DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication

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ABSTRACT

We have analysed DNA from African cassava mosaic virus (ACMV)-infected *Nicotiana benthamiana* by two-dimensional agarose gel electrophoresis and detected ACMV-specific DNAs by blot-hybridisation. ACMV DNA forms including the previously characterised single-stranded, open-circular, linear and supercoiled DNAs along with five previously uncharacterised heterogeneous DNAs (H1 – H5) were resolved. The heterogeneous DNAs were characterised by their chromatographic properties on BND-cellulose and their ability to hybridise to strand-specific and double-stranded probes. The data suggest a rolling circle mechanism of DNA replication, based on the sizes and strand specificity of the heterogeneous single-stranded DNA forms and their electrophoretic properties in relation to genome length single-stranded DNAs. Second-strand synthesis on a single-stranded virus-sense template is evident from the position of heterogeneous subgenomic complementary-sense DNA (H3) associated with genome-length virus-sense template (VT) DNA. The position of heterogeneous virus-sense DNA (H5), ranging in size from one to two genome lengths, is consistent with its association with genome-length complementary-sense template (CT) DNA, reflecting virus-sense strand displacement during replication from a double-stranded intermediate. The absence of subgenomic complementary-sense DNA associated with the displaced virus-sense strand suggests that replication proceeds via an obligate single-stranded intermediate. The other species of heterogeneous DNAs comprised concatemeric single-stranded virus-sense DNA (H4), and double-stranded or partially single-stranded DNA (H1 and H2).

INTRODUCTION

The genome of the whitefly-transmitted geminivirus African cassava mosaic virus (ACMV; also known as cassava latent virus) is composed of two single-stranded (ss) DNAs of similar size (DNAs A and B, formerly DNAs 1 and 2; (1)). DNA A encodes genes responsible for the replication of the virus (2) since it alone is capable of self-replication when introduced into protoplasts. Although not essential for DNA replication, and because both DNAs are required to initiate a wild-type infection (3), the two essential gene products of DNA B are thought necessary for transport of the virus or viral DNA around the plant (2,4).

Gene expression from both components is bidirectional (5). DNA A encodes the coat protein gene on the virion-sense DNA and at least three overlapping complementary-sense genes (AC1–3) have been identified. Mutational analysis (6) has indicated that gene AC1 is essential for DNA replication since mutants are unable to replicate in *Nicotiana tabacum* protoplasts but can be complemented *in trans* in plants. A mutant containing a lesion in gene AC2 retained the ability to replicate in protoplasts to produce both single- and double-stranded DNA forms but was unable to infect plants, suggesting its involvement in virus spread within the plant. Recent studies on a related geminivirus, tomato golden mosaic virus (TGMV), have shown that this gene is able to transactivate the expression of the coat protein gene (7). Gene AC3 mutants are infectious but produce lower levels of virus and greatly attenuated symptoms when compared with a wild-type infection (6), suggestive of a role in the modulation of virus proliferation. Recent results (8) have indicated that TGMV gene AC3 is necessary for efficient DNA replication.

The coding regions diverge from an intergenic region on both genomic components. Within the intergenic region is a sequence of approximately 200 bases that is highly conserved between the components, referred to as the common region (1). Located within the common region is a nonanucleotide sequence that is conserved in all geminiviruses, sequenced to date. In view of its conservation, location and similarity to sequences involved in the replication of bacteriophage ssDNA (9), the nonanucleotide has tentatively been assigned a role in geminivirus replication.

In addition to the genomic ssDNAs (virus-sense DNA), ACMV-infected plant material also contains several other double-stranded (ds) DNA forms including supercoiled (sc), open-circular (oc) and linear DNA. The predominant single- and double-stranded forms are genome length although dimeric forms

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have also been identified (10). Little is known about the replicative intermediates involved in geminivirus replication although the presence of concatemeric forms is thought to be indicative of a rolling circle mechanism. In the present study, additional ACMV DNA forms have been identified by resolving DNA from ACMV-infected plants by two-dimensional agarose gel electrophoresis and detecting viral DNAs by blot-hybridisation with strand-specific probes. We present data consistent with a rolling circle mechanism for geminivirus DNA replication which involves an obligate ssDNA intermediate. The suggested model for geminivirus DNA replication is compared with strategies established for the replication of bacteriophage and plasmid ssDNAs.

