

A DNA primer associated with banana bunchy top virus

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Banana bunchy top virus (BBTV) genomic ssDNA is capable of complementary strand synthesis *in vitro* without the addition of exogenous primers. We have demonstrated that the self-priming of BBTV can be attributed to a population of endogenous primers which are bound to the genomic DNA within the virions. The primer molecules appeared to be composed entirely of DNA and are heterogeneous in size. The primers were cloned, sequenced and

shown to map to a region within the major common region and extend 5' of this conserved region. These primers were found to be associated with multiple components of the genome and were capable of full-length complementary strand synthesis *in vitro*. Interestingly, most of the cloned primers appeared to be derived from BBTV DNA-5; no function has yet been determined for the putative protein of the large ORF within this component.

Introduction

Banana bunchy top virus (BBTV) has 18–20 nm isometric virions and a multicomponent genome of circular ssDNA (Harding *et al.*, 1991, 1993; Burns *et al.*, 1994, 1995). The genome of BBTV consists of at least six components, all approximately 1 kb in size. The components are also characterized by the presence of two conserved regions located within the intergenic region of each component; the stem-loop common region (CR-SL) and the major common region (CR-M) (Burns *et al.*, 1995).

The CR-SL incorporates a region of 69 nucleotides which is 62% identical between all components and incorporates a putative stem-loop structure. The CR-M, located 5' of the CR-SL, incorporates a 66–92 nucleotide region which is 76% identical between components (Burns *et al.*, 1995). Analysis of the viral transcripts has revealed the presence of a single open reading frame (ORF) in the virion sense of each component and also a second internal ORF within BBTV DNA-1 (P. Beetham, G. Hafner, R. Harding & J. Dale, unpublished results). Based on these characteristics, BBTV appears to belong to a previously undescribed group of circular ssDNA plant viruses which may include subterranean clover stunt virus (SCSV) (Boevink *et al.*, 1995), coconut foliar decay virus (CFDV) (Rohde *et al.*, 1990), faba bean necrotic yellows virus (FBNYV) (Katul *et al.*, 1995) and milk vetch dwarf virus (MDV) (Sano *et al.*, 1993).

There is little information about the replication of BBTV or other viruses within this possible new group. The geminiviruses are the only characterized group of ssDNA plant

viruses and the replication of these viruses is also not completely understood. Mutational analysis and expression studies have indicated that only one virus encoded protein is essential for geminivirus replication. This replication protein (Rep) is encoded by the C1–C2 spliced gene for the subgroup I (SI) geminiviruses, the C1 gene of the subgroup II (SII) geminiviruses and the AC1 gene of the subgroup III (SIII) geminiviruses (Brough *et al.*, 1988; Elmer *et al.*, 1988; Hanley-Bowdoin *et al.*, 1990; Etesami *et al.*, 1991; Lazarowitz, 1992; Rybicki, 1994). This presents a limitation for the initiation of geminivirus replication as all of these Rep genes are not able to be transcribed from the viral ssDNA genome. Rather, the encapsidated ssDNA genome must be first converted to a transcriptionally active double-stranded form prior to transcription and translation of its replication proteins. This limitation appears to be overcome by at least two separate mechanisms. The SI geminiviruses have a small complementary strand DNA molecule of about 80 nucleotides associated with the virion DNA. This DNA molecule is complementary to the small intergenic region of the ssDNA genome and is thought to act as a primer for complementary strand synthesis (Donson *et al.*, 1984). No such primer has been isolated from the SII and SIII geminiviruses although there is one report of a putative RNA primer associated with African cassava mosaic virus (SIII geminivirus) (Saunders *et al.*, 1992). This primer appears to be formed only after uncoating of the virus and maps to the large intergenic region.

The BBTV-like viruses appear to have a similar dilemma to the geminiviruses, in that the viral genes are not transcriptionally active as an ssDNA genome. Hence, these viruses should also have a mechanism of complementary strand synthesis upon entry into the host cell. Chu & Helms (1988)

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reported that the encapsidated viral ssDNA of SCSV was able to self-prime second-strand synthesis but molecular analysis did not indicate the nature of this priming mechanism.

In this paper, we report the isolation and characterization of a DNA primer associated with BBTV virions capable of self-priming complementary to virion-sense DNA *in vitro*.

