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RNA-primed complementary-sense DNA synthesis of the geminivirus African cassava mosaic virus

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ABSTRACT

The plant DNA virus African cassava mosaic virus (ACMV) is believed to replicate by a rolling circle mechanism. To investigate complementary-sense DNA (lagging strand) synthesis, we have analysed the heterogenous form of complementary-sense DNA (H3 DNA) from infected *Nicotiana benthamiana* by two-dimensional agarose gel electrophoresis and blot hybridisation. The presence of an RNA moeity is demonstrated by comparison of results for nucleic acids resolved on neutral/alkaline and neutral/formamide gels, suggesting that complementary-sense DNA synthesis on the virus-sense single-stranded DNA template is preceded by the synthesis of an RNA primer. Hybridisation with probes to specific parts of ACMV DNA A genome indicates that synthesis of the putative RNA primer initiates between nucleotides 2581 – 221, a region that includes intergenic sequences that have been implicated in geminivirus DNA replication and the control of gene expression.

INTRODUCTION

The genome of the geminivirus African cassava mosaic virus (ACMV), a plant virus with a restricted dicotyledonous host range, comprises two single-stranded (ss) DNA components (DNAs A and B) of a similar size encapsidated in twinned quasi-isometric particles [1,2]. Extracts of ACMV-infected *Nicotiana benthamiana* contain monomeric, concatemeric and subgenomic forms of both ssDNA and supercoiled, open-circular and linear double-stranded (ds) DNA [3]. In addition, we have identified and characterised a complex population of virus-specific heterogenous DNA forms by chromatography on BND-cellulose and blot hybridisation of samples resolved by two-dimensional neutral/alkaline agarose gel electrophoresis [4]. Our results are consistent with rolling circle replication for ACMV DNA and represent the first direct evidence for such a mechanism in eukaryotes. Studies on the replicational release of the monopartite geminivirus beet curly top virus (BCTV) from tandem repeat sequences support this idea [5].

The initial event following infection and uncoating of the virion DNA is presumed to be the host-directed synthesis of complementary-sense DNA on the ssDNA template to produce a transcriptionally active intermediate (see Figure 5). The inability to detect complementary-sense DNA (lagging strand) synthesis associated with newly synthesised virus-sense ssDNA, generated by rolling circle replication, suggested the latter to be an obligate intermediate of replication [4]. It is likely that complementary-sense DNA synthesis on this replicative intermediate proceeds by a similar mechanism to that on ssDNA following uncoating. The encapsidated viral DNA of geminiviruses that infect monocotyledonous plants is predominantly single-stranded but is associated with a complementary-sense DNA fragment of approximately 80 nucleotides containing 5′ terminal ribonucleotides [6–9]. The ability to synthesise full-length dsDNA *in vitro* using viral DNA extracted from purified virus particles suggested that the fragment might serve as a primer for at least the first round of DNA synthesis *in vivo*. We have been unable to detect an analogous fragment associated with ACMV particles [3], suggesting that primer synthesis occurs immediately after uncoating in infected cells.

On the basis of electrophoretic mobility on neutral/alkaline agarose gels, we have identified subgenomic-sized DNAs (heterogenous DNA H3) as intermediates in complementary-sense DNA synthesis [4]. In the present study we investigate complementary-sense DNA synthesis by comparing viral DNA forms that have been fractionated on neutral/alkaline and neutral/formamide agarose gels and detected by blot hybridisation using probes to specific regions of the genome.

MATERIALS AND METHODS

Virus source and propagation

ACMV was derived from infectious clones (pJS092 and pJS094) of a Kenyan isolate [2] and propagated in *N. benthamiana* as described [4]. Virus was held and manipulated under MAFF licence numbers PHF 1185A/68(21) and PHF 1185B/17(111) under the Plant Pests (Great Britain) Order 1980.

