive material after treatment with the nuclease. The results indicated that most (92%) of the newly synthesized DNA molecules are single-stranded and become acid-soluble after digestion with S1 nuclease. These molecules occur either free (40%) and are directly digested by the enzyme, or are associated with RNA by base-pairing (52%) and are susceptible to S1 nuclease only after RNase A treatment in a buffer of low ionic strength i.e. under conditions in which base-paired RNA can be digested. The remaining S1 nuclease-resistant material (8%) probably arises from the DNA synthesis performed on the circular and linearized forms of the CaMV genome and on the residual contaminating cellular DNA sequences (Mazzolini et al., 1985).

**Presence of viral RNA in the purified viroplasms**

The observation that a significant part of the viral DNA of negative polarity synthesized in the purified viroplasms is associated with endogenous RNA sequences by base-pairing implies that CaMV RNA should be present in these organelles. Indeed, a preferential association of the intracellular viral RNA with the viroplasms has been demonstrated by comparing the relative

---

**Fig. 1.** Analysis of the $^{32}P$ DNA synthesized in the purified viroplasms by centrifugation in a caesium sulphate density gradient. Nucleic acids $^{32}P$-labelled during endogenous DNA synthesis in the purified viroplasms were isolated and fractionated by centrifugation in an isopycnic caesium sulphate density gradient without (○) or after (●) RNase A treatment. Identical amounts of radioactive DNA were analysed in each case. D and R indicate the position in the gradient of dsDNA (density 1.42 g/ml) and ssRNA (density 1.62 g/ml), respectively. (△) Density of caesium sulphate (g/ml).
CaMV genome replication in viroplasms

Fig. 2. Relative enrichment of viral RNA sequences in purified viroplasms. Autoradiograph obtained after hybridization of cloned CaMV DNA 32P-labelled by nick translation, against purified RNA dot-blotted in decreasing amounts onto nitrocellulose sheets according to Thomas (1980) and isolated from uninfected leaves (lane 1), CaMV-infected leaves (lane 2) and viroplasms purified from the same leaf sample as in lane 2 (lane 3). Lanes 4 and 5 represent control filters corresponding to lanes 2 and 3 respectively, except that the purified RNA samples were digested with RNase prior to dot-blotting. X represents the maximum quantity of purified RNA blotted onto nitrocellulose and corresponds to 2 μg.

intensity of the hybridization signals observed when using nick-translated CaMV DNA as a probe against RNA purified from either CaMV-infected leaves or viroplasms isolated from the same leaf extract. As shown in Fig. 2 a significantly higher hybridization signal was observed on the autoradiograph corresponding to the RNA extracted from viroplasms (Fig. 2, lane 3) as compared to total RNA isolated from infected leaves (Fig. 2, lane 2). This indicates that the presence of viral RNA in the isolated viroplasms is not due to non-specific trapping of the whole cellular RNA by these organelles during cell fractionation but to a specific association.

Additional information on the viral RNA present in the viroplasms was obtained by performing other hybridization experiments. First, the RNA extracted from the viroplasms was radioactively labelled and used as a probe against Southern blots of restriction digests of cloned CaMV DNA (Fig. 3a). The autoradiography patterns revealed that the viral RNA sequences associated with viroplasms encompass the whole genome. Second, the size of the corresponding RNA molecules was estimated using Northern blots of the RNA associated with the viroplasms which were probed with cloned, nick-translated CaMV DNA (Fig. 3c). Although no viral RNA molecules of discrete size could be detected, probably indicating that some degradation had occurred, large viral RNA molecules were present with an estimated upper size limit close to 8 kb, i.e. the size of the CaMV genome. Those results strongly suggest that the CaMV RNA associated with the viroplasms corresponds to the major 35S RNA.

