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Replication of *in vitro* constructed viroid mutants: location of the pathogenicity-modulating domain of citrus exocortis viroid

Jane E.Visvader[1] and Robert H.Symons

Adelaide University Centre for Gene Technology, Department of Biochemistry, University of Adelaide, Adelaide, SA 5000, Australia

[1]Present address: Molecular Biology and Virology Laboratory, The Salk Institute, PO Box 85800, San Diego, CA 92138, USA

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Sequence variants from field isolates of citrus exocortis viroid (CEV) that cause either mild or severe symptoms on tomato plants have previously been classified into two groups, A and B. These groups differ primarily in two domains, P_L and P_R, of the proposed native structure. Infectivity studies with full-length cDNA clones of variants from each class have now directly confirmed the original correlation between Class A sequences and the severe phenotype and between Class B sequences and the mild phenotype. Direct evidence for this correlation could only be obtained by using individual sequence variants since field isolates of CEV have been shown to contain a mixture of RNA species. The construction and infectivity of chimaeric cDNA clones derived from mild and severe sequence variants of CEV has demonstrated that novel, infectious viroid molecules can be generated *in vitro*, and that P_L is the pathogenicity-modulating domain. The role of the P_R domain is not known but infectivity experiments with one chimaeric cDNA clone suggest that it may influence the efficiency of the infection or replication process of the viroid in the plant.

Key words: citrus exocortis viroid/chimaeric cDNA clones/infectivity studies

Introduction

Viroids, the smallest known pathogens of higher plants, are distinguished from conventional viruses by their unusual structure and their apparent lack of coding capacity. They consist of single-stranded, circular RNA molecules of 246-375 nucleotides and exist as highly base-paired, rod-like structures (Diener, 1983; Sänger, 1984; Riesner and Gross, 1985). Their ability to replicate and exert pathogenic effects, without encoding proteins, indicates that viroid functions are mediated through sequence and structural signals alone. One approach to investigating the relationship between viroid structure and function has been the sequence determination of naturally occurring viroid isolates which differ in pathogenicity (Gross *et al.*, 1981; Schnölzer *et al.*, 1985; Visvader and Symons, 1985). An alternative approach has been to construct viroid cDNA mutants for infectivity studies (Visvader *et al.*, 1985; Owens *et al.*, 1986).

The sequence analysis of five different field isolates of citrus exocortis viroid (CEV) by construction of full-length cDNA clones has identified 15 sequence variants in these isolates which can be classified into two groups of sequence, Class A and Class B (Figure 1; Visvader and Symons, 1985), which differ primarily in two structurally distinct domains, P_L and P_R, of the proposed native structure of CEV. Each field isolate produced either mild or severe disease symptoms when inoculated onto tomato seedlings. On the basis of these infectivity results, it was possible to correlate Class A sequences with severe symptoms and Class B sequences with mild symptoms (Visvader and Symons, 1985). It was concluded that nucleotide differences in either one or both of these domains are responsible for the variation in pathogenicity.

Here we report the construction of chimaeric cDNA clones derived from mild and severe variants of CEV. Infectivity studies with these clones have shown that the P_L domain is the pathogenicity-modulating domain.

Results

Biological properties of clones of Class A and Class B sequence variants of CEV

The presence of a mixture of sequence variants in three field isolates of CEV (CEV-A, CEV-J, CEV-DE30; Visvader and Symons, 1983, 1985) has important implications for the correlation of nucleotide differences in sequence variants with variation in pathogenicity. Direct evidence for this type of correlation can only be obtained by using single species for infectivity studies. The finding that certain full-length monomeric cDNA clones of CEV are infectious (Visvader *et al.*, 1985) has allowed individual variants from Class A and Class B to be directly tested for symptom expression on tomato plants.

