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Infectious mutants of cassava latent virus generated *in vivo* from intact recombinant DNA clones containing single copies of the genome

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ABSTRACT

Intact recombinant DNAs containing single copies of either component of the cassava latent virus genome can elicit infection when mechanically inoculated to host plants in the presence of the appropriate second component. Characterisation of infectious mutant progeny viruses, by analysis of virus-specific supercoiled DNA intermediates, indicates that most if not all of the cloning vector has been deleted, achieved at least in some cases by intermolecular recombination in vivo between DNAs 1 and 2. Significant rearrangements within the intergenic region of DNA 2, predominantly external to the common region, can be tolerated without loss of infectivity suggesting a somewhat passive role in virus multiplication for the sequences in question. Although packaging constraints might impose limits on the amount of DNA within geminate particles, isolation of an infectious coat protein mutant defective in virion production suggests that packaging is not essential for systemic spread of the viral DNA.

INTRODUCTION

Cassava latent virus (CLV) is typical of the whitefly-transmitted geminiviruses, having a genome comprising two similar sized circular single-stranded (ss) DNAs (DNAs 1 and 2 of 2779 and 2724 nucleotides respectively (1)), encapsidated in characteristic twinned quasi-isometric particles. Cloned copies of the genome are infectious when excised from the cloning vector and mechanically inoculated to Nicotiana benthamiana and have been used to demonstrate the essential role of both DNA components in infectivity (2). Whereas excision of single cloned copies of the genomic components of the closely related geminivirus tomato golden mosaic virus (TGMV) was considered to be essential for infectivity (3), we report here the potential to produce systemic infection in plants using intact recombinant clones and characterise a number of progeny viral DNAs.

MATERIALS AND METHODS

Construction of clones

The construction from a West Kenyan isolate of CLV of full length copies of DNA 1 (pJS092, cloned at MluI (734)) and DNA 2 (pJS094, cloned at PstI (245)) in bacteriophage M13 vectors has been described (2). Virus-specific supercoiled (sc) DNA (4) was extracted from plants infected following inoculation with cloned CLV DNA and used to construct full length copies of DNA 2 in either orientation in Escherichia coli plasmid pUC8, cloned at its unique PstI site (pCLV010 (+) and pCLV011 (-); + denotes virus sense DNA in the same orientation as M13 DNA) or EcoRV (2550) site (pCLV014 (+) and pCLV015 (-)). The positions of these restriction endonuclease sites within the CLV genome are indicated in Figure 1.

Infectivity studies

Recombinant M13 RFs and E. coli plasmids were digested with either the appropriate restriction endonuclease to excise the cloned insert or single cutting restriction endonucleases PstI (pJS092) or BamHI (pJS094, pCLV010, pCLV011, pCLV014 and pCLV015) to produce linearised chimeric DNA. Complete digestion was verified by agarose gel electrophoresis and the DNA was subsequently phenol extracted, precipitated from ethanol and redissolved to a concentration of 1mg/ml with respect to the DNA insert. Two week old Nicotiana benthamiana plants, maintained

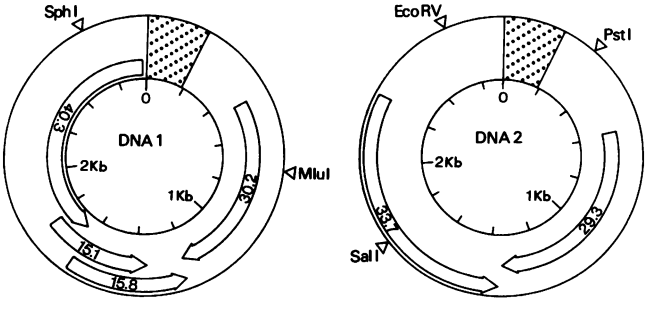

FIGURE 1. Organisation of the CLV genome. The coding capacities of proposed functional ORFs are given in kilodaltons. Sequences common to both components are denoted by the stippled regions. The positions of selected restriction endonuclease sites relevant to the discussion are indicated.

at 25°C, were mechanically inoculated with 1 µg of each CLV DNA insert or the molar equivalent of chimeric DNA.

Characterisation of progeny virus

Virus-specific scDNA was purified from plants (4) infected using selected chimeric DNA inocula and analysed by agarose gel electrophoresis after digestion using SphI. Full length copies of the progeny virus DNA components were cloned from the scDNA. To circumvent the potential problem of loss of the original cloning site, the DNA components were cloned at a second single cutting restriction endonuclease site, either SphI (2581) in DNA 1 (pCHI001 and pCHI002) or SalI (1753) in DNA 2 (pCHI003 - pCHI011). The infectivity of progeny clones was tested as described above after excision of inserts using the appropriate restriction endonuclease. Total nucleic acids were extracted from systemically infected plants (4) following inoculation using progeny clones and analysed on 1.4% agarose gels (5). Gels were Southern blotted and probed specifically for either genome component using nick translated M13 RFs of clone pJS057 containing a BglII fragment (804-1410) of DNA 1 or pJS055 containing a HindIII fragment (1494-2281) of DNA 2.