MATERIALS AND METHODS

Virus source and propagation

ACMV, derived from infectious clones of a Kenyan isolate (pJS092 and pJS094; (3)) was propagated in *Nicotiana benthamiana* grown under glasshouse conditions at 25°C with supplementary lighting to give a 16 hour photoperiod. Plants were mechanically inoculated at the five or six leaf stage with 4μg of each cloned DNA (equivalent to 1μg of each genomic component) following excision of the clone inserts using the appropriate restriction enzyme. Systemically-infected leaves were harvested 10 days post-inoculation and total nucleic acid was isolated using procedures described by Covey and Hull (11).

Analysis of DNA

Nucleic acid was digested with ribonuclease A (50μg/ml) and T_1 (100U/ml) in 50mM Tris-HCl (pH 7.9), 5mM $MgCl_2$. DNAs containing a single-stranded moiety were separated from dsDNA by benzoylated-naphthoylated DEAE (BND)-cellulose chromatography (12). Samples containing 10μg of DNA were loaded into a circular well positioned at one corner of a 20×20 cm 1.2% agarose slab gel. Electrophoresis in the first dimension was in 40mM Tris-acetate, 20mM sodium acetate, 2mM EDTA (pH 7.5) at 1.25V/cm for 24 hours. Gels were washed in alkaline buffer (30mM NaOH, 2mM EDTA) for 45 min. Electrophoresis in the second dimension was in alkaline buffer at 1.25V/cm for 24 hours at 90° orientation to the first dimension. After electrophoresis the separated DNAs were transferred to Hybond N (Amersham International) following depurination treatment in 0.25N HCl for 20 min, denaturation in 200mM NaOH, 600mM NaCl for 45 min and neutralisation in 1M Tris-HCl (pH 7.6), 1.5M NaCl for 45 min.

The transcription plasmid pAL001 was constructed by subcloning the DNA A-specific fragment *Dra*I(221)-*Sph*I(2581) from a clone containing a partial repeat of the genomic component (pCLV1.3A; (13)) into pBS(−) (Stratagene). Strand-specific riboprobes were generated by either T3 or T7 RNA transcription of pAL001. Double-stranded probes were obtained by random priming of pAL001 (14). When necessary, probes were removed from blots by sequential incubation at 45°C for 30 min in 0.4M NaOH followed by 200mM Tris-HCl (pH 7.5), 0.1×SSC, 0.1% SDS. Successful probe removal was verified by autoradiography prior to reprobing.

RESULTS

Analysis of viral DNAs

DNA isolated from ACMV-infected *N. benthamiana* leaves and examined by blot-hybridisation following neutral agarose gel electrophoresis has been shown to contain ssDNA as well as open-circular, linear and supercoiled double-stranded forms of ACMV DNA (10). This complex DNA population can be analysed in greater detail by performing blot-hybridisation following separation by two-dimensional (neutral/alkaline) agarose gel electrophoresis. To simplify the analysis and to avoid the detection of both genomic DNAs, we selected a DNA A double-stranded hybridisation probe that does not contain the nucleotide sequence of the common region and hence is specific to this genomic component. The characterised DNA forms mentioned above were readily resolved as discrete species with apparent molecular weights corresponding to genome length DNAs (Figure 1A). OcDNA migrated more slowly than linear DNA in the first/neutral dimension which, in turn, was slower than scDNA. SsDNA migrated ahead of the scDNA in this dimension. In the second/denaturing dimension, the ocDNA form was resolved into two components which, by analogy with the different forms of ocDNA present in cauliflower mosaic virus-infected *Brassica*