Sequence analysis of full-length clones of isolate CEV-A in the vectors pBR322 (Visvader and Symons, 1983) and M13mp93 showed the presence of minor sequence variants, including two designated CEV-A(1) and CEV-A(2). CEV-A(1), referred to as CEV-AM in Visvader and Symons (1983), is 371 nucleotides long and differs from the reference sequence CEV-A (Visvader *et al.*, 1982) by three nucleotide changes, at positions 234 (U→A), and 263-264 (AG→CU). Variant CEV-A(2) has not been described previously and differs from CEV-A by the deletion of a G nucleotide at position 70 and is therefore 370 nucleotides in size. Full-length *Bam*HI cDNA inserts of these variants were recloned into the plasmid vector pSP6-4 (Melton *et al.*, 1984). A pSP6-4 clone containing a viroid insert in the plus orientation is defined as one which produces RNA transcripts with the same polarity as the viroid sequence. A mixture of plasmid DNA and plus pSP6-4 RNA transcripts was used for infectivity studies as this was found to be more infectious than DNA alone (Visvader *et al.*, 1985).

The infectious nature of the *Bam*HI plus CEV-A(2) clone and its pSP6-4 RNA transcripts was reported in Visvader *et al.* (1985) where it was referred to as CEV-A. This clone produced severe symptoms on tomato plants, characterized by severe stunting and epinasty, and similar results were obtained here as well as with the *Bam*HI plus clone of another variant, CEV-A(1) (Table I). Both variants are Class A sequences and thus directly confirm the correlation between Class A sequence and the severe phenotype.

To test the correlation between Class B sequence and the mild

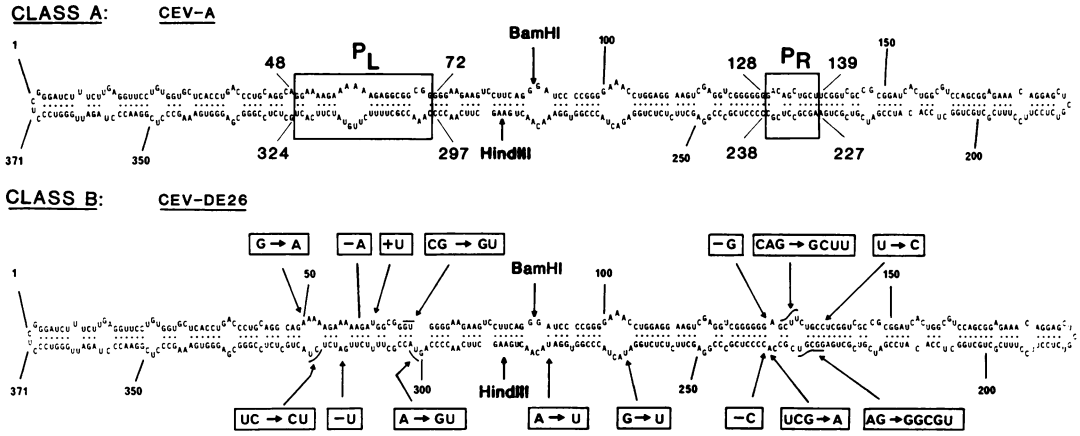

Fig. 1. The primary and proposed secondary structures of the Class A and Class B reference sequences, CEV-A and CEV-DE26 (Visvader and Symons, 1985). The nucleotide changes in CEV-A necessary to give the sequence of CEV-DE26 are boxed in CEV-DE26. The P_L and P_R domains of CEV (Visvader and Symons, 1985) are indicated for CEV-A, together with the nucleotide positions on the outside of the boundaries of the two domains. The *Bam*HI site used for the construction of full-length cDNA clones and the *Hin*dIII site used in the construction of the chimaeric clones are indicated.

Table I. Infectivity of cDNA clones plus RNA transcripts

Full-length cloned insert (nucleotide positions)[a]	Inoculum[b] Amount (μg) of DNA and RNA (approx.) inoculated per tomato seedling		Infectivity and symptom expression[c] Experiment number				
	DNA	RNA	1	2	3	4	5
Wild-type CEV clones							
CEV-A(2) (89−88)	1	0.5	4/4S	5/5S			
CEV-A(1) (90−89)	1	0.5	5/5S				
CEV-DE30(a) (90−89)	6	6	5/5M	6/6M			
Chimaeric cDNA clones							
CEV-DE30 P_L/CEV-A P_R (90−89) (M_L/S_R)	2	1	4/4M	4/4M	4/4M	5/5M	
CEV-A P_L/CEV-DE30 P_R (89−88) (S_L/M_R)	2	1	2/20S 18/20M	4/20S 16/20M	4/20S 16/20M	4/20S 16/20M	
	6.5	9					11/20S 9/20M
CSV left/CEV-A right	1	1	0/5				