Insertions and/or deletions within the cloned progeny DNAs were characterised by subcloning into appropriately linearized M13 vectors DNA 1 fragments BamHI (291) - BamHI (485), BamHI (485) - BglII (804), and BglII (804) - BglII (1410) from clones pCHI001 and pCHI002 (to cover the entire coat protein open reading frame (ORF) (6)) and DNA 2 fragments TaqI (2498) - TaqI (338) from clone pCHI003, EcoRV (2550) - HpaI (416) from clones pCHI004 - pCHI006, TaqI (2409) - TaqI (338) from clones pCHI008 and pCHI009 and HindIII (2281) - HindIII (1494) from clones pCHI010 and pCHI011. Sequences were determined by the dideoxy-termination procedure of Sanger et al. (7,8) and data compiled using the computing methods of Staden (9).

Immuno-detection of virus and coat protein in plants

The presence of virus particles in extracts of systemically infected N. benthamiana was investigated by immunosorbent electron microscopy (ISEM) (10) using an antiserum raised in rabbits and directed against purified CLV virions (11).

To detect truncated and un-assembled versions of the coat

protein, tissue samples were subjected to PAGE followed by Western blotting (12,13). Systemically infected tissues were homogenised in 2 volumes of 50mM Tris HCl (pH 8.0), 60mM KCl and 6mM 2-mercaptoethanol and the homogenate filtered through muslin. The filtrate was clarified by centrifugation at 10,000xg for 10 min at 4°C. Proteins were denatured by the addition of a 1/3 volume of 4 x SDS sample buffer (14) and heated at 90°C for 5 min. Samples equivalent to 75mg of starting tissue were analysed on SDS/linear gradient polyacrylamide (7.5 - 25%), bisacrylamide (0.2 - 0.125%) slab gels with 4.5% acrylamide, 0.09% bisacrylamide stacking gels using the buffer system of Laemmli (14). Gels were either stained with Coomassie brilliant blue or electroblotted to nitrocellulose sheets (12,13). Blots were probed with 1:500 dilutions of either of two antisera directed against CLV (11,15) which had been cross-adsorbed (16) against an acetone powder of healthy N. benthamiana. Bound antibodies were located with goat anti-rabbit IgG-alkaline phosphatase (17).

Isolation and mapping of virus-specific poly(A)$^+$ RNA

Total RNA was extracted from systemically infected N. benthamiana (6), 14 days after inoculation with cloned DNAs. Residual virion ssDNA was removed by incubation with RNase-free DNase I (25 $\mu g/ml^{-1}$) for 60 min at 4°C and the deproteinised RNA fractionated by two cycles of oligo(dT)-cellulose chromatography.

Nuclease S_1 mapping (18) was employed to characterise the transcription products of the coat protein mutant clone pCHI001. Poly(A)$^+$ RNA (200ng) was hybridised (19) at 48°C for 3h with excess of pJS092 ssDNA containing a full length copy of virion (+) sense DNA 1, or with complementary (-) sense clone pJS107, containing an EcoRI fragment of DNA 1 (2732-1868). Heteroduplexes were incubated with S_1 nuclease (50U) and the protected DNA fragments fractionated on alkaline 1.1% agarose gels (19). After neutralisation and transfer to nitrocellulose, blots were probed with gel purified cloned DNA 1 insert labelled to high specific activity with [α-^{32}P]dCTP by the 'oligolabelling' method of Feinberg and Vogelstein (20,21).

RESULTS

Infectivity of chimeric DNAs

Previous experiments have indicated that inoculation using either DNA 1 or 2 alone does not produce a systemic infection in N. benthamiana (2). All of the inocula used for the experiments summarised in Table 1 therefore contain a single copy of each DNA component in one of three possible forms; as an excised insert (pJS092 (MluI); pJS094 (PstI)), a linearised chimeric DNA (pJS092 (PstI); pJS094, pCLV010, pCLV011, pCLV014 and pCLV015 (BamHI)) or undigested chimeric scDNA. All three forms of clones pJS092 and pJS094 are infectious (Table 1A). In general, both primary (necrotic lesions on inoculated leaves) and systemic symptoms (leaf curling of newly expanding leaves) appear more rapidly when the inocula contain excised DNA inserts rather than linearised chimeric DNA or undigested scDNA. Furthermore, the success rate of infection is enhanced if linearised chimeric DNA