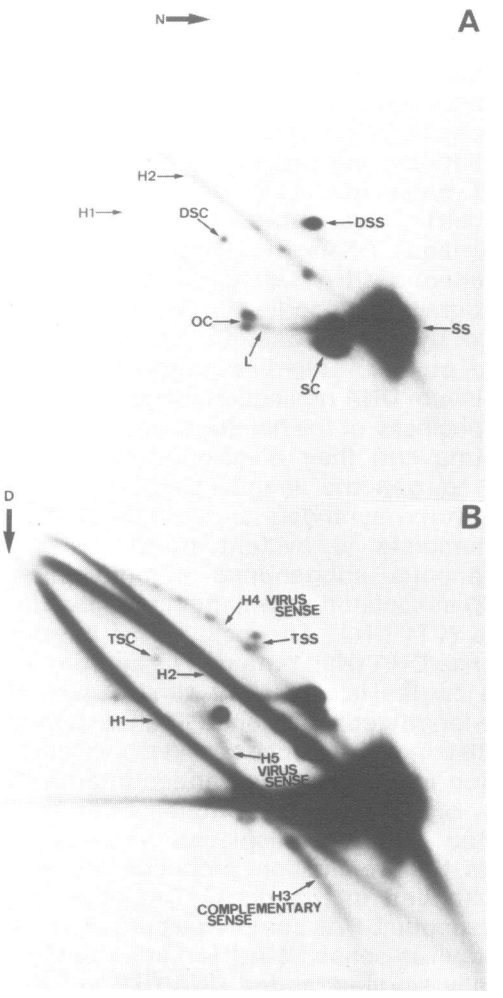

Figure 1. Two-dimensional agarose gel electrophoresis of ACMV DNA forms extracted from infected *N. benthamiana* and detected by blot-hybridisation with a double-stranded probe specific for ACMV DNA A. Panel B is a longer exposure of panel A. DNA was applied to a single well located at the top left of these and subsequent gels and electrophoresis was in neutral buffer in the first dimension (N) and under denaturing conditions in the second dimension (D). The positions of open-circular (OC), linear (L), supercoiled (SC), single-stranded (SS) and dimeric and trimeric forms of supercoiled (DSC and TSC) and single-stranded (DSS and TSS) DNAs are indicated. The characterisation of heterogeneous DNAs H1−H5 are discussed in the text.

leaves (15), correspond to single-stranded linear (faster migating) and circular DNAs. Similar DNA forms that co-migrated with scDNA in the first dimension probably result from nicking of the latter before fractionation in the second dimension.

Dimeric forms of ssDNA and scDNA, which routinely appear when cloned DNAs are used as a source of inoculum (10), were also evident (DSS and DSC respectively, Figure 1A). Additional concatemeric forms of ssDNA and scDNA (see below for characterisation) were visible following extended autoradiography of the blot (Figure 1B). Two classes of heterogeneous DNAs (H1 and H2), one of which intersects the ssDNA, are clearly visible in Figure 1A. H2 DNA branches and probably comprises two separate forms. At least three other classes of heterogeneous DNAs (H3, H4 and H5) became evident after a longer exposure (Figure 1B). An identical pattern is obtained when plants are inoculated using infected sap, indicating that the observed DNA forms are not a consequence of introducing the virus as linerised cloned DNA.