Analysis of virus-specific nucleic acids

Total nucleic acids were isolated from systemically infected leaves as described [4]. Nucleic acids were resolved by 1.2% or 1.4% two-dimensional (neutral/alkaline) agarose gel electrophoresis and blotted onto Hybond N (Amersham International) membranes [4]. Nucleic acids resolved by two-dimensional neutral/formamide agarose gels were either untreated (control) or digested with a combination of ribonuclease A (50μg/ml) and ribonuclease T_1

(100U/ml) (Boehringer) by incubation in 50mM Tris-HCl, 5mM $MgCl_2$ (pH 7.9) at 37°C for 2 hours. Nucleic acids were recovered by phenol-chloroform extraction and ethanol precipitation prior to electrophoresis. The first dimension of two-dimensional (neutral/formamide) agarose gel electrophoresis was carried out using 1.2% gels containing 40mM Tris-acetate, 20mM sodium acetate, 2mM EDTA (pH 7.5), electrophoresed at 1.25V/cm for 24 hours. The gel strip was incubated in a 50% (v:v) solution of formamide in 20mM MOPS, 8mM sodium acetate, 1mM EDTA (pH 7.0) for 15 minutes at 65°C. Following equilibration, melted 1.2% agarose in formamide-containing buffer was poured around the first dimension strip. Electrophoresis was continued for 24 hours at 1.25V/cm at 90° orientation to the first dimension and nucleic acids were blotted onto Hybond N membrane without prior treatment of the gel.

Strand-specific probes

Complementary-sense nucleic acid was detected with RNA probes to ACMV produced by run-off transcription from the T7 promoter of pBS(−) (Stratagene). The probes are summarised in Figure 1. ACMV nucleotide numbering throughout is according to [1]. Clone pAL001, containing the DNA A-specific fragment *Dra*I(221)−*Sph*I(2581) [4], was linearised with *Sph*I (probe A), *Hinc*II (probe C) and *Eco*RV (probe D). Clones pAL006 and pAL008 are derivatives of pAL001 in which the DNA A-specfic fragments *Dra*I(221)−*Eco*RI(1938) for pAL006 and *Dra*I(221)−*Cla*I(2403) for pAL008 have been removed. Probes B and F were generated by run-off transcription of pAL006 and pAL008 respectively, following linearisation with *Sph*I. Clone pAL002 was constructed by subcloning the DNA A fragment *Sph*I(2581)−*Dra*I(221) from pCLV1.3A [9] into pBS(−) digested with *Sph*I and *Sma*I. Probe E was generated by run-off transcription from the T3 promoter following linearisation of pAL002 with *Kpn*I (located within the polylinker, downstream of the ACMV insert).

Isolation of heterogenous DNA

Following neutral/alkaline two-dimensional agarose gel electrophoresis and ethidium bromide staining to locate the positions of the predominant viral DNA forms, the region of the gel containing the H3 DNA was excised and the DNA purified using a Prep-a-Gene kit (Bio-Rad). The 5′ end of the DNA was labelled using [γ-^{32}P]ATP (111 TBq/mmol; NEN) and polynucleotide kinase (Gibco BRL) and the size of the DNA was estimated on a 1.2% alkaline agarose gel against ^{32}P-labelled pJS092 (full-length DNA A clone) digested with *Mlu*I and ^{32}P-labelled kilobase DNA markers (Gibco BRL).

RESULTS

Detection of complementary-sense H3 DNA

Previous analysis of DNA A from ACMV-infected *N. benthamiana* by BND-cellulose chromatography, two-dimensional (neutral/alkaline) electrophoresis and blot hybridisation revealed the presence of a number of different heterogenous DNA forms [4]. In particular, when a strand-specific probe corresponding to nucleotides 221−2581 (Figure 1, probe A) was used, a heterogenous form of complementary-sense DNA was identified (H3 DNA). The electrophoretic mobility of H3 DNA was dependent upon its association with its circular template during the first dimension from which it was denatured