[a]Refer to Figure 3 for cloning sites. All cloned inserts were in the plus orientation.
[b]The amount of RNA in each inoculum was estimated by electrophoresing an aliquot of each transcription reaction, together with a known quantity of CEV RNA, on a polyacrylamide gel and staining with toluidine blue.
[c]Infectivity is shown as the number of tomato plants with mild (M) or severe (S) symptoms over the number of inoculated plants. Nucleic acid extracts were prepared from pooled leaves from the plants of each experiment and the infectivity results confirmed by Northern hybridization analysis. The size of the progeny viroid was always indistinguishable from that of CEV-A. Mock-inoculated control plants from all experiments (not shown) were symptomless and uninfected by Northern hybridization analysis.

phenotype, a full-length *Bam*HI plus clone of CEV-DE30 (variant a) was constructed in pSP6-4. Tomato seedlings inoculated with a mixture of plasmid DNA and *in vitro* synthesized RNA transcripts were essentially symptomless (Table I).To determine whether these very mild symptoms could be correlated with changes in sequence rather than variation of the viroid level in the infected plants, equal amounts of partially purified nucleic acid extracts were prepared from plants (Table I) infected with cDNA clones of Class A and Class B sequences, CEV-A(2) and CEV-DE30(a), respectively. Northern blot hybridization analysis (Figure 2) showed that the level of CEV in plants with severe symptoms (track 1) was less than that in plants with mild symptoms (track 3). Thus the mild symptoms could be attributed to the CEV variant with a Class B sequence and not to a low level of infection.

Construction and infectivity of chimaeric cDNA clones between mild and severe variants of CEV

Full-length chimaeric CEV cDNA clones with the two possible arrangements of the P_L and P_R domains derived from mild and severe variants were constructed (Figure 3A and B) to investigate the relative effects of the two domains on viroid pathogenicity. One chimaeric clone in the vector pSP6-4, designated M_L/S_R, contained the left-hand region of a mild variant, CEV-DE30(a), adjoined to the right-hand region of a severe variant, CEV-A(2), through the highly conserved central region (Haseloff *et al.*, 1982). The reverse chimaera, S_L/M_R, was constructed using the same variants. The construction of these chimaeric clones involved the ligation of an equimolar mixture of two *Bam*HI−*Hin*dIII fragments with *Bam*HI-cut pSP6-4, so that the fragments were joined at the *Hin*dIII site in the central region (Figures 1, 3A