Detection of strand-specific DNAs

Since ACMV must produce virus-sense ssDNA for encapsidation and possibly for spread, we decided to further characterise the complex viral DNA pattern using DNA A-specific probes that are also strand-specific. When probed for virus-sense DNA, the pattern of DNA forms detected (Figure 2A) is almost identical to that detected using the double-stranded probe. Only one class of heterogeneous DNA (H3, Figure 1B), which migrates faster than genomic length DNA in the second dimension, failed to hybridise with this probe, indicative of complementary-sense DNA. In contrast, when probed for complementary-sense DNA (Figure 2B), the pattern of DNA forms is less similar but nevertheless related to that seen when using the double-stranded probe. Most noticeable is the absence of heterogeneous DNA H4, which must therefore comprise virus-sense DNA. The distinct forms within this heterogeneous DNA, seen in Figures 1B and 2A, are characteristic of concatemers that increase in size by genome length increments. The series of discrete DNAs migrating slightly slower than these concatemeric forms in the second dimension are also virus-sense. By virtue of their mobility, we conclude that they are circular ssDNAs and the heterogeneous DNA H4 comprises linear single-stranded forms, which are probably breakdown products of the circular forms. The heterogeneous DNA H5 is not detected when the blot is probed for complementary-sense DNA (Figure 2B) implying that it too must comprise virus-sense DNA. The independent hybridisation

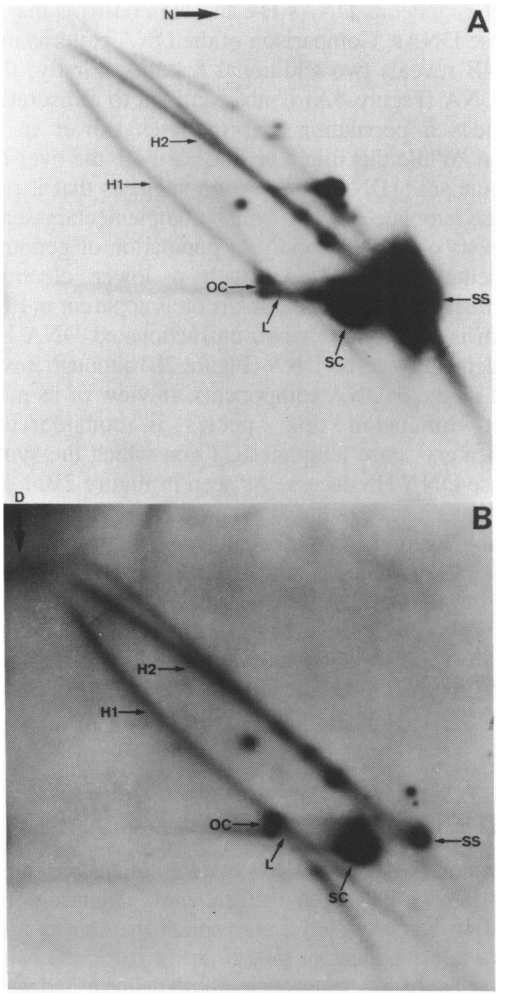

Figure 2. Analysis of ACMV DNA forms fractionated by two-dimensional agarose gel electrophoresis (reprobed blot from Figure 1) using DNA A-specific single-stranded probes for the detection of virus-sense DNA (A) and complementary-sense DNA (B). DNA forms are labelled according to Figure 1.