Labelling of the viral replication intermediate sa-DNA in the purified viroplasms

Previous analyses by electrophoresis of the nucleic acids 32P-labelled upon in vitro DNA synthesis in viroplasms have revealed that in addition to the circular and linear forms of the CaMV genome, a third molecule, with an estimated size of 650 nucleotides, was labelled
Fig. 3. Characterization of the viral RNA present in purified viroplasms. (a) RNA isolated from purified viroplasms was radioactively labelled by a kinase reaction and used as a probe for hybridization to Southern blots of AccI, ClaI or EcoRI digests of cloned CaMV DNA (pLW414). Lanes 1, ethidium bromide pattern of the restriction digests after agarose gel electrophoresis; lanes 2, corresponding autoradiograph after hybridization. (b) Restriction map of plasmid pLW414 for the enzymes used in (a). In each case, restriction fragments are numbered in order of decreasing size. The shaded area corresponds to vector DNA. A1 and A2 indicate positions of the single-stranded interruptions on the viral genome. (c) The RNA extracted from purified viroplasms was electrophoresed under denaturing conditions in 1% agarose without (lane 2) or with (lane 3) RNase A pretreatment. After electrophoresis the RNA was blotted onto nitrocellulose sheets and probed with radioactively labelled cloned CaMV DNA. Lane 1, ethidium bromide pattern of size markers (Bethesda Research Laboratories' 1 kb' ladder) run in the same gel. Size of markers (in kb) is indicated on the left.

(Mazzolini et al., 1985). The location of this molecule on the CaMV genome was determined. For that purpose the corresponding radioactive DNA was purified and hybridized against Southern blots of different restriction digests of cloned CaMV DNA. As shown in Fig. 4(a), the radioactive DNA molecule hybridized to a well-defined region, close to the single-stranded interruption Δ1. An identical position has been ascribed to the CaMV replication intermediate sa-DNA which consists of ssDNA, 600 nucleotides in length, covalently linked at its 5' end to a tRNA molecule (Turner & Covey, 1984). In order to confirm that the small DNA molecule labelled in vitro corresponded to sa-DNA, the nucleic acids 32P-labelled during endogenous DNA synthesis were fractionated by agarose gel electrophoresis under denaturing conditions. Analysis of the dried gel by autoradiography revealed that a band was present at the expected position of sa-DNA (Fig. 4c, lane 4). Furthermore, RNase A treatment of the nucleic acids prior to electrophoresis resulted in a faster migration of the labelled material (compare lanes 3 and 4 of Fig. 4) indicating that the corresponding DNA is covalently associated with an RNA of an estimated size of 70 nucleotides.

Previously it has been shown that a chemical cleavage reaction specific to polynucleotides containing the modified base 7-methyl guanosine and present at a well defined position in the tRNA molecules increases the electrophoretic mobility of sa-DNA (Turner & Covey, 1984). A similar effect of such chemical treatment was observed with the radioactive DNA molecule (Fig. 4c, lane 2); although incomplete, this cleavage reaction resulted in a faster migration of the molecule at a position intermediate between those of the native and the RNase A-treated molecule.
CaMV genome replication in viroplasms

Fig. 4. \(^{32}\)P-labelling of the replication intermediate sa-DNA upon DNA synthesis in isolated viroplasms. (a) The short DNA molecule preferentially labelled during endogenous DNA synthesis in viroplasms was used as a probe for hybridization to Southern blots of ClaI and AccI restriction digests of CaMV DNA (pLW414). Lanes 1, ethidium bromide pattern of the gel after electrophoresis of the digests. Lanes 2, corresponding autoradiograph after hybridization. (b) Restriction map of plasmid pLW414 for the enzymes used in (a). The shaded area corresponds to vector DNA. The positions on the viral genome of the single-stranded interruptions, \(\Delta 1\) and \(\Delta 2\), and of the replication intermediate sa-DNA are indicated. (c) \(^{32}\)P-labelled DNA synthesized in the purified viroplasms was electrophoresed under denaturing conditions in a vertical agarose gel without any treatment (lane 4), after digestion with RNase A (lane 3) or chemical cleavage at 7-methyl guanosine residues (lane 2). After electrophoresis the gel was dried and autoradiographed. Only the relevant part of the gel is presented. Lane 1, radioactive markers (Bethesda Research laboratories 123 bp ladder) \(^{32}\)P-labelled in the presence of polynucleotide kinase and run in the same gel. Size of markers (in nucleotides) is indicated on the left.