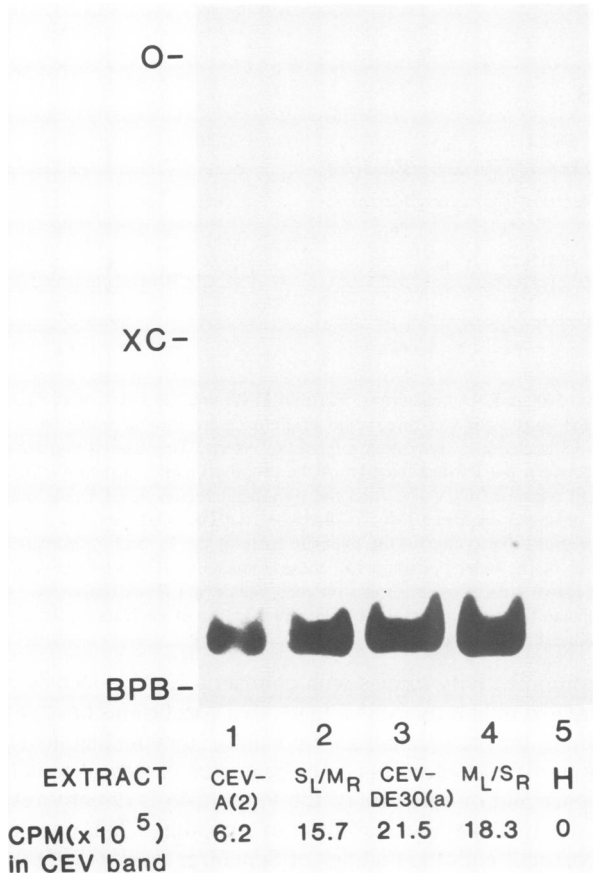

Fig. 2. Comparison of the level of CEV in tomato plants infected with cDNA clones plus RNA transcripts of two wild-type sequence variants [CEV-A(2) and CEV-DE30(a)] and of two chimaeric CEV constructs (M_L/S_R and S_L/M_R), by Northern hybridization analysis. A mixture of plasmid DNA and RNA transcripts was used for each infectivity experiment. Nucleic acid extracts were prepared from ~15 g of plant tissue, 5 weeks after inoculation, pooled from individual experiments of Table I. Glyoxalated nucleic acid extracts were electrophoresed in 1.9% agarose gels, transferred to Genescreen (New England Nuclear) and probed with [^{32}P]RNA transcripts containing full-length minus CEV sequence. The Genescreen was then autoradiographed for 15 min and each of the CEV bands located was excised and counted in Triton–toluene scintillation fluid. Leaf extracts used were: **track 1**, CEV-A(2); **track 2**, CEV S_L/M_R (severe symptoms); **track 3**, CEV-DE30(a); **track 4**, CEV M_L/S_R; **track 5**, healthy (H). The positions of the origin (O) and the marker dyes, xylene cyanol FF (XC) and bromophenol blue (BPB), are indicated.

and B). The M_L/S_R clone contained an insert of 371 bp, with the 174-bp *Bam*HI–*Hin*dIII fragment from CEV-DE30(a) linked to the 197-bp *Bam*HI–*Hin*dIII fragment from CEV-A(2). The S_L/M_R clone contained an insert of 370 bp with the 173-bp *Bam*HI–*Hin*dIII fragment of CEV-A(2) joined to the 197-bp *Bam*HI–*Hin*dIII fragment of CEV-DE30(a).

A mixture of plasmid DNA and RNA transcripts derived from each of these *Bam*HI plus chimaeric CEV clones was infectious on tomato plants (Table I). The M_L/S_R clone and RNA transcripts elicited mild symptoms in all plants in four separate experiments (Table I). Inoculation of tomato seedings with a nucleic acid extract prepared from plants of one of these experiments (Expt. 3, Table I) also produced only mild symptoms. However, the S_L/M_R clone and RNA transcripts elicited both mild and severe symptoms (Table I), the ratio of mild to severe being dependent on the amount of inoculum used per tomato seedling. (This latter aspect is considered further below.) Northern hybridization analysis (Figure 2) of nucleic acid extracts prepared from pooled plants of a single experiment (only plants with severe symptoms were used for S_L/M_R) showed that the two chimaeric clones (tracks 2 and 4) gave levels of viroid a little lower than that of the clone of the mild variant CEV-DE30(a) (track 3) but higher than that of the severe variant CEV-A(2) (track 1). These infectivity results show that the P_L domain has a direct role in determining whether the symptoms are mild or severe while the P_R domain does not. Hence, P_L has been identified as the pathogenicity-modulating domain.

It was most important to determine if any sequence changes had occurred during replication of the CEV variants derived from the chimaeric clones. Hence full-length cDNA clones in the vector M13mp93 were prepared (Visvader and Symons, 1985) from the progeny viroid purified from pooled plants infected with the M_L/S_R clone and the S_L/M_R clone (Expt. 1, Table I). The complete sequences of the inserts were determined (Sanger *et al.*, 1980) and found to be identical to those of the chimaeric cDNAs.