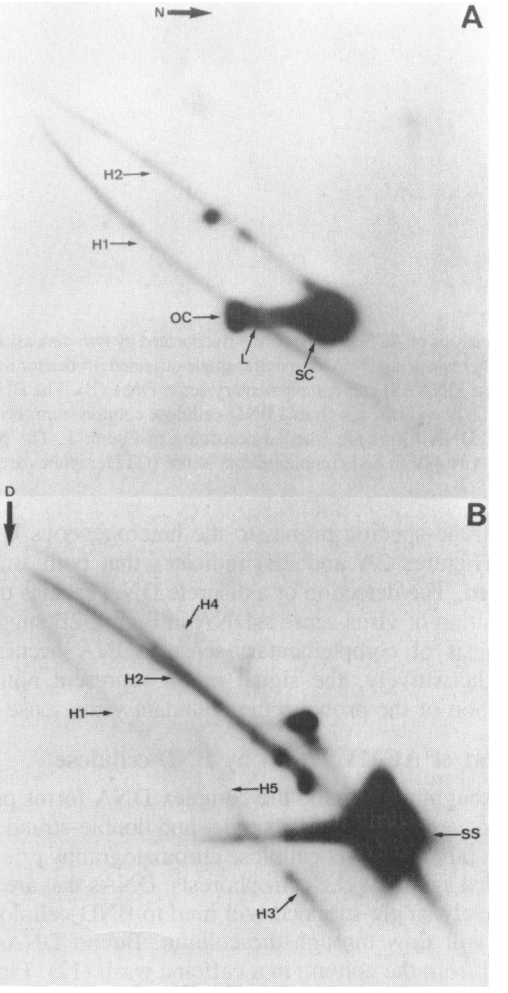

Figure 3. Analysis of ACMV DNA forms fractionated by two-dimensional agarose gel electrophoresis using a DNA A-specific double-stranded probe. DNA was fractionated by BND-cellulose chromatography prior to electrophoresis. (A) flow-through (double-stranded DNA) fraction; (B) bound fraction (containing single-stranded moiety). DNA forms are labelled according to Figure 1.

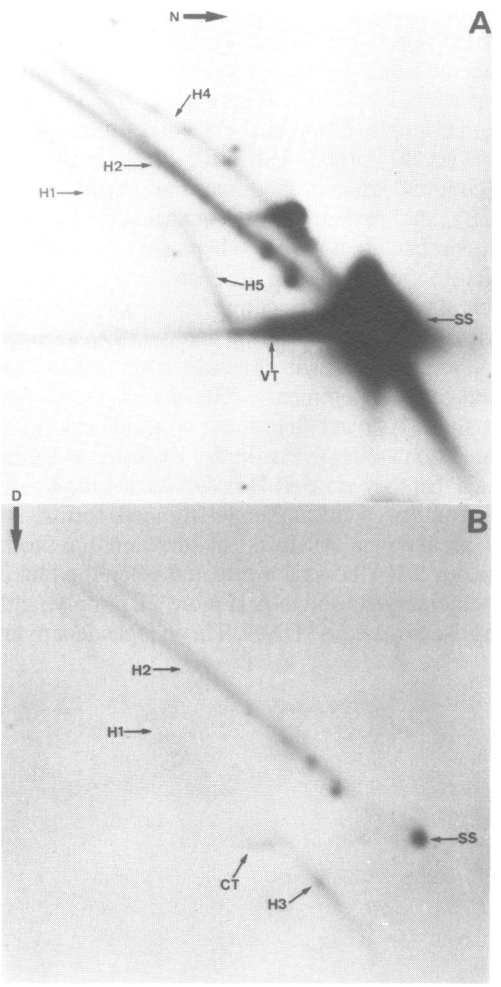

Figure 4. Analysis of ACMV DNA forms fractionated by two-dimensional agarose gel electrophoresis using DNA A-specific single-stranded probes for the detection of virus-sense DNA (A) and complementary-sense DNA (B). The DNA samples were from the bound fraction from a BND-cellulose column (reprobed blot from Figure 3B). DNA forms are labelled according to Figure 1. The positions of the putative virus-(VT) and complementary-sense (CT) templates are indicated.

of both strand-specific probes to the heterogeneous DNAs H1 and H2 (Figures 2A and 2B) indicates that both strands are represented. The detection of a discrete DNA species migrating to the position of virus-sense ssDNA in Figure 2B suggests that the synthesis of complementary-sense ssDNA occurs at low levels. Alternatively, the signal might represent non-specific hybridisation of the probe to the abundant virus-sense ssDNA.

