STRUCTURES OF VIROIDS AND VIRUSOIDS

AND THEIR FUNCTIONAL SIGNIFICANCE

A thesis submitted to the University of Adelaide for the degree of Doctor of Philosophy

by

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"Be admonished, of making many books there is no end; and much study is a weariness of the flesh."

Ecclesiastes 12:12

STATEMENT

This thesis contains no material which has been previously submitted for an academic record at this or any other University and is the original work of the author, except where due reference is made in the text. I consent to this thesis being made available for photocopying and loan.

PAUL K. KEESE

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ABBREVIATIONS

ASBV	avocado sunblotch viroid
CCCV	coconut cadang-cadang viroid
cDNA	complementary DNA
CEV	citrus exocortis viroid
CPFV	cucumber pale fruit viroid
CTiV	coconut tinangaja viroid
CVaV	citron variable viroid
dNTP	deoxyribonucleotide triphosphate
$\Delta G(KJmol^{-1})$	free energy (Kilojoules/mole)
HSV	hop stunt viroid
LTSV	lucerne transient streak virus
PSTV	potato spindle tuber viroid
RNase	ribonuclease
SCMoV	subterranean clover mottle virus
SNMV	solanum nodiflorum mottle virus
STobRV	satellite RNA of tobacco ringspot virus
TASV	tomato apical stunt viroid
T_m	midpoint temperature of the melting curve
TPMV	tomato planta macho viroid
VTMoV	velvet tobacco mottle virus

SUMMARY

Two approaches were used to investigate the possible common ancestry of viroids and virusoids. One approach examined evolution among viroids. To this end, two viroids were sequenced, coconut tinangaja viroid and a sequence variant of coconut cadang-cadang viroid that arises late in infection and is composed of a series of four partial duplications. These two viroids are 62% sequence homologous and share a common structural plan of five domains with all other viroids except avocado sunblotch viroid. These five domains correspond to different functional signals and indicate that viroid evolution may have involved intermolecular RNA rearrangements. The partial duplications of coconut cadang-cadang viroid may be a present day expression of the mechanism responsible for such rearrangements.

The second approach involved the structural and functional analysis of virusoids. Two virusoids from separate isolates of lucerne transient streak virus were sequenced and found to be 98% homologous. Although these virusoids can form a similar rod-like secondary structure to viroids, there is low sequence homology with viroids other than avocado sunblotch viroid. Blot hybridisation analysis of nucleic acids from plants infected with virusoids was used to detect greater-than-unit-length virusoid and complementary RNA forms and is

consistent with models of rolling circle replication previously proposed for viroids and virusoids. In contrast to viroids, however, virusoids appear dependent upon a helper virus for replication. Infectivity studies and hybridisation analysis support the view that virusoids are plant viral satellite RNAs and as such show most homology with the satellite RNA of tobacco ringspot virus.

From these two approaches it is concluded that viroids have had two separate origins and although somewhat structurally and functionally analogous to virusoids, common ancestry may exist between only one group of viroids, namely avocado sunblotch viroid, and virusoids.

CHAPTER 1

INTRODUCTION

1-1 Viroids

Viroids (Table 1-1) are molecular parasites of flowering plants inducing virus-like symptoms. However, three features significantly distinguish viroids from viruses.

1) Viroids lack mRNA activity. This has two important consequences. Firstly, there is no viroid-coded protein coat; a virus-coded protein coat has normally been associated with virus survival and spread. Secondly, viroids rely completely on host factors for their replication in contrast to viruses which encode a viral-specific polymerase in all cases where definitive results have been obtained.

2) Viroids are of low molecular weight. Composed of 246 nucleotide residues, CCCV (Haseloff et al., 1982) is less than one-tenth the size of the genomes of the smallest known viruses and bacteriophages such as maize streak virus, a single-