The symptoms observed after inoculation of tomato seedlings with the chimaeric clone, S_L/M_R, were found to vary with the concentration of nucleic acid used in the inoculum (Table I). When 2 μg of plasmid DNA and ~1 μg of RNA transcripts were inoculated on to each tomato seedling, only two to four plants, out of a total of 20 in each of four experiments, showed the severe symptoms of leaf epinasty and stunting. However, when the concentrations of plasmid DNA and RNA transcripts were increased by 3-fold and 9-fold, respectively, 11 of the 20 plants showed severe symptoms (Expt. 5, Table I). In a further trial, five tomato seedlings were inoculated with a nucleic acid extract prepared from pooled plants of Experiment 4, Table I, and all exhibited severe symptoms, presumably because of the high level of viroid in the inoculum. To investigate the basis for the difference in symptom expression among plants inoculated with the reverse chimaera (S_L/M_R), nucleic acid extracts were prepared from each of three plants showing mild symptoms and of three plants with severe symptoms (Expt. 5, Table I) and the levels of CEV compared by Northern hybridization analysis (Figure 4). Extracts from plants with severe symptoms gave viroid levels 9–20 times greater than those in extracts of plants wth mild symptoms. Hence, at least in the case of infection with the chimaeric variants, S_L/M_R, symptom expression was dependent on the level of viroid which accumulated in the plant.

This variation of symptom expression with viroid concentration is surprising in view of the results of Visvader and Symons (1985) which indicated that differences in symptom expression between naturally occurring mild and severe CEV isolates (each containing a mixture of sequence variants) were independent of the viroid level in infected plants. The data demonstrate that infectivity trials with *in vitro* constructed mutants should be interpreted with caution and they emphasize the importance of multiple experiments using inocula of different concentrations.

Discussion

Despite their remarkably small size, viroids contain different regions which appear to regulate different functions. These regions include three adjacent nucleotides in the highly conserved central region where processing of CEV precursors is predicted to occur (Visvader *et al.*, 1985) as well as a pathogenicity-modulating domain described here for CEV and a similar domain for potato spindle tuber viroid (PSTV) considered by H.L.Sänger and his colleagues (Sänger, 1984; Schnölzer *et al.*, 1985).