Separation of ACMV DNAs by BND-cellulose

We also sought to examine the complex DNA forms present in infected leaves by separating single- and double-stranded forms from each other by BND-cellulose chromatography prior to two-dimensional agarose gel electrophoresis. DNAs that are partially or completely single-stranded will bind to BND-cellulose while dsDNAs will flow through the column. Bound DNAs can be recovered from the column in a caffeine wash (12). Figures 3A and 3B show the hybridisation of a DNA A-specific double-stranded probe to the flow-through and bound DNA respectively. As would be anticipated the open-circular, linear and supercoiled forms are double-stranded and do not bind to the column, and so can be detected in Figure 3A but not 3B. The results also suggest this to be the case for the heterogeneous DNA H1, although traces of this DNA (or a co-migrating DNA) can be seen in Figure 3B. In contrast, both linear and circular forms of the monomeric and concatemeric (H4 DNA) virus-sense ssDNAs, previously identified from their mobility and hybridisation to strand-specific probes, are only detected in the bound fraction (Figure 3B). This is also the case for the heterogeneous complementary-sense DNA H3 and virus-sense DNA H5, implying that they are at least partially single-stranded in their non-denatured forms. The results for the heterogeneous DNA H2 are more ambiguous because this DNA is represented in both the bound and flow-through fractions although one branch is more highly represented in Figure 3B, suggesting that it is at least partially single-stranded.

In order to confirm our observations we used DNA A strand-specific probes to analyse the BND-cellulose bound fraction. The pattern of hybridisation when probed for virus-sense DNA (Figure 4A) is very similar to that observed when the same fraction is hybridised to a double-stranded probe (Figure 3B). All DNA forms detected in Figure 3B are present with the exception of heterogeneous DNA H3, confirming it as complementary-sense DNA. In contrast, when probed for complementary-sense DNA (Figure 4B), the heterogeneous DNA H3 was readily detectable. The inability of this probe to hybridise to the heterogeneous DNAs H4 and H5 confirms that they are virus-sense DNAs. Comparison of the DNA patterns in Figures 4A and 4B reveals two additional features. Firstly, the virus-sense ssDNA (Figure 4A) is not confined to a discrete species but includes a population that migrates slower in the first dimension. While this might be due partly to the over-exposure of the abundant ssDNA, its location suggests that it represents virus-sense template (VT) on which complementary-sense DNA H3 synthesis occurs. Secondly, a population of genome length complementary-sense DNAs with a lower electrophoretic mobility than VT in the first dimension is apparent in Figure 4B. This form is also visible when unfractionated DNA is probed for complementary-sense DNA (Figure 2B) although it is partially obscured by the ocDNA components. In view of its mobility in the first dimension this species is considered to be complementary-sense template (CT) on which the synthesis of virus-sense DNA H5 occurs. As seen in Figure 2B, a low level of a DNA species migrating to the position of virus-sense ssDNA is detectable using the complementary-sense probe (Figure 4B).

Finally, we used the strand-specific probes to detect DNA forms in the BND-cellulose flow-through fraction. The pattern of hybridisation was identical for both probes (data not shown) and was identical to that seen when the double-stranded probe was used (Figure 3A) confirming that these DNAs contain both strands.

DISCUSSION

The separation of ACMV DNAs by two-dimensional agarose gel electrophoresis and their subsequent detection by blot-hybridisation has provided a convenient method to analyse the replicative intermediates of this virus. In particular, along with the previously characterised open-circular, linear and supercoiled dsDNA forms (10), we have been able to identify several novel DNA species. By separating the DNAs into double- and single- or partially single-stranded fractions prior to electrophoresis and hybridising blots with double-stranded and strand-specific probes,