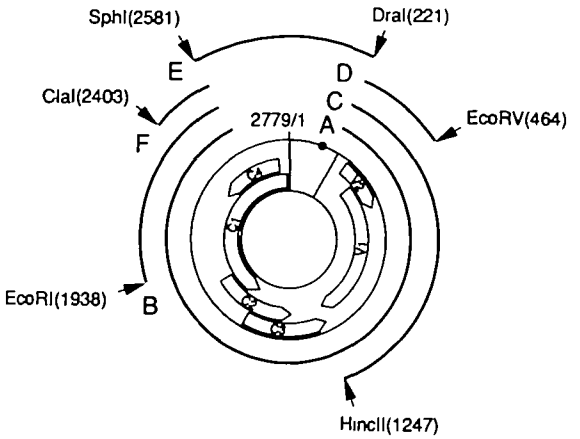

Figure 1. Location of ACMV DNA A complementary-sense probes (A−F) relative to virus- (V) and complementary-sense (C) open reading frames. The positions of the common region (shaded) and conserved nonanucleotide TAATATTAC, (black dot) are indicated. Nucleotide numbering is according to [1].

in the second dimension. To identify the part of the genome involved in the initiation of complementary-sense DNA synthesis we hybridised similar blots with probes for specific regions of complementary-sense DNA A (Figure 1). Using probe B, corresponding to nucleotides 1938−2581, the heterogenous DNA forms H1, H2 and H3 and the major dsDNA forms consisting of open-circular, linear and supercoiled DNAs were detected (Figure 2A). These DNAs were identified and characterised previously [4] with the use of probe A. On both occasions, the probes specific to complementary-sense DNA also detected ssDNA, although in reduced amounts compared with probes specific for virus-sense DNA. The pattern of DNA forms detected by probe B, in particular complementary-sense H3 DNA, was very similar to that detected by probe A. When blots were hybridised with a probe specific for complementary-sense nucleotides 221−1247 (Figure 1, probe C), most of the DNA forms that were previously detected by probe B were visualised (Figure 2B). To maximise detection of ssDNA forms the sample was resolved on a 1.4% gel and the DNA was subjected to milder depurination *in situ*, resulting in a reduction in the supercoiled DNA signal in Figure 2B. However, unlike probe B, probe C did not hybridise to the low molecular weight forms of H3 DNA. Using a probe specific to complementary-sense nucleotides 221−464 (Figure 1, probe D) the pattern of hybridisation was similar except that only the highest molecular weight forms of H3 DNA were detected (Figure 2C). A prominent signal (labelled X in Figure 2) is present within the H3 DNA following hybridisation with each probe, suggesting that a discrete DNA form is generated during the synthesis of full-length complementary-sense DNA.

The results show that progressively smaller H3 DNA forms are detected using probes D, C and B. In addition, a similar pattern of hybridisation to probe B was seen when probe F encompassing nucleotides 2403−2581 was used (data not shown). These results strongly suggest that the origin of complementary-sense DNA synthesis is located between nucleotides 2403 and 221. To investigate this idea we selected a probe specific to complementary-sense nucleotides 2581−221 (Figure 1, probe E).