Table 1. Infectivity of chimeric DNA

Inoculum		Number of infected/inoculated plants		
A				
		I	II	III
pJS092 (MluI)	pJS094 (PstI)	5/5	5/5	7/10
pJS092	pJS094 (PstI)	1/5	-	5/10
pJS092 (PstI)	pJS094 (PstI)	-	-	6/10
pJS092 (MluI)	pJS094	2/5	-	2/10
pJS092 (MluI)	pJS094 (BamHI)	-	-	7/10
pJS092	pJS094	0/5	1/5	-
pJS092 (PstI)	pJS094 (BamHI)	-	1/5	-
B				
		I	II	
pJS092 (MluI)	pJS094 (PstI)	2/3	7/10	
pJS092 (MluI)	pCLV010	0/3	0/5	
pJS092 (MluI)	pCLV010 (BamHI)	3/3	5/5	
pJS092 (MluI)	pCLV011	2/3	1/5	
pJS092 (MluI)	pCLV011 (BamHI)	2/3	3/5	
pJS092 (MluI)	pCLV014	0/3	0/5	
pJS092 (MluI)	pCLV014 (BamHI)	1/3	2/5	
pJS092 (MluI)	pCLV015	0/3	0/5	
pJS092 (MluI)	pCLV015 (BamHI)	1/3	2/5	

- = not done

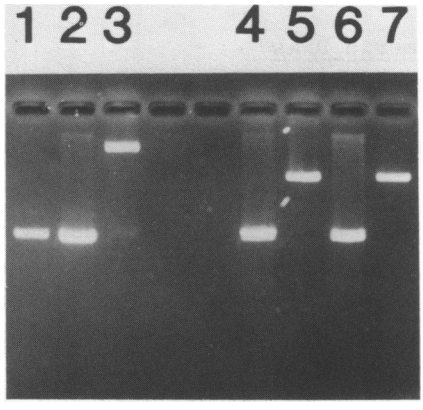

FIGURE 2. Agarose gel electrophoresis of marker dsDNA from CLV (native West Kenyan isolate) (1), progenitor chimeric dsDNAs pJS092 (3), pCLV001 (5) and pCLV014 (7) and progeny dsDNAs from infection of chimeric clones pJS092 (2), pCLV011 (4) and pCLV014 (6). All samples were linearised using SphI prior to electrophoresis.

rather than its undigested scDNA form is used (Table 1A and B). Infected plants showed identical symptoms regardless of the form of the DNA in the inocula.

Characterisation of progeny virus DNA

Virus-specific scDNA was isolated from plants systemically infected using linearised chimeric DNAs 1 (pJS092 (PstI)) or 2 (pCLV010 (BamHI) or pCLV014 (BamHI)) together with the excised full length insert of the appropriate second DNA component. The size of the progeny virus genome was estimated by agarose gel electrophoresis of the scDNA after digestion using SphI; there are single SphI sites in both DNAs 1 and 2 (positions 2581 and 1303 respectively) and no sites in either M13mp8 or pUC8 and consequently both progenitor and progeny scDNA should be linearised under such conditions. In each case, the progeny DNA had reverted to a size similar to that produced during normal virus infection (Figure 2), suggesting that most if not all of the cloning vector moiety had been excised from each genomic component during the process of infection. The extent of the deletions and/or insertions was investigated by cloning full length copies of the DNA component in question at a second

Table 2. Summary of progenitor chimeric constructs and their progeny

Progenitor chimeric DNA				Progeny DNA			
					Characteristics[a]		Size
Clone	DNA component	Site	Vector	Clone	Deletion	Insertion	difference
pJS092	1	MluI (734)	M13mp8/ MluI[b]	pCHI001[c]	658-733	-	-76
				pCHI002	658-733	-	-76
pCLV010	2	PstI (245)	pUC8	pCHI003	(103/170)-329	(103/170)-242 (DNA 1)	-88
				pCHI004[d]	(103/170)-256	(103/170)-319 (DNA 1)	+63
				pCHI005[e]	(103/170)-258	(103/170)-177 (DNA 1)	-81
				pCHI006	207-286	-	-80
pCLV014[f]	2	EcoRV (2550)	pUC8	pCHI008	2457-2552	33 bps (pUC8)[g]	-63
				pCHI009	2457-2552	33 bps (pUC8)	-63
				pCHI010	2457-2552	33 bps (pUC8)	-63
				pCHI011	2457-2552	33 bps (pUC8)	-63

[a] Unless specified the cloning vector was completely excised. Numbering according to Stanley and Gay (1983).
[b] For construction see Stanley (1983).
[c] C to T (399) transition.
[d] G to A (403) transition.
[e] T to A (103) transversion.
[f] DNA 2 insert in pCLV014 has lost its PstI (145) site.
[g] pUC8 sequences located immediately upstream of the polylinker SmaI site.

single-cutting restriction endonuclease site and subcloning and sequencing fragments traversing the original cloning site of the chimeric DNA. The results are summarised in Table 2. Of the three progenitor chimeric DNAs, only the MluI (734) cloning site of pJS092 lies within a putative coding region, namely the 30.2K ORF encoding the coat protein (1,6) (Figure 1). Clones pCHI001 and pCHI002, constructed from pJS092 progeny scDNA, have identical deletions; the whole of the M13 vector has been excised together with 76 nucleotides (658-733) of the CLV genome located immediately upstream of the MluI cloning site which itself remains intact. The deletion serves to change the reading frame within the coat protein gene, resulting in a putative truncated 8.8K coat protein polypeptide (Figure 3). The entire sequence between BamHI (291) - BglII (1410) was determined to ensure that no other alterations had occurred within the coat protein ORF. Only a single C to T transition was detected at nucleotide 399, external to the coat protein ORF.