Methods

■ Virus and viral DNA purification. An Australian isolate of BBTV was collected from Nambour, Queensland in infected banana plants (*Musa sp.* cv. Cavendish). BBTV was purified from banana midrib tissue as described by Harding *et al.* (1991). Viral DNA was extracted from virions in 0.07 M sodium phosphate buffer pH 7.2 by mixing with 0.1 vol. 100 mM Tris-HCl, 500 mM NaCl, 50 mM EDTA pH 8.0, 0.1 vol. 10% SDS, 30 µg proteinase K (Boehringer Mannheim) and incubating at 37 °C for 1 h. The reaction was heated at 65 °C for 10 min, emulsified with an equal volume of phenol-chloroform (1:1), centrifuged and the aqueous phase re-extracted with an equal volume of chloroform. DNA was precipitated from the aqueous phase by the addition of 0.1 vol. 3 M sodium acetate pH 5.2, 2 vols 100% ethanol, 40 µg glycogen (Boehringer Mannheim) and incubated at -80 °C for 12 h. The DNA was pelleted by centrifugation, washed with 70% ethanol and finally resuspended in sterile distilled water.

■ Self-priming of the BBTV genome. Complementary strand DNA was synthesized using virion DNA as a template without the addition of exogenous primers. Virion DNA (1 µg) was incubated with [α -³²P]dCTP (50 µCi, 3000 Ci/mmol), 2.5 units Sequenase (US Biochemical) in 40 mM Tris-HCl, 20 mM MgCl₂, 50 mM NaCl pH 7.5 containing 5 mM dithiothreitol, 200 µM dATP, dGTP, dTTP in a total volume of 20 µl. The reaction was incubated at 37 °C for 30 min and chased with 1 µl of 'cold' 10 mM dCTP and further incubation at 37 °C for 30 min. Non-polymerase and random-primed control reactions were set up as for the self-priming reaction, but without the addition of Sequenase, or with the addition of 4 µg random hexamers, respectively. Specified reactions did not contain [α -³²P]dCTP. The reaction products were extracted with phenol-chloroform (1:1), re-extracted with chloroform and precipitated with 2 vols 100% ethanol. Pellets were resuspended

in denaturing buffer (47% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol FF), heated at 95 °C for 5 min and then quenched on ice for 3 min. The products were analysed by electrophoresis in an 8 M urea, 6% denaturing polyacrylamide gel and exposure to X-ray film.

■ 5' and 3' end-labelling of viral DNA. Virion DNA was first treated with calf intestinal alkaline phosphatase (Pharmacia) prior to 5' end-labelling with [γ -³²P]ATP (100 µCi, 4000 Ci/mmol; Brestac) using T4 polynucleotide kinase (Stratagene) according to the manufacturer's instructions. 3' end-labelling using terminal transferase (Boehringer Mannheim) and [α -³²P]ddATP (Brestac) followed the procedure described by Yousaf *et al.* (1984).

To determine the composition of the labelled products, half of the reaction was treated with 0.3 M NaOH, 1 mM EDTA at 65 °C for 30 min to remove any ribonucleotides (Hayes *et al.*, 1988). Following alkaline hydrolysis, the reactions were neutralized. Both the untreated and alkaline hydrolysed samples were extracted as done with the self-primed extension reactions and aliquots were electrophoresed in an 8% denaturing polyacrylamide gel against an M13 sequencing ladder as a size standard. Gels were then dried and exposed to X-ray film.

■ Cloning and sequencing of primer molecules. Virion DNA (1 µg) was digested with 1 and 10 units of mungbean nuclease (Gibco BRL) in 100 µl reactions at 37 °C for 15 min. The reactions were then extracted twice with phenol-chloroform (1:1) before precipitation at -80 °C for 1 h in 70% ethanol and 20 µg glycogen. DNA was collected by centrifugation, washed in 70% ethanol and resuspended in sterile distilled water. The products of the mungbean nuclease digestion were then blunt-end cloned (Upcroft & Healey, 1987) into dephosphorylated, *Sma*I-digested pGEM-3Zf(+) vector (Promega) or the Ready-To-Go pUC18 *Sma*I/BAP+ligase kit (Pharmacia). The ligations were then electroporated into *E. coli* DH5 α (Dower *et al.*, 1988) and potential recombinant clones were identified and sequenced using an Applied Biosystems 373A DNA sequencer.