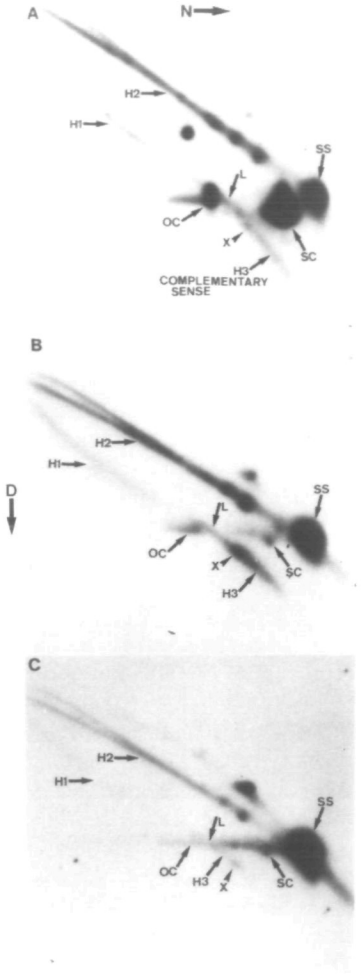

Figure 2. Two-dimensional neutral/alkaline agarose gel electrophoresis of ACMV DNA A forms detected by blot hybridisation using probes specific to complementary-sense nucleotides 1938−2581 (A), 221−1247 (B) and 221−464 (C). DNA was applied to a well located at the top left of these and subsequent gels. Electrophoresis was in neutral buffer in the first dimension (N) and under alkaline denaturing conditions in the second dimension (D). The positions of open-circular (OC), linear (L), supercoiled (SC) and single-stranded (SS) DNAs and the heterogenous DNAs H1, H2 and H3 are indicated. The characterisation of the form labelled X within H3 DNA is discussed in the text.

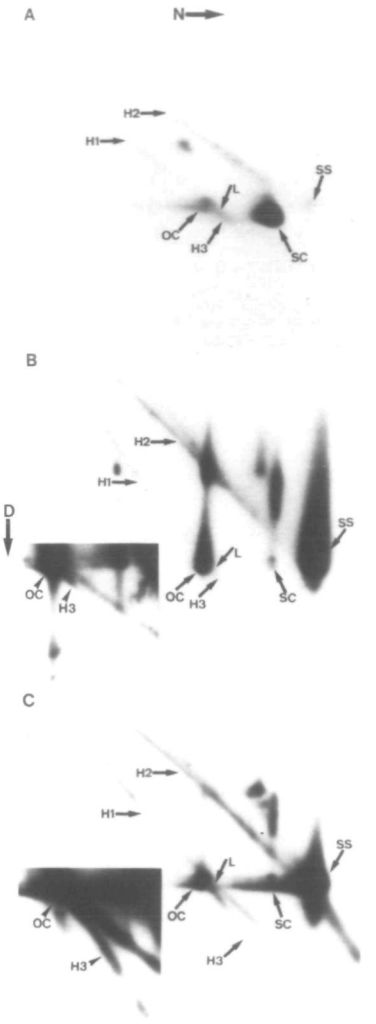

Figure 3. Analysis of ACMV DNA A forms fractionated by two-dimensional agarose gel electrophoresis detected using probe E, specific for nucleotides 2581−221. Electrophoresis was in neutral buffer in the first dimension (N) and under alkaline (A) or formamide (B and C) denaturing conditions in the second dimension (D). The sample resolved in (B) was treated with ribonucleases prior to electrophoresis. The insets in B and C are longer exposures of the blot in the region of H3 DNA. DNA forms are labelled according to Figure 2.

Unexpectedly, only high molecular weight forms of H3 DNA were detected with this probe (Figure 3A), reminiscent of the hybridisation pattern produced using probe D.

Complementary-sense H3 DNA forms in neutral/formamide gels

All previous analyses had been accomplished by resolving viral DNAs on two-dimensional neutral/alkaline agarose gels. Under these conditions any RNA species associated with the replicative intermediates would be degraded during electrophoresis in the second dimension. In order to preserve RNA species we fractionated the nucleic acids in the second dimension using denaturing conditions that are frequently used to resolve RNA. When probe E was hybridised to blots of nucleic acids that had been pretreated with ribonuclease (Figure 3B) the predominent forms of H3 DNA were high molecular weight. The reduced level of supercoiled DNA detected by probe E was due to the fact that the gel was neither depurinated nor treated with alkali prior to northern transfer and consequently the binding efficiency of this species to Hybond N was reduced. The hybridisation pattern of probe E to nucleic acids that had not been pretreated with ribonuclease was similar except that low molecular weight H3 DNA forms were additionally detected (Figure 3C). Comparison of the over-exposed autoradiographs (insets of Figures 3B and 3C) clearly shows the low molecular weight forms of H3 DNA associated with the untreated sample. The ability of probe E to detect low molecular weight forms of H3 DNA only when samples have not been subjected to alkaline conditions or ribonuclease digestion suggests that the nucleic acids which hybridise to the probe (nucleotides 2581−221) are predominantly RNA. The extremely faint signal associated with the treated sample maybe attributed either to incomplete ribonuclease

digestion or weak hybridisation to a small fragment of complementary-sense DNA located immediately downstream of the primer.