The endogenous viral DNA synthesis may occur in virion-related particles

In view of obtaining further information on the nucleoprotein complexes involved in viral DNA synthesis, purified viroplasms were treated with urea and Triton X-100, after incubation in the presence of radioactive DNA precursors. The resulting suspension was centrifuged to equilibrium in a caesium chloride density gradient. As shown in Fig. 5, the acid-insoluble radioactive material was located in the gradient at the level of a sharp peak in a region of density close to that of purified CaMV virions. These results indicate that the newly synthesized DNA molecules are present in nucleoprotein complexes of a well defined structure. The apparent resistance of these complexes to the treatment with urea and Triton X-100, their estimated density and their reactivity against anti-virion antibodies in ELISA (data not shown) also suggest that they could correspond to virion-related particles. The characterization of these structures is in progress.

DISCUSSION

Previous results from our laboratory have indicated that the CaMV DNA synthesis performed in the purified viroplasms is highly strand-specific, producing DNA sequences mostly of polarity opposite to that of the viral transcripts (Mazzolini et al., 1985). The further characterization of such DNA synthesized in vitro has revealed that most of the labelled molecules are single-stranded and are partly associated with RNA by base-pairing. An
asymmetric synthesis of the viral DNA strands was also observed with a subcellular fraction obtained from CaMV-infected turnip leaves (Thomas et al., 1985) whereas other purified CaMV replication complexes appear to be able to synthesize both DNA strands actively (Pfeiffer et al., 1984; Marsh et al., 1985). This indicates that positive strand DNA synthesis may be rate-limited in some in vitro systems, resulting in the accumulation of negative strand replication intermediates. This could reflect the partial loss or inactivation of factors essential for positive strand synthesis, owing presumably to the procedure for purification of the replication complexes and/or the incubation conditions used during the endogenous DNA synthesis. A similar situation has been described for hepatitis B virus, an animal DNA virus which replicates its genome by reverse transcription. Replication complexes obtained from virus-infected liver cells appear to synthesize mostly ssDNA of negative polarity which can be associated with RNA by base complementarity (Summers & Mason, 1982).

An uncoupling of the CaMV DNA strand synthesis has also been observed in vitro. Indeed, negative strand ssDNA of a size up to that of the viral genome and occurring either free or hybridized to viral RNA was detected in infected plants (Marsh et al., 1985; Thomas et al., 1985). Analogous replication intermediates were also observed in the case of hepatitis B virus (Mason et al., 1982; Miller et al., 1984). These results suggest that in retroid DNA viruses positive strand DNA synthesis could occur mainly on a completed negative strand DNA.

A 650 nucleotide DNA molecule is labelled preferentially in the viroplasms upon endogenous DNA synthesis (Mazzolini et al., 1985). The results presented indicate that this molecule corresponds to the negative strand replication intermediate sa-DNA. The newly labelled molecule was found to map at the expected position of sa-DNA on the CaMV genome and also appears like sa-DNA to be covalently linked to tRNA. This RNA is probably the primer used for the RNA-dependent DNA synthesis of CaMV negative strand DNA as in the case for animal retroviruses (Taylor, 1977). Significant amounts of sa-DNA with or without its attached tRNA primer are present in infected plants (Covey et al., 1983; Turner & Covey, 1984) as compared to the other detected replication intermediates. It is assumed that this molecule accumulates because of the pause caused by the template switch taking place during reverse transcription of the 35S RNA. The labelling of sa-DNA observed in the purified viroplasm fraction may also result from a rate-limiting template switch.
The polarity and the association with RNA of the DNA molecules labelled upon endogenous DNA synthesis suggest that these molecules are mostly reverse transcription products of an endogenous viral RNA. An RNA-dependent DNA polymerase activity able to use poly(rC)-oligo(dG), a template–primer rather specific to reverse transcriptase, was previously detected in the purified viroplasms (Mazzolini et al., 1985). The poly(rC)-directed as well as the endogenous DNA polymerase activity were inhibited to the same extent by ammonium-21-tungsto-9 antimoniate (data not shown), a drug which was shown to impair lymphadenopathy-associated virus reverse transcriptase (Dormont et al., 1985). However the influence of such a compound on the plant cellular DNA polymerase activities remains to be studied.