The PstI (245) cloning site of pCLV010 is located within an apparently intergenic region of DNA 2 (Figure 1), downstream of the common region. Four unique clones (pCHI003-pCHI006), all of which had lost their original PstI cloning site, were constructed from pCLV010 progeny scDNA of which at least three (pCHI003 -pCHI005) were produced by intermolecular recombination *in vivo* between DNAs 1 and 2. In the case of pCHI003, the recombination

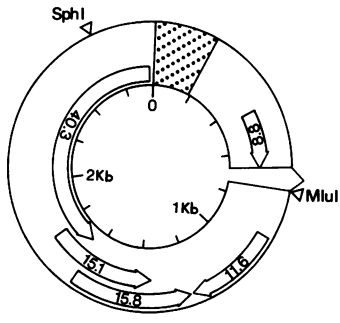

FIGURE 3. Organisation of DNA 1 deletion mutant pCHI001. The position of the 76 nucleotide deletion adjacent to the MluI site is indicated.

event has deleted all of the pUC8 cloning vector together with a fragment of DNA 2 comprising nucleotides within the common region (originating somewhere between nucleotides 103-170) downstream as far as nucleotide 329, replacing it with a fragment of DNA 1 encompassing an identical part of the common region and extending downstream as far as nucleotide 242. Due to the near identical nature of the sequences within the common region of DNAs 1 and 2, only partial delimitation of the 5' termini of the fragments is possible on the basis of nucleotide differences at nucleotides 102 and 171 (1). The 3' termini of both fragments precede the sequence PyGGGCCA (where Py is a pyrimidine). There is a net decrease in the size of DNA 2 by 88 nucleotides. In the case of pCHI004, the pUC8 cloning vector has been excised and the DNA 2 fragment 103/170-256 is replaced by the larger DNA 1 fragment 103/170-319, resulting in a net increase of 63 nucleotides in DNA 2. The sequence CGGTG occurs immediately downstream of the 3' site of recombination in each DNA component. A similar DNA 2 deletion to that of pCHI004 occurs in pCHI005 ((103/107)-258) but is replaced by the smaller DNA 1 fragment (103/170)-177 located entirely within the common region resulting in a net decrease in the size of DNA 2 of 81 nucleotides including sequences located at the extreme 3' terminus of the common region. Again, a homologous sequence GTGGTCCCCGC is present immediately downstream of the 3' site of recombination in both DNA compo-

nents. Finally, in addition to the removal of the pUC8 cloning vector, there is an 80 nucleotide deletion from within the DNA 2 insert of pCHI006 from the region immediately downstream of the common region (207-286).

The EcoRV site of pCLV014 is located upstream of the common region of DNA 2 in an apparently intergenic location (Figure 1). Four identical progeny clones (pCHI008-pCHI011) were generated from this progenitor chimeric DNA. A 96 nucleotide fragment (2457-2552) has been deleted from the DNA 2 insert together with most of the pUC8 cloning vector. A residual 33 nucleotide fragment, originating from immediately upstream of the pUC8 polylinker SmaI site replaces the deleted DNA 2 fragment, resulting in a net loss of 63 nucleotides from the DNA 2 insert. In addition, all four progeny clones have a second deletion of 42 nucleotides (215-257) across the PstI (245) site, an identical deletion to that present in the progenitor clone pCLV014; pCLV014 was constructed from scDNA isolated from N. benthamiana infected using cloned inserts from pJS092 and pJS094, the latter excised using PstI. The deletion across the PstI site presumably occurred during this single passage through the host by exonuclease activity prior to circularisation of the input DNA.

Infectivity of progeny virus DNA

Inserts of the clones constructed from progeny scDNA, described in Table 2, were excised and mechanically inoculated to N. benthamiana in the presence of infectious cloned insert of the appropriate second DNA component. The results summarised in Table 3 show that all progeny clones are infectious.

In each case, symptoms of infection were indistinguishable from those produced using an inoculum containing inserts of the parental clones pJS092 and pJS094. While typical virus particles were detected in leaf extracts from plants systemically infected using pJS092 and pJS094 inserts, ISEM failed to detect particles in infected tissue following inoculation with inserts of the coat protein mutant clone pCHI001 and pJS094.