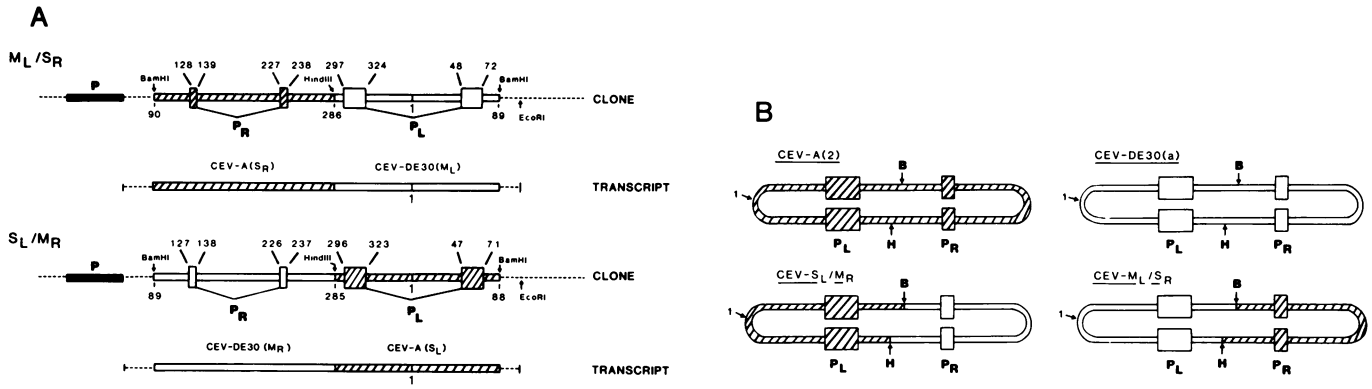

Fig. 3. A. Schematic diagram of chimaeric viroid cDNA clones in the plus orientation and their RNA transcripts. Plasmid DNA was restricted with *Eco*RI prior to transcription by SP6 RNA polymerase. The *Bam*HI and *Hin*dIII sites used for the construction of three chimaeric clones are shown. Initiation of transcription was from 34 nucleotides upstream from the inserts, while termination was 18 nucleotides downstream from the inserts. The *Bam*HI and *Hin*dIII sites used for the construction of three chimaeric clones are shown. $\mathbf{M_L/S_R}$, chimaera between the left-hand region of the proposed native structure of CEV-DE30(a) and the right-hand region of CEV-A(2). $\mathbf{S_L/M_R}$, chimaera between the left-hand region of CEV-A(2) and the right-hand region of CEV-DE30(a). In each RNA transcript, the pSP6-4 plasmid sequence is represented as a broken line, the promoter sequence P by a filled box, the CEV-A(2) sequence by a hatched box and the CEV-DE30(a) sequence by an open box. The two single-stranded regions that comprise the opposite parts of the P_L and P_R domains of either mild or severe variants are boxed and joined by a bracket. The nucleotide numbers of the outer boundaries of these domains are indicated on each vector and nucleotide position 1 of CEV, which is at the left-hand end of the proposed native structures, is shown on each vector and RNA transcript.
B. Schematic diagram of two parental and two chimaeric viroids in circular form to indicate the relative positions of the two halves of each molecule on either side of the *Bam*HI (B) and *Hin*dIII (H) sites and of the P_L and P_R domains. Residue 1 is indicated.

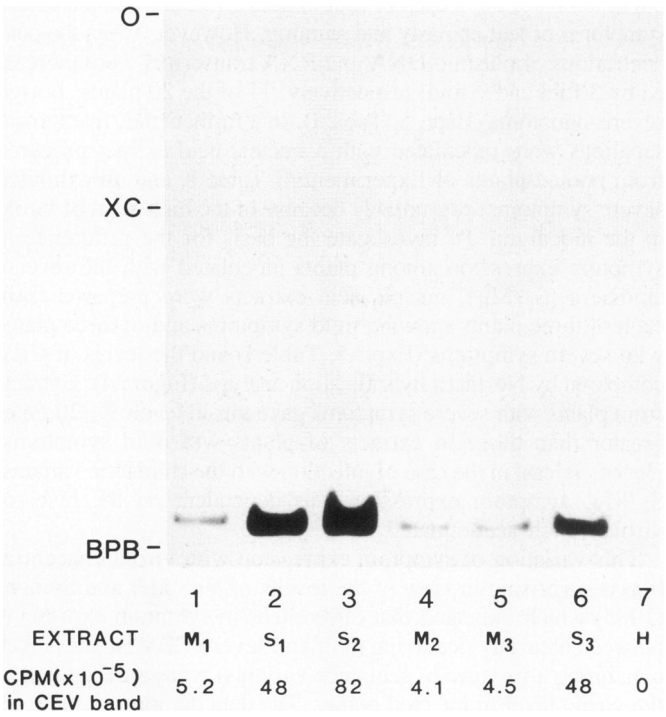

Fig. 4. Comparison of the level of CEV in tomato plants of Experiment 5, Table I, showing mild or severe symptoms by Northern hybridization analysis. Twenty tomato seedlings were inoculated with a mixture of plasmid DNA and RNA transcripts of the chimaeric clone, S_L/M_R. Nucleic acids were extracted 5 weeks after inoculation from 3 g of leaf tissue derived from individual plants, three showing mild symptoms and three severe. Glyoxalated nucleic acid extracts were electrophoresed on a 1.9% agarose gel, transferred to Genescreen (New England Nuclear) and probed with [^{32}P]RNA transcripts derived from a *Bam*HI full-length cDNA clone of CEV-A(2) in the minus orientation. The gel was autoradiographed and each of the bands excised and counted in Triton–toluene scintillation fluid. **Tracks 1, 4** and **5**, extracts from three plants showing mild symptoms (M1, M2, M3); **tracks 2, 3** and **6**, extracts from three plants showing severe symptoms (S1, S2, S3); **track 7**, healthy (H). The positions of the gel origin (O) and marker dyes, xylene cyanol FF (XC) and bromophenol blue (BPB), are indicated.