■ Construction of component- and strand-specific transcription vectors for each BBTV component. The primers outlined in Table 1 were designed and synthesized from the published sequences of

Table 1. Primers used to generate component- and strand-specific probes to BBTV

This table indicates the name, sequence and location of the primers for each respective component of BBTV.

BBTV DNA-	Primer name	Sequence	Location
1	BT1RepEx	5' GATGGCGCGATATGTGG 3'	129-144
	BTRepFD	5' GAGCTCTCAGCAAGAAACCAAC 3'	989-974
2	BT2R3	5' CCTAATCCTGTCAAGGA 3'	266-282
	BT2F3	5' CGTTAGTATACGTTCCG 3'	574-558
3	BT3V.Exp	5' TAGATCCATGGTCAGACAAGAA 3'	206-229
	BT3C.Exp	5' ATAAAGCTTTCAAACATGATATGT 3'	749-726
4	BT4V.V3	5' ATGGCATTAAACAACAGA 3'	279-295
	BT4C.C2	5' GAACATAGGTCCAGCGT 3'	629-613
5	BT5V.Exp	5' AAGAGCCATGGAGTTCTGGGAATC 3'	233-256
	BT5C.Exp	5' GATATAAGCTTAGAGTTAATGTTAC 3'	734-711
6	BTP2B2.17	5' GATCTATTGAAGCTGTG 3'	426-442
	BTP2R1.17	5' CTAACCTCCATGTCTCT 3'	666-651

the BBTV components (Harding *et al.*, 1993; Burns *et al.*, 1995). BBTV DNA was amplified in 50 μ l reactions containing 20 pmol of each primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs and 1.0 unit *Taq* polymerase (Cetus). BBTV virion DNA (0.1 ng) was added to each PCR reaction and subjected to one initial cycle of heat denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 42 °C for 1 min and 72 °C for 2 min and a final extension step at 72 °C for 10 min. The regions amplified corresponded to either part of the ORF or the entire ORF for each component. The amplified products were cloned using the pGEM-T cloning kit (Promega) as recommended by the manufacturer. Clones with appropriately sized inserts were screened for orientation by restriction digestion analysis and sequenced. Clones were selected such that in each case a virion-sense transcript was transcribed from a *SalI*-linearized template using T7 RNA polymerase.

■ Transcription and hybridization of component-specific and strand-specific RNA probes to self-primed extension reactions.

Unlabelled self-primed extension reactions and cloned full-length BBTV component controls were electrophoresed in 2% agarose gels in Tris-borate-EDTA (Sambrook *et al.*, 1989). DNA was transferred from the agarose gel to Hybond-N+ membrane (Amersham) using alkaline transfer conditions (0.4 M NaOH) in the Posiblot pressure blotter (Stratagene) as per the manufacturer's instructions. The membranes were then rinsed briefly in 5 \times SSPE and air dried. Virion sense RNA probes were transcribed separately from each of the *SalI*-linearized transcription vectors as described by Hafner *et al.* (1995). Membranes were hybridized and processed essentially as described by Hafner *et al.* (1995) except that the hybridization temperature was 42 °C.

Results

Self-primed extension of BBTV genomic DNA

Purified viral DNA was used in self-primed extension reactions together with a DNA-dependent DNA polymerase (Sequenase) to determine whether BBTV genomic DNA was capable of synthesizing complementary strand DNA *in vitro*. Products of approximately 1 kb, similar in size to full-length BBTV components, were generated in reactions with DNA-dependent DNA polymerase but without the addition of exogenous primers (Fig. 1, lane 3). The reaction with added exogenous random primers and DNA polymerase generated a population of products, the most abundant of which was approximately 1 kb (Fig. 1, lane 2) whereas no labelled products were detected in reactions without DNA polymerase (Fig. 1, lane 1). In addition, the self-primed extension products produced one predominant band that migrated around 950 nucleotides when digested with *AccI* (Fig. 1, lane 4) and 750 nucleotides when digested with *XbaI/HincII* (Fig. 1, lane 5). However, additional products of distinct sizes were detected after prolonged exposure of the self-primed extension products which had been digested with *AccI* (Fig. 1, lane 7) or *XbaI/HincII* (Fig. 1, lane 8). It was unlikely that these bands were the result of the DNA polymerase terminating prematurely during full-length complementary strand synthesis due to their distinct sizes and their absence in the undigested self-primed extension reaction (Fig. 1, lane 6). Furthermore, the predominant products seen in the shorter exposure (Fig. 1,