Analysis of viral DNA forms

Tissue infected using inserts of the parental clones pJS092 and pJS094 contained virus specific DNA forms previously identified (4) as circular single-stranded (css) and supercoiled (sc),

Table 3. Infectivity of progeny DNA

Inoculum		Number of plants inoculated/infected	
		I	II
pJS092 (MluI)	pJS094 (PstI)	5/5	30/30
pCHI001 (SphI)	pJS094 (PstI)	4/5	27/30
pCHI002 (SphI)	pJS094 (PstI)	4/5	-
pJS092 (MluI)	pCHI003 (SalI)	3/5	-
pJS092 (MluI)	pCHI004 (SalI)	3/5	8/30
pJS092 (MluI)	pCHI005 (SalI)	3/4	-
pJS092 (MluI)	pCHI006 (SalI)	4/4	-
pJS092 (MluI)	pCHI008 (SalI)	2/4	-
pJS092 (MluI)	pCHI009 (SalI)	3/4	-
pJS092 (MluI)	pCHI010 (SalI)	4/4	-
pJS092 (MluI)	pCHI011 (SalI)	4/4	-

- = not done

linear (lin) and open circular (oc) double-stranded molecules (Figure 4). Other minor bands reflected the presence of dimeric forms of all these DNA species (4). Tissue from plants infected with inserts of pJS092 and the recombinant clone pCHI004 contained proportionally less scDNA and, presumably as a consequence less linear and dsDNA forms which largely arise from nicking of the scDNA molecules (5). A striking feature of infections caused by the coat protein mutant clone pCHI001 was the marked reduction in the amount of circular ssDNA of both genomic components, although the quantity of scDNA was comparable with that synthesized in parental type infections.

Investigation of the expression of coat protein mutant pCHI001

While 30K molecular weight coat protein antigen was readily detected on Western blots of tissue extracts from plants infected with inserts of parental clones pJS092 and pJS094 (Figure 5), there was no evidence of a similar sized or truncated protein reacting with either of the anti-CLV sera in extracts from plants infected with inserts of clones pCHI001 and pJS094. The minor 15K molecular weight antigen which was detected in tissue infected with the parental clones is thought not to represent an additional structural component of the capsid but to have arisen through degradation of the 30K molecular weight

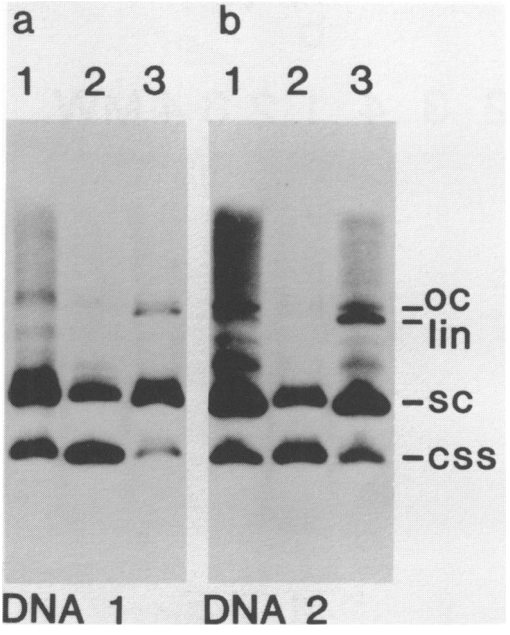

FIGURE 4. Southern blot of total nucleic acids extracted from plants infected using inserts of clones pJS092 and pJS094 (1), pJS092 and pCHI004 (2) and pCHI001 and pJS094 (3). Probes were specific to either DNA 1 (a) or DNA 2 (b).

protein especially since no equivalent product was detected in pCHI001 infected tissue.

In order to determine if the absence of truncated coat protein was a consequence of perturbed transcription of clone pCHI001, the orientation, size and abundance of polyadenylated RNAs isolated from tissue infected with the coat protein mutant were compared with the major polyadenylated transcripts of DNA 1 which we have previously identified (6). Transcription of the complementary (-) sense of pCHI001 progeny was similar to that of DNA 1. Two fragments of clone pJS092 were protected from S_1 nuclease digestion corresponding to transcripts of 1.5Kb and 0.6Kb (Figure 6). The most abundant transcript of DNA 1 is the virion (+) sense RNA which maps between nucleotides 270 and 1240 and encodes the coat protein (6). This RNA protects a single