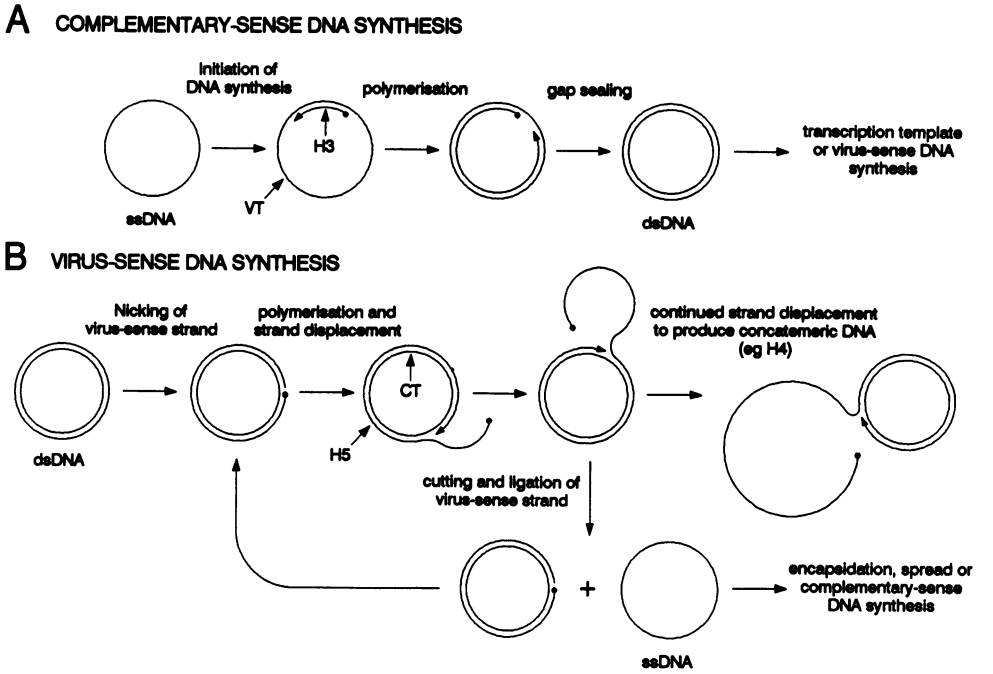

Figure 5. Model for geminiviral replication. The properties of heterogenous DNAs H3−H5 are discussed in the text. VT and CT refer to virus- and complementary-sense DNA templates respectively. The 5′ termini of elongating strands are indicated by black dots.

we have been able to characterise the different DNA forms. Several features of geminivirus DNA replication can be deduced from this analysis.

Synthesis of complementary-sense DNA

On infection of a plant and following uncoating of the genomic components, the encapsidated single-stranded DNA must be converted to a transcriptionally active double-stranded form prior to gene expression by the synthesis of complementary-sense DNA. Since purified single-stranded geminiviral DNA is infectious (16,17) this must be entirely under the control of host-encoded proteins. The heterogeneous DNA H3 comprises subgenomic length complementary-sense DNA. Its retarded electrophoretic mobility in the first dimension is consistent with its association with the genome length virus-sense DNA (VT, Figure 4A). Together, these DNAs are believed to comprise the intermediates in the synthesis of complementary-sense DNA (Figure 5A). Such intermediates would have single-stranded regions under non-denaturing conditions and so would be expected to bind to BND-cellulose (Figures 4A and 4B). The origin of complementary-sense DNA synthesis remains to be investigated. By probing the heterogeneous DNA with specific parts of the genome it should be possible to identify the region involved in the initiation of this process. Once second strand synthesis is completed, the gap in the complementary-sense strand must be sealed prior to the production of scDNA.

Synthesis of virus-sense DNA

The results are consistent with a rolling circle mechanism of DNA synthesis (18) for the production of virus-sense ssDNA. To produce virus-sense DNA from the double-stranded template by this mechanism, a nick must be introduced into the virus-sense strand. The 3′ terminus of the nicked DNA acts as a primer for DNA synthesis, displacing the original virus-sense strand as the template strand is copied (Figure 5B). During a single round of replication, genome length complementary-sense template DNA (CT, Figure 4B) would be associated with virus-sense DNA ranging in size from one to two genome lengths (heterogeneous DNA H5). The relative mobilities of these DNA species are consistent with their association in this manner. The displaced virus-sense strand would facilitate binding of the intermediates to BND-cellulose (Figures 4A and 4B). The fact that the proposed replicative intermediates H3 and H5 accumulate to relatively low levels, compared to the amount of product that is eventually generated, would suggest that DNA synthesis on either the virus- or complementary-sense template is not rate limiting.