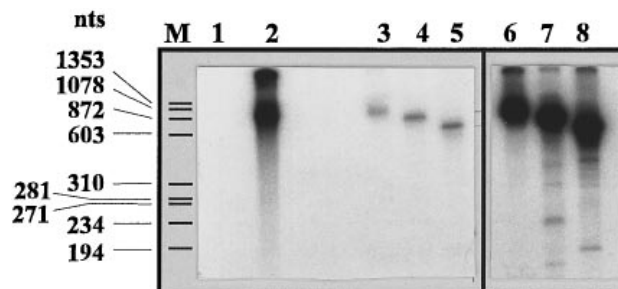


Fig. 1. Self-primed extension of BBTV genomic DNA. Purified BBTV DNA was used in self-primed extension assays with [α -³²P]dCTP and Sequenase. Controls, which consisted of a reaction with no Sequenase (lane 1) and a reaction to which random hexamers had been added (lane 2), were electrophoresed alongside the self-primed extended BBTV reaction (lane 3). Self-primed extension products were also digested with either *AccI* (lane 4) or *HincII* and *XbaI* (lane 5). Following extension the products were purified, denatured and electrophoresed on a 6% denaturing polyacrylamide gel. Lanes 6, 7 and 8 represent prolonged exposures of lanes 3, 4 and 5, respectively. A *HaeIII* digest of ϕ X174 DNA was used as a molecular mass marker (lane M).

lanes 4 and 5) have a restriction profile consistent with BBTV DNA-5.

End-labelling of viral DNA

DNA extracted from purified virions was 3' end-labelled with [α -³²P]ddATP. A heterogeneous population of DNA molecules was apparent after electrophoresis of these products in an 8% denaturing polyacrylamide gel (Fig. 2). The major DNAs were estimated to be between 73 and 77 nucleotides in length when compared to a M13 sequencing ladder after allowing for the additional ddATP molecule added by the labelling reaction. There was no apparent change in the migration patterns when the 3' end-labelled reaction was alkaline hydrolysed to remove any possible RNA moiety present. However, a reduction in band intensity was observed, possibly due to the differential precipitation between this reaction and the untreated sample. This reduction also correlated with a decrease in the amount of labelled DNA molecules migrating at the top of the gel, which were thought to be linear genomic length ssDNA, which was formed possibly as a result of nicking during the viral DNA purification. The same population of endogenous primers was observed when viral DNA was 5' end-labelled (result not shown).

Cloning and sequencing of primer molecules

Purified viral DNA was digested with mungbean nuclease to remove the ssDNA genome of BBTV leaving only the small dsDNA regions in order to clone the possible endogenous primers responsible for initiating second strand synthesis. These digestion products were cloned and the inserts from 41 clones were sequenced. More clones containing larger inserts