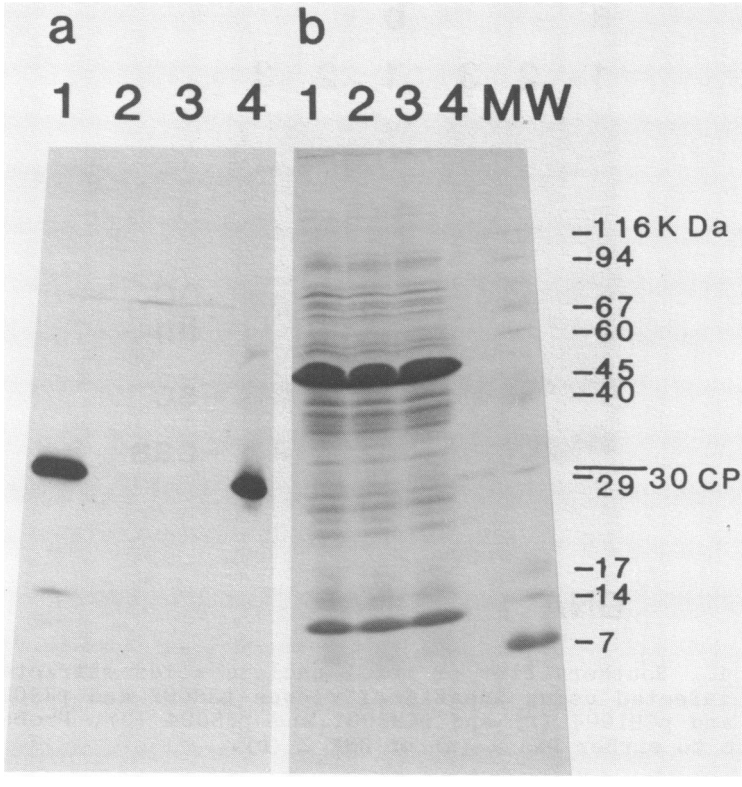

FIGURE 5. PAGE fractionation of total protein extracts from plants either inoculated using inserts of clones pJS092 and pJS094 (1) or pCHI001 and pJS094 (2) or mock inoculated (3). Samples were co-electrophoresed with purified CLV coat protein (4) and molecular weight markers (MW). Gels were either Western blotted and probed with antisera raised against purified CLV (a) or stained with Coomassie brilliant blue (b).

0.96Kb fragment of clone pJS107 which spans the entire coat protein gene. Poly(A)$^+$ RNA transcribed from clone pCHI001 progeny protected two fragments of pJS107 of 0.38Kb and 0.50Kb, reflecting the S_1 nuclease sensitive discontinuity in the heteroduplex caused by the 76 nucleotide deletion in the coat protein mRNA. No reduction in the abundance of the modified transcript relative to normal coat protein mRNA was observed.

DISCUSSION

Intact recombinant DNA clones containing single copies of

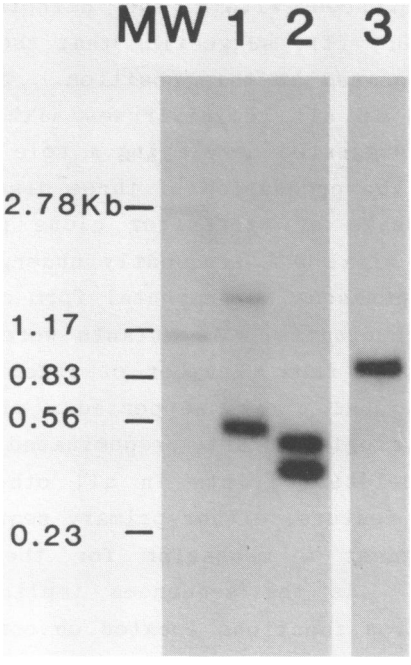

FIGURE 6. Southern blot of S_1 nuclease-resistant fragments of ssDNA clones pJS092 (1) and pJS107 (2,3) protected by virus-specific RNAs isolated from plants infected using inserts of clones pCHI001 and pJS094 (1,2) and pJS092 and pJS094 (3).

either CLV DNAs 1 or 2 can elicit a systemic infection when mechanically inoculated to host plants in the presence of the appropriate second genomic component. Systemic infection is associated with the removal of most, if not all, of the cloning vector to produce infectious progeny DNA components of approximately the same size as those normally associated with CLV infection. Analysis of progeny scDNA reveals that, at least in some instances (clones pCHI003-pCHI005), deletion of the vector sequences had been mediated by intermolecular recombination *in vivo* between the two DNA components. In each case, homologous sequences are implicated in the mechanism of recombination; the upstream crossover point is located within the extensive homologies to be found within the common region while the downstream site precedes short, variable length sequences found in both components. These downstream sequences show significant

homologies to sequences within the hairpin loop structure (nucleotides 133-165) (1), suggesting that the upstream site of recombination is located at this position. This structure has so far been found in all geminiviruses with a dicotyledonous host range and is suggested as playing a role in replication of the genome (22). The production of three distinct recombinants from a single passage of progenitor clone pCLV010 (Table 2) suggests that the viral DNA frequently undergoes recombination although in most instances the parental form of the genome must outcompete the recombinants. As mutants were constructed from scDNA that was pooled from a number of infected plants, it is not known if individual plants supported a mixed population of mutants or if a single mutant predominated. The sequences adjacent to the deletion points in all other progeny clones reveal no unifying feature, either primary sequence or secondary structure, to suggest a mechanism for the delimitation of deleted sequences. As the sequences implicated in encoding viral DNA replication functions located on complementary strand DNA 1 (5) are unperturbed in each progenitor DNA, deletions other than those arising from intermolecular recombination might have occurred during DNA replication. This would imply that the chimeric scDNA is transcriptionally active. The inability to infect plants using intact clones of both genomic components of TGMV (3) may be due to the location of the cloning site of DNA A (equivalent to CLV DNA 1) within the complementary strand 40.3K ORF, serving to disrupt the replicative function.