During the replication of ssDNA bacteriophages and plasmids that are known to replicate via a ssDNA intermediate, a virus-encoded protein is responsible for the cleavage of the virus-sense strand at a specific site in order to initate DNA synthesis (19,20). By analogy, the initiation of ACMV replication could be mediated by the action of gene AC1 which is the only virus-encoded gene essential for DNA replication (6). The site at which nicking occurs is unknown but, on the basis of homologies with the gene A cleavage site of bacteriophage ΦX174, has been suggested to be within the conserved nonanucleotide sequence located within the common region (9). It has been proposed that nicking within the origin of replication during the initiation or termination of rolling circle replication is responsible for locating deletion endpoints in ssDNA bacteriophages and plasmids (21−23). A similar explanation has been proposed to explain the generation of deletion endpoints within the conserved nonanucleotide in A-CMV coat protein mutants (24). Furthermore, the nonanucleotide is located at the apex of a potential hairpin loop. Such a structure might be responsible for the production of a localised single-stranded region within the dsDNA which is required for the assembly of the replication complex (25, and references therein).

We have found no evidence for heterogeneous complementary-sense DNA associated with the growing virus-sense strand,

suggesting that virus-sense ssDNA is an obligate intermediate in DNA synthesis in a manner similar to that found for single-stranded DNA plasmids (20) and bacteriophages (19). We propose that after a full round of replication the nascent strand is cleaved and religated to produce a circular ssDNA and the double-stranded template DNA (Figure 5B). Again, by analogy with ssDNA bacteriophage replication, this might involve the product of gene AC1. The circular ssDNA might then enter into DNA replication as a template for the generation of additional dsDNA or it might be sequestered for the purpose of encapsidation. The regulation of this process might be achieved by the production of coat protein, its absence early in infection leading to the channelling of the DNA back into replication, its presence in the latter stages of infection leading to encapsidation. Alternatively, the DNA might be sequestered by other ssDNA-binding proteins for the purpose of spread throughout the plant. In common with the ssDNA bacteriophages, but unlike the ssDNA plasmids (26), synthesis of virus-sense strand appears to be continuous since failure to process the nascent strand leads to the production of concatemeric forms of virus-sense ssDNA (H4 DNA, Figure 1B). This suggests that the mechanism for processing genome length ssDNA is not closely regulated.

The origin and significance of the heterogeneous DNAs H1 and H2 is not understood. The results of Figures 2A and 2B clearly show that they contain similar amounts of both virus- and complementary-sense DNAs. Their binding characteristics to BND-cellulose suggest that H1 DNA is primarily double-stranded while H2 DNA contains a single-stranded moeity. The observed electrophoretic differences of H1 and H2 DNA could be a consequence of the association of either one with proteinaceous material. Concatemeric double-stranded forms can be produced by complementary-sense DNA synthesis on virus-sense DNA templates that result from inefficient processing (H4 DNA), as described in Figure 5B. It is also possible that they represent artefacts produced by the association of concatemeric complementing strands *in vitro* following the isolation of DNA from replication complexes. The heterogenous populations could arise from errors during replication, recombination and degradation of concatemeric forms. The exact nature of these and other heterogeneous DNA species is currently under investigation.

The generation of a double-stranded intermediate represents a key step in the replication cycle of ACMV. It not only provides the template for the generation of virus-sense DNA but, in association with host factors, will convert to transcriptionally active scDNA. Recent evidence concerning caulimoviruses suggests that the regulation of supercoiled DNA is dependent upon the host genotype and that its control markedly affects viral symptomatology (15). At present it is not known whether geminivirus hosts influence viral DNA replication in a similar way. Studies are in progress to investigate this possibility.

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