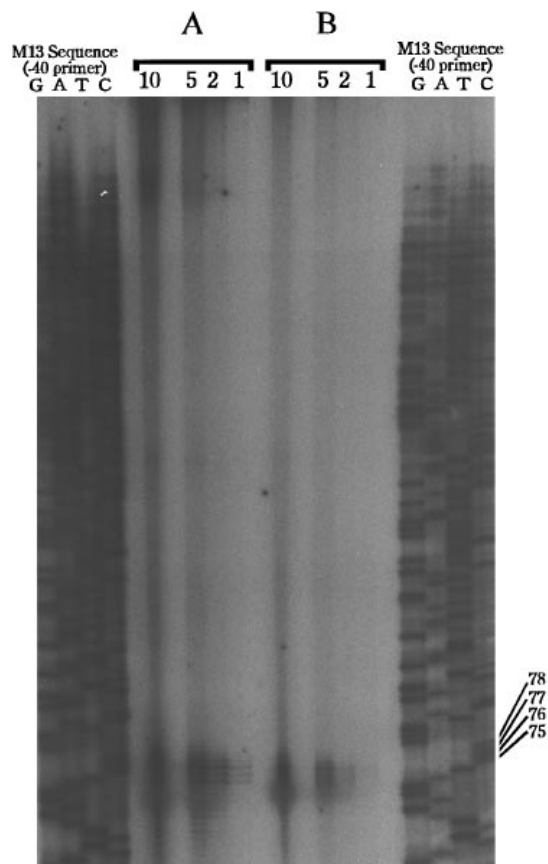


Fig. 2. End-labelled BBTV virion DNA. BBTV virion DNA was 3' end-labelled and either untreated (A) or alkaline hydrolysed to remove any RNA molecules (B). Aliquots of 10, 5, 2 and 1 μ l were electrophoresed in an 8% denaturing polyacrylamide gel.

were obtained from the digest with 1 unit of mungbean nucleasethan that with 10 units of mungbean nuclease suggesting that the primers were digested by higher concentrations of this enzyme. All inserts were clearly derived from BBTV sequences (Fig. 3A). The cloned inserts ranged in size from 19 to 97 nucleotides, with the majority around 80 nucleotides. The sequences of the cloned inserts were similar and all sequences initiated from within the CR-M of the BBTV components but with a variable initiation site. All but three of the primers extended past the end of the CR-M into the non-conserved regions and more than 70% of the primers terminated within a two nucleotide region, especially when the lower concentration of mungbean nuclease was used. Interestingly, all sequences that extended past the CR-M had homology to BBTV DNA-5 only. The position of these primers is illustrated diagrammatically in Fig. 3(B).

Identification of BBTV components which were capable of self-primed extension

Self-primed extension products from BBTV genomic DNA were hybridized with the six BBTV component- and strand-specific probes to determine which of the six BBTV com-

ponents were self-primed. Inserts from full-length clones of each BBTV component were isolated, purified and adjusted to equimolar amounts to use as controls in each of the hybridizations. To test for self-primed extension of BBTV DNA-1 (Fig. 4, panel 1), the cloned insert of BBTV DNA-1 (lane A), the cloned inserts of BBTV DNA-2 to 6 (lane B), DNA extracted from purified virions (lane C), and an unlabelled self-primed extension reaction (lane D) were electrophoresed in a 2% agarose gel using TBE buffer. The gel was then Southern transferred onto a nylon membrane and hybridized with an RNA probe designed to specifically hybridize with the complementary sense strand of BBTV DNA-1. This same procedure was repeated for BBTV DNA-2 to 6 except that for each blot, lane A contained the homologous cloned component insert and lane B contained the other five heterologous cloned component inserts. The labelled transcripts for each component hybridized to their respective homologous inserts and not to any of the DNA in the lanes containing the combination of the other five heterogeneous inserts, demonstrating that the probes were component specific. In addition, the probes did not hybridize to the purified virus demonstrating that the probes hybridized only with the complementary sense strand. The hybridization of the probes to the self-primed extension products indicated that BBTV DNA-1, 2, 4, 5 and 6 were capable of self-primed extension. No self-primed extension of BBTV DNA-3 was detected while the level of self-primed extension products synthesized appeared to be greatest for BBTV DNA-5, which was several fold higher than the products for all the other components. Furthermore, considering this data and the results from the labelled self-primed extensions shown in Fig. 1, the size of the addition restriction products in reactions digested with *AccI* (Fig. 1, lane 7) or *HincII/XbaI* (Fig. 1, lane 8) were consistent with the primer of the other BBTV components initiating from within the CR-M.

Discussion

We have demonstrated, using self-primed extension reactions, that BBTV genomic ssDNA is capable of *in vitro* self-primed complementary strand DNA synthesis. A population of endogenous DNA primers approximately 80 nucleotides long was subsequently detected in the viral genomic DNA. The initiation site for the primers was located within the CR-M although the precise site was variable. The majority of primers extended 5' of the CR-M and terminated within a two nucleotide region. Interestingly, the sequence of all primers which extended 5' of the CR-M had homology to BBTV DNA-5.