Size estimation of H3 DNA

H3 DNA isolated from a neutral/alkaline two-dimensional agarose gel was 5'end-labelled with ^{32}P and electrophoresed on an alkaline agarose gel together with size markers (Figure 4). The prominent form of H3 DNA, labelled X, was smaller than DNA A (2779 nucleotides), ranging from approximately 1600 to 2000 nucleotides in length. On a longer exposure, DNA with a higher molecular weight than that of X could be detected (data not shown).

DISCUSSION

The identification of the region of the genome involved in ACMV complementary-sense DNA synthesis was made possible by the resolution of the virus-specific nucleic acids by two-dimensional agarose gel electrophoresis and detection of complementary-sense H3 DNA with strand-specific probes corresponding to defined regions of the genome. Comparison of hybridisation patterns of nucleic acids fractionated under conditions that either preserve or degrade RNA suggests that complementary-sense DNA synthesis commences with the synthesis of RNA which acts as a primer. Probes B and F (nucleotides 1938−2581) but not probe E (nucleotides 2581−221) detected low molecular weight forms of H3 DNA under alkaline conditions, suggesting that complementary-sense DNA synthesis starts somewhere within nucleotides 1938−2581. Probes A (nucleotides 221−2581), C (nucleotides 221−1247) and D (nucleotides 221−464) show a progressive decrease in ability to detect low molecular weight forms consistent with this idea. However, after fractionation of nucleic acids under denaturing conditions that do not degrade RNA, probe E additionally detected low molecular weight forms of H3 DNA, indicating that the RNA moiety maps to this region of the genome. The reduction of the probe E hybridisation signal following treatment of the nucleic acids with ribonuclease supports this conclusion.

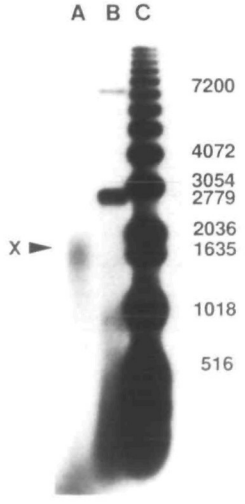

Figure 4. Alkaline agarose gel electrophoresis of ^{32}P-labelled H3 DNA (A), *Mlu*I digested pJS092 DNA (B) and kilobase DNA markers (C). X indicates the prominent form found within H3 DNA.

Following the uncoating of the virion ssDNA, the first step in DNA replication is the synthesis of a second strand on the ssDNA template leading to the production of a transcriptionally active dsDNA form (Figure 5). This process must be achieved entirely by host-encoded proteins since deproteinised geminivirus ssDNA is infectious [11−13]. In this respect, the replication of ACMV complementary-sense DNA resembles minus strand replication of *Escherichia coli* bacteriophages [14−16] and lagging strand synthesis of *Staphylococcus aureus* plasmids [17] on ssDNA rolling circle intermediates, processes that are initiated by host-encoded RNA polymerases. Our study shows that synthesis of RNA on the ACMV ssDNA template commences in a region of the genome encompassing intergenic sequences and including the common region [1]. The common region is a sequence of near identity present in both genomic components that plays a central role in bidirectional gene expression [18]. The common region (or its equivalent in monopartite viruses) is retained in all naturally-occurring subgenomic forms of ACMV [3] and the closely related virus tomato golden mosaic (TGMV) [19], and in the monopartite geminivirus BCTV [20,21], implying that it contains *cis*-acting elements essential for viral DNA synthesis. Within this region is a nonanucleotide sequence, TAATATTAC (nucleotides 146−154 in ACMV), located within