Analysis of the infectious clones constructed from scDNA derived from progenitor chimeric DNAs pCLV010 and pCLV014 indicates that significant stretches of the intergenic region of DNA 2 (nucleotides 178-329 and 2457-2552 respectively) can be deleted without adversely affecting the potential for mechanical transmission of the cloned viral DNA, systemic spread of the virus or symptom production. The contribution of these sequences to virus transmission by the natural insect vector Bemisia tabaci has not yet been investigated. The deletion in progeny clone pCHI005 encroaches on the extreme 3' terminus of the common region although the adjacent hairpin loop structure (nucleotides 133-165) (1) remains intact. The lack of homology

between the deleted DNA 2 sequences and those that replace them, either from DNA 1 or cloning vector pUC8, suggests that these parts of the intergenic region of DNA 2 play a passive role in virus multiplication, possibly serving to maintain a DNA component size suitable for encapsidation. A progeny size variation of only 2-3% with respect to the progenitor DNA components, resulting from the deletions and rearrangements summarised in Table 2, suggests that a positive size selection mechanism exists for encapsidation of the DNA within the geminate particles. However, while encapsidation is probably essential for virus transmission in the field by B. tabaci, characterisation of progeny derived from chimeric clone pJS092 indicates that encapsidation is not a prerequisite for systemic spread of the viral DNA within the host plant. The progeny clones in question (pCHI001 and pCHI002) have undergone a deletion within the coat protein gene to produce a putative truncated form of the coat protein of 8.8K molecular weight shown in Figure 3. Both clone inserts are infectious when mechanically inoculated to host plants in the presence of pJS094 insert and systemic spread of the viral DNA elicits symptoms typical of CLV infection. As might be anticipated, neither geminate particles nor intact coat protein were found in systemically infected material although they were readily detected in control plants inoculated with a mixture of pJS092 and pJS094 inserts.

More surprising was the failure to detect the predicted 8.8K molecular weight truncated coat protein by Western blotting using either of the two independently prepared polyvalent antisera. It is arguable that particles or modified proteins might escape detection by the methods used since these rely on the conservation of epitopes characteristic of the assembled capsid. However, the predicted amino-terminal product contains at least one hydrophilic region (23) which might be expected to represent an epitope (24) especially as terminal residues of proteins frequently possess antigenic activity (25). Absence of the 8.8K molecular weight product could not be attributed to perturbed transcription of the coat protein gene since S_1 nuclease mapping indicated that transcription of pCHI001 progeny was similar to that previously observed for DNA 1 (6) except where it was

consistent with the predicted deletion in the coat protein mRNA. Since the truncated version of the coat protein may be turned over rapidly in plants, we have attempted to detect the product in vitro using synthesising systems programmed with pCHI001-specific RNA. So far the results of these studies have been inconclusive. The coat protein mRNA is translated with low efficiency in both rabbit reticulocyte lysate (6) and wheat germ extracts (unpublished). Furthermore, the resolution of proteins of molecular weight <12K synthesised in reticulocyte lysates is dramatically reduced by the presence of endogenous globin necessitating alternative fractionation procedures such as immunoprecipitation. While low molecular weight products synthesised in wheat germ extracts can be resolved more easily, translation of the native coat protein mRNA gives rise to several low molecular weight polypeptides presumably by premature termination of translation (unpublished).

The inability of clone pCHI001 progeny to synthesise intact coat protein is associated with a marked reduction in the quantity of circular ssDNA present in infected tissues. While it is possible that the unencapsidated circular ssDNA is more susceptible to nuclease degradation, this finding is also consistent with our previous suggestion (5) that, by analogy with bacteriophage ØX174, the accumulation of progeny viral ssDNA depends on the availability of coat protein to encapsidate the DNA making it unavailable as a template for further - strand synthesis leading to RF formation.

While the possible contribution of the truncated coat protein to virus multiplication remains unknown, systemic spread of non-encapsidated viral nucleic acid is not without precedent; mutants of tobacco mosaic virus defective in coat protein synthesis (26) and an uncoated RNA component of members of the tobravirus group (27) retain the capacity for systemic invasion of host plants. Furthermore, an Angolan isolate of CLV, defective for particle production, has recently been described (28), although in this case the possibility that particles are produced which are unstable under the conditions of isolation has not been ruled out. The fact that all mutants described in Table 2 fall within a reasonably well defined size range suggests that

while encapsidation might not be essential for systemic spread, mutants of a size suitable for encapsidation are conferred with a selective advantage.