Using component- and strand-specific probes, it was demonstrated that BBTV DNA-1, 2, 4, 5 and 6 were capable of self-primed extension. The highest concentration of extension products was associated with BBTV DNA-5 while the amounts of products associated with BBTV DNA-1, 2, 4 and 6 were relatively similar. Interestingly, no products were detected for

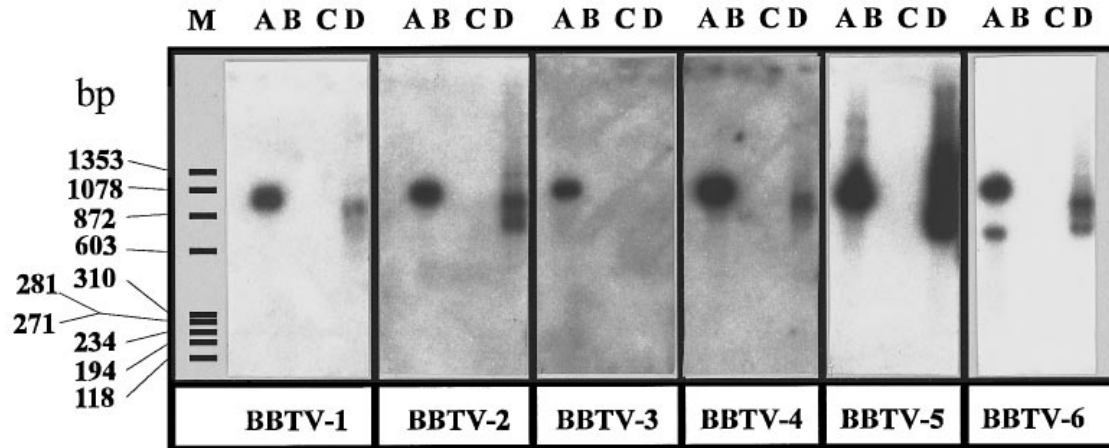


Fig. 4. Hybridization of BBTV component- and strand-specific probes to self-primed extension products. Positive control insert (lanes A), negative control inserts (lanes B), purified viral ssDNA (lanes C) and self-primed viral DNA (lanes D) were electrophoresed through a 2% agarose gel alongside a *Hae*III digest of ϕ X174 (lane M). The nucleic acids were then transferred to Hybond-N+ membrane and probed with respective component- and strand-specific probes.

BBTV DNA-3. It is unlikely that this result was due to differing concentrations of the BBTV genomic components since we have shown that viral DNA isolated from purified BBTV contains similar amounts of BBTV DNA-1 to 6 (S. Prasad, R. M. Harding & J. L. Dale, unpublished results). It is more likely, however, that BBTV DNA-3 is capable of self-priming but that the concentration of extension products in the reaction was extremely low. The relatively efficient self-priming of BBTV DNA-5 compared to BBTV DNA-1, 2, 3, 4 and 6 may indicate that the protein encoded by this component is important early in the infection process. As yet, however, the function of the protein encoded by BBTV DNA-5 is unknown.

DNA primers have also been associated with some of the SI geminiviruses. Maize streak virus – Nigerian isolate (MSV-N; Donson *et al.*, 1987) and Kenyan isolate (MSV-K; Howell, 1984), *Digitaria* steak virus (DSV; Donson *et al.*, 1987), *Chloris* striate mosaic virus (CSMV; Anderson *et al.*, 1988), wheat dwarf virus (WDV; Hayes *et al.*, 1988) and tobacco yellow dwarf virus (TobYDV; Morris *et al.*, 1992) have all been shown to contain a small complementary strand primer associated with the virion DNA which is capable of complementary strand synthesis *in vitro*. Both these SI geminiviruses and BBTV have primers around 80 nucleotides which are located within the intergenic regions of the viral genomes. However, while the primers associated with BBTV appear to have a variable 5' initiation site, those of the monopartite geminiviruses have a defined 5' end to which several ribonucleotides are attached. It is possible that the variable 5' initiation sites of the BBTV primers are artefacts resulting from mungbean nuclease digestion but this is unlikely since the 3' ends are more highly conserved using identical concentrations of the enzyme. It has been postulated that the ribonucleotides attached to the 5' end of the monopartite geminivirus primers may be remnants of a larger RNA molecule used to prime the DNA primer's synthesis (Donson *et al.*, 1984). Although no ribonucleotides

were associated with the BBTV primers, it is possible that they were present but were removed prior to encapsidation or degraded during the experimental procedures.