In addition to an RNA-dependent DNA polymerase activity, the purified viroplasms contain viral RNA of heterogeneous size. Heterodisperse CaMV RNA was also detected in other replication complexes isolated from infected plants (Pfeiffer et al., 1984; Thomas et al., 1985). The upper limit observed for the size of the viral RNA present in our subcellular fraction suggests that this RNA could originate mainly from the 35S RNA, the presumed template used for reverse transcription. The observed association of this viral RNA with the viroplasms is in accordance with an active transport from the nuclei to these inclusion bodies. This step may involve the viral capsid protein, recently shown to bind RNA (Fuetterer & Hohn, 1987) and which shares the cysteine–histidine box (Covey, 1986), a sequence involved in the replication and packaging of the genomic RNA of retroviruses (Meric & Spahr, 1986; Prats et al., 1988).

The apparent degradation of a part of the CaMV RNA found in the isolated viroplasms could be caused by contaminating RNases originating from the host cell. However, despite the different subcellular fractionation steps, the presence of high Mr RNA indicates that this RNA is in a partially nuclease-resistant form. A significant protection against nuclease treatments was noticed previously for the DNA syntheses performed in CaMV replication complexes (Guilfoyle et al., 1983; Thomas et al., 1985). We also observed that pretreatment of the viroplasms with a pancreatic RNase decreased the overall endogenous DNA synthesis by only 40% (data not shown). These results can be correlated with the parallel observation that exogenous template–primers are poorly used by isolated CaMV replication complexes (Pfeiffer et al., 1984; Mazzolini et al., 1985; Thomas et al., 1985) and they suggest that the reverse transcription of CaMV occurs in protected structures. This feature is general to retroviruses and is presumably important for these infectious agents (Fuetterer & Hohn, 1987). In particular it must allow the RNA template to be protected against cellular nucleases and also to reduce the artefactual reverse transcription of host cell RNAs which could compete to some extent with the viral genomic RNA.

All retroviruses as well as hepatitis B virus were found to reverse transcribe the viral RNA inside the capsid (Summers & Mason, 1982; Fuetterer & Hohn, 1987). The hypothesis that CaMV replication could also occur in virion-related particles came from the observation that purified virions could retain an associated DNA polymerase activity able to introduce nucleotides into endogenous linearized viral DNA (Menissier et al., 1984). This hypothesis was further strengthened by other results showing that replication complexes isolated from CaMV-infected plants migrated in sucrose gradients at a position close to that of viral particles and that replication intermediates of the CaMV DNA were associated with highly purified virions (Marsh & Guilfoyle, 1987). In agreement with these results is our observation that most of the DNA synthesized in vitro is found, after disruption of viroplasms by treatment with urea and Triton X-100, at a position close to that of purified virions in a caesium chloride gradient. However the difference in caesium chloride banding of the radioactivity, as compared to that of purified viral particles, suggests that CaMV replication could occur in structures different from mature virions. This is consistent with the data of Marsh & Guilfoyle (1987) who showed that the particles containing the single-stranded subgenomic CaMV DNA molecules have a density slightly lower than that of virions. This suggests that CaMV replication occurs in viral precursor particles and must be completed before capsid maturation is achieved. In this respect CaMV behaves differently from the other retro-element hepatitis B virus for which maturation, i.e. coating and export, of the viral particle occurs before the completion of synthesis of the encapsidated viral genome (Tiollais et al., 1985).
REFERENCES


CaMV genome replication in viroplasms


(Received 15 May 1989)