From infectivity studies wtih chimaeric CEV clones between severe and mild sequence variants, two conclusions have emerged. First, the recombinants were viable, showing that novel, infectious viroid molecules can be generated *in vitro*. Second, the sequence and/or structure of the P_L domain is directly responsible for modulating the severity of symptoms. This conclusion is consistent with the findings of Schnölzer *et al.* (1985) where the sequence analysis of naturally occurring isolates of PSTV showed that sequence changes in the corresponding P_L region of PSTV correlated with variation in symptom expression. It remains to be determined how the P_L domain modulates pathogenicity.

It may be of functional significance that the two classes of nucleotide differences (A and B) in the sequence variants of CEV have evolved so that one class of P_L domain is always linked to the same class of P_R domain. However, as demonstrated here with *in vitro* constructed mutants, constructs containing a P_L domain from one class joined to a P_R domain from another class are infectious. It is feasible that, although these hybrid constructs are infectious, they may have a replication disadvantage compared with the homologous species and have, therefore, not been detected *in vivo* (Visvader and Symons, 1985).

The P_R domain falls within the most variable domain between all PSTV-like viroids as defined by Keese and Symons (1985) and will subsequently be referred to as the V domain. The function, if any, of this V domain remains to be determined, although it has been implicated as a site for RNA rearrangements between viroids (Keese and Symons, 1985). Here infectivity experiments (Figure 4) with the chimaeric CEV S_L/M_R clone indicate that the efficiency of the initial infection or replication process has been affected, accounting for the observed variation in the level of progeny viroid found between different plants. It is possible that the reduced infectivity of the S_L/M_R chimaeric clone is due to the disruption of long-range interactions that may occur between distant parts of the viroid molecule (Riesner *et al.*, 1979, 1983; Riesner and Gross, 1985).

Infectivity studies with site-specific mutants of viroids have suggested that there is considerable sequence and structural specificity governing the viability of viroids. Point mutants of

CEV at positions 96 (G→U) of CEV-A(2) (Visvader *et al.*, 1985) and 351 (G→U) of CEV-A and a four base deletion mutant (−AGCU, nucleotides 178−181) at the right-hand end loop of CEV-A(2) (unpublished data) as well as several site-specific mutants of HSV (Ishikawa *et al.*, 1985) and PSTV (Owens *et al.*, 1986) have all proved to be non-infectious. In contrast, chimaeric molecules between the two different classes of CEV sequence were infectious, suggesting that the approach involving the interchange of structural domains between different isolates and different viroid species may allow a more useful analysis of viroid function. However, our first attempt to construct an infectious interspecies chimaeric viroid in which the 164-bp *Bam*HI−*Hin*dIII fragment from the left half of chrysanthemum stunt viroid, which shares ~60% sequence homology with CEV (Visvader *et al.*, 1982; Haseloff and Symons, 1981; Gross *et al.*, 1982), was joined to the 197-bp *Bam*HI−*Hin*dIII fragment from the right-half of CEV-A(2) was not successful (unpublished). Likewise, Owens *et al.* (1986) were not successful in obtaining infectious cDNA clones prepared in a similar way using PSTV and the related tomato apical stunt viroid (Kiefer *et al.*, 1983). Taken together, the data indicate that there are very rigid sequence and structural requirements, most of which have probably still to be defined, which are necessary for the successful construction *in vitro* of infectious viroid mutants.

Materials and methods

Construction of wild-type CEV cDNA clones

The field isolates of CEV, CEV-A and CEV-DE30 have been described in Visvader *et al.* (1981) and Visvader and Symons (1985). The construction of full-length cDNA copies of CEV-A(2) using the unique *Bam*HI site (Figure 1) in the vectors pBR322, M13mp93 and pSP6-4, and of CEV-A(1) in pBR322 has been described (Visvader and Symons, 1983; Visvader *et al.*, 1985). The CEV-A(1) insert was recloned into pSP6-4. CEV-A(1) and CEV-A(2) have previously been referred to as CEV-AM (Visvader and Symons, 1983) and CEV-A (Visvader *et al.*, 1985), respectively.