Multiple components of BBTV are self-primed and the primers appear to be initiated from within the CR-M. This suggests that one of the potential roles of the CR-M may be to direct the synthesis of this primer. The CR-M appears to be divided into three separate domains (Fig. 3*a*). The 55 nucleotides at the 5' end of the CR-M (Domain I) are 80% conserved amongst all components of BBTV, except BBTV DNA-1 which has a 26 nucleotide deletion in this region. Most, or all of this region, was found in the cloned primer sequences especially when low concentrations of mungbean nuclease were used. The 3' end of the CR-M contains an 18 nucleotide region (Domain III) which is greater than 94% conserved between all components. This domain contains a G-C rich inverted repeat which has the potential to form a small stem-loop structure, with a common trinucleotide G[A/C/T]A loop. This domain lies upstream of the apparent DNA primer initiation sites. Separating these two highly conserved regions is a 19 nucleotide gap (Domain II) which has only 26% similarity between components. Over 66% of the primers cloned using low concentrations of mungbean nuclease initiated within this region. Domain III appears to be similar to the single-strand initiation (*ssi*) signals in some bacterial plasmids such as RSF1010 (Honda *et al.*, 1988) and R1162 (Lin & Meyer, 1987). The *ssi* signals associated with these broad host range plasmids are known to direct priming of the complementary strand synthesis on the ssDNA template, using plasmid-encoded enzymes which specifically recognize the particular *ssi* signals. The plasmid is able to replicate independently of the host *dnaA*, *B*, *C*, *G*, *T* and *rpoB* genes through the use of plasmid-encoded helicase (RepA), primase (RepB') and initiation protein (RepC) (Haring & Scherzinger, 1989; Honda *et al.*, 1989). The plasmid is unable to replicate

normally without either of the *ssi* signals or the three plasmid-encoded proteins, unless the *ssi* signals are replaced with other types of *ssi* signals that use different priming mechanisms such as the primosome assembly sites for ϕ X174 (Honda *et al.*, 1991) or G-site from phage G4 (Honda *et al.*, 1992; Tanaka *et al.*, 1994). The *ssiA* signal associated with RSF1010 consists of a highly conserved 40 nucleotide sequence (Honda *et al.*, 1988). Twenty-two nucleotides of this *ssiA* signal form a G-C rich stem-loop structure with a trinucleotide GAA loop sequence. Mutational analysis has shown that both the nucleotide sequence and base-pairing in the *ssiA* region are essential for *ssiA* activity (Miao *et al.*, 1993; Honda *et al.*, 1993). The replicational ability of the plasmid was reduced when a base was mutated in the stem, but mutating a base in the opposing 3' stem to its complement restored its replicational ability. This indicates a structural, rather than functional, role of some nucleotides. The GAA loop sequence has also been shown to be essential for the *ssiA* activity (Honda *et al.*, 1993). One of the findings based on various *ssi* signals, regardless of mode of action, is that specific stem-loop structures are an essential requirement for their priming ability. Priming of RSF1010 and RI162 both occur 3' of this stem-loop structure.

It is possible, therefore, that BBTV uses virus encoded proteins (e.g. primase) to direct the synthesis of an RNA primer around Domain III of the CR-M. This RNA primer could then be used by DNA polymerase to generate the DNA primer isolated during this study. This would require an RNA primer of around 20–40 nucleotides in length and could explain the variable initiation site of the BBTV DNA primer. The formation of the short dsDNA fragment in the CR-M may then act as a signal for encapsidation of the virions and explain the relatively precise termination site.

Further work is needed to determine the sequence and structural requirements of these possible *ssi* signals in BBTV and the mechanisms by which they function. It is possible that the ssDNA plant viruses have different *ssi* signals than those presently identified to initiate second-strand synthesis. Since some DNA primases are known to bind to one sequence of a template and then migrate down to the region where the primer is initiated (Sheaff & Kuchta, 1993), it may be possible that the *ssi* signals in plant ssDNA viruses may be located anywhere in the viral genome rather than in close proximity to the primer initiation sites.

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