Mutation of the coat protein of cauliflower mosaic virus (CaMV), type member of the only other known group of DNA-containing plant viruses, generally destroys infectivity of the viral DNA (29,30). The requirement for packaging of CaMV DNA within virus particles, which serves to limit the size of the DNA that can spread systemically though the plant, impairs the use of the caulimoviruses as vectors with which to introduce foreign DNA into host plants. It remains to be seen if the whole of the coat protein of CLV can be deleted without adversely affecting systemic spread of the viral DNA and, if so, whether or not a replacement gene will be stably maintained during virus replication. Should this be the case, as DNA 1 encodes all of the functions necessary for self-replication (5) and expression of the CLV coat protein appears to be under the control of the major viral promoter (6), geminiviruses will make a significant contribution to the development of an autonomously replicating vector with which to study transient gene expression in plants.

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REFERENCES

1. Stanley,J. and Gay,M.R. (1983). Nature (London) **301**, 260-262.
2. Stanley, J. (1983). Nature (London) **305**, 643-645.
3. Hamilton,W.D.O., Bisaro,D.M., Coutts,R.H.A. and Buck,K.W. (1983). Nucleic Acids Res. **11**, 7387-7396.
4. Stanley,J. and Townsend,R. (1985). Nucleic Acids Res. **13**, 2189-2206.
5. Townsend,R., Watts,J. and Stanley,J. (1986). Nucleic Acids Res. **14**, 1253-1265.
6. Townsend,R., Stanley,J., Curson,S.J. and Short,M.N. (1985). EMBO J. **4**, 33-37.

7. Sanger,F., Nicklen,S. and Coulson,A.R. (1977). Proc. Natl. Acad. Sci. USA **74**, 5463-5467.
8. Sanger, F., Coulson, A.R., Barrell, B.G., Smith,A.J.H. and Roe, B.A. (1980). J. Molec. Biol. **143**, 161-178.
9. Staden,R. (1980). Nucleic Acids Res. **8**, 3673-3694.
10. Roberts, I.M., Robinson, D.J. and Harrison, B.D. (1984). J. Gen Virol. **65**, 1723-1730.
11. Stanley, J., Townsend, R. and Curson, S.J. (1985). J. Gen. Virol. **66**, 1055-1061.
12. Towbin,H., Staehelin,T. and Gordon,J. (1979). Proc. Natl. Acad. Sci. USA **76**, 4350-4354.
13. Burnette, W.N. (1981). Ann. Biochem. **112**, 195-203.
14. Laemmli, U.K. (1970). Nature **227**, 680-685.
15. Sequeira, J.C. and Harrison, B.D. (1982). Ann. Appl. Biol. **101**, 33-42.
16. Otsuki, Y. and Takebe, I. (1969). Virology **38**, 497-499.
17. Blake,M.S., Johnson,K.H., Russell-Jones,G.J. and Gotschlich E.C. (1984). Ann. Biochem. **136**, 175-179.
18. Berk, A.J. and Sharp, P.A. (1977). Cell **12**, 721-732.
19. Favaloro, J.M., Treisman, R.H. and Kamen, R.I. (1980). Methods Enzymol. **65**, 718-749.
20. Feinberg, A.P. and Vogelstein, B. (1983). Ann. Biochem. **132**, 6-13.
21. Feinberg, A.P. and Vogelstein, B. (1984). Ann. Biochem. **137**, 266-267.
22. Stanley, J. (1985). Adv. Virus Res. **30**, 139-177.
23. Hamilton,W.D.O., Stein,V.E., Coutts, R.H.A. and Buck, K.W. (1984). EMBO J. **3**, 2197-2205.
24. Hopp, T.P. and Woods, K.R. (1981). Proc. Natl. Acad. Sci. USA **78**, 3824-3828.
25. Westhof,E., Altschuh,D., Moras,D., Bloomer,A.C., Mandragon, A., Klug,A. and van Regenmortel,M.H.V. (1984). Nature **311**, 123-126.
26. Siegel,A. Zaitlin,M. and Sehgal, O.P. (1962). Proc. Natl. Acad. Sci. USA **48**, 1845-1851.
27. Sanger, H.L. (1969). J. Virol. **3**, 304-312.
28. Robinson, D.J., Harrison, B.D., Sequeira, J.C. and Duncan, G.H. (1985). Ann. Appl. Biol. **105**, 483-493.
29. Dixon, L.K., Koenig, I. and Hohn, T. (1983). Gene **25**, 189-199.
30. Daubert, S., Shepherd, R.J. and Gardner, R.C. (1983). Gene **25**, 201-208.