Monomeric clones containing *Bam*HI inserts of isolate CEV-DE30 in pSP6-4 were constructed using the procedure outlined in Visvader and Symons (1983). Briefly, double-stranded cDNA was synthesized (Gould and Symons, 1982) from polyadenylated linear viroid using oligo(dT)$_{10}$ as the primer for first strand synthesis by reverse transcriptase (Molecular Genetic Resources, Florida) and self-priming for second strand synthesis, also by reverse transcriptase. The starting linear viroid was purified from infected plants (Visvader *et al.*, 1982) and each molecule was assumed to contain a single nick which could occur at a number of sites in the molecule. The double-stranded cDNA was restricted with *Bam*HI and *Hin*dIII and two fragments of 174 bp and 197 bp were purified by polyacrylamide gel electrophoresis. An equimolar mixture of the two fragments was ligated with *Bam*HI-cleaved pSP6-4 and then used to transform *Escherichia coli* MC1061.

Construction of chimaeric CEV cDNA clones

*Bam*HI−*Hin*dIII fragments of CEV were purified by gel electrophoresis after cleavage of cDNA clones containing full-length inserts of CEV-A(2) or CEV-DE30(a) with *Bam*HI plus *Hin*dIII. An equimolar mixture of two *Bam*HI−*Hin*dIII fragments derived from different variants was then ligated with *Bam*HI-cleaved pSP6-4 to generate chimaeric clones between mild and severe variants.

Screening and sequencing of in vitro constructed clones

Single-stranded phage DNA of all clones in M13mp93 was sequenced by the dideoxynucleotide chain termination technique (Sanger *et al.*, 1980) using the M13 sequencing primer (17-mer). Potential chimaeric clones in pSP6-4 were restricted with *Bam*HI plus *Hin*dIII to identify the structure of the insert. The orientation of chiameric cDNA clones in pPS6-4 was determined by restricting the DNA with *Ava*I and comparing the sizes of the resulting fragments on a non-denaturing polyacrylamide gel relative to those predicted for each orientation. In addition, some pSP6-4 clones were partially sequenced by the dideoxy method using a pSP6-specific primer (17-mer; BRESA, Adelaide).

Infectivity experiments

RNA transcripts were synthesized with SP6 RNA polymerase (BRESA, Adelaide) after cleavage at the *Eco*RI site downstream from the inserts. All transcription reactions were monitored by electrophoresis of aliquots of each reaction mixture on denaturing polyacrylamide gels. Each inoculum consisted of a mixture of recombinant plasmid DNA and *in vitro* synthesized RNA transcripts in 0.1 M potassium phosphate, pH 6.8, containing 0.1 vol of 37 mg/ml bentonite suspension. One-week-old tomato seedlings (*Lycopersicon esculentum* cv. Grosse Lisse) at the cotyledon stage were dusted with carborundum and then inoculated by rubbing the suspension on the cotyledons and any primary leaves. All plants were grown in a controlled temperature growth room with 14 h at 28°C under artificial light and for 10 h at 22°C in the dark. The tomato plants were monitored for stunting and leaf epinasty, symptoms characteristic of CEV infection. Symptoms were designated as severe where there was pronounced epinasty and stunting, and as mild when there were essentially no symptoms. Partially purified nucleic acid extracts were prepared (Hutchins *et al.*, 1985) from 3−15 g of leaves and analysed by the Northern blot hybridization technique using agarose gels (Hutchins *et al.*, 1985) to confirm the presence or absence of progeny viroid.

Purification and sequencing of viroids

Progeny viroid was purified from nucleic acid extracts (Palukaitis and Symons, 1980) of tomato leaves harvested 5−6 weeks after inoculation. Full-length double-stranded cDNA was synthesized from the purified viroid by the sequential use of two oligonucleotide primers (Visvader and Symons, 1985). After cloning of the cDNA into the *Sma*I site of M13mp93, the inserts were completely sequenced by the dideoxynucleotide chain termination technique of Sanger *et al.* (1980).

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