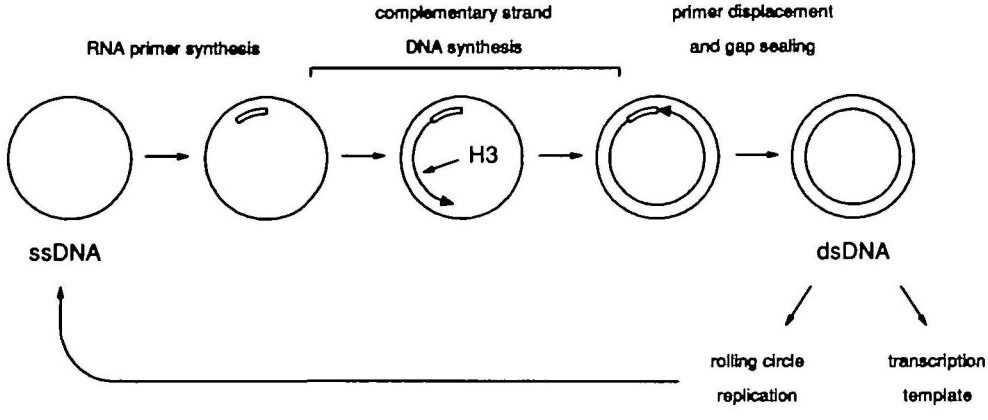

Figure 5. Model for complementary-sense DNA synthesis. The various stages are discussed in the text. The complementary-sense RNA primer open box and H3 DNA synthesised on virus-sense ssDNA are indicated.

the loop of a putative hairpin-loop structure, that is conserved in all geminiviruses. The nonanucleotide was suggested to play a role in DNA replication because of its homology with a *cis*-acting element that participates in rolling circle replication of bacteriophage DNA [23]. Indeed, nicking within the ACMV origin of replication prior to virus-sense strand displacement was invoked to explain the recombination hot-spot that occurs between nucleotides 152 and 155 [23]. It is possible that the hairpin-loop structure serves to cause local destabilisation within the dsDNA, an effect that is believed to play an important role in rolling circle replication of bacterial plasmids [24]. Protein AC1, the only ACMV-encoded protein essential for viral DNA replication [25], might contribute to the unwinding process or function by nicking the dsDNA. Recently, it has been shown that TGMV protein AL1 (the homologue of ACMV AC1) binds to the left side of the common region [26], although the binding site does not include the conserved nonanucleotide. *Cis*-acting elements that participate in the production of the RNA primer and the initiation of DNA synthesis remain to be investigated, but our data are consistent with the idea that primer synthesis initiates within the common region and it is possible that the hairpin-loop structure plays a role in the initiation of complementary-sense DNA synthesis. In this respect, it is interesting to note that sequences involved in the conversion of ssDNA to dsDNA in bacteriophages and bacterial plasmids that replicate via single-stranded intermediates are located within regions of strong secondary structure [27].

Primed by the nascent RNA, complementary-sense DNA synthesis will proceed on the circular ssDNA template until the 5′ end of the RNA primer is reached (Figure 5). At this point, displacement of the RNA primer will be necessary to allow DNA synthesis to continue to completion, to produce dsDNA on closure of the termini of the newly synthesised strand. Virus-sense ssDNA will be produced from the dsDNA by rolling circle replication [4] and the *de novo* synthesised ssDNA will once again serve as a template for complementary-sense strand synthesis as described here. The accumulation of a prominant DNA form (labelled × in Figure 2) within the H3 DNA population might result from the slowing of DNA synthesis during displacement of the RNA primer. If this is the case, the size of the DNA suggests that the primer could be at least 700 nucleotides in length. Alternatively, the hairpin-loop structure within the common region might present a temporary barrier to DNA synthesis. Without a method suitable for the isolation of the non-denatured double-stranded form containing the heterogenous H3 nucleic acid, we have not yet been able to characterise the RNA/DNA hybrid and so determine the exact length of the RNA primer and the point at which DNA synthesis is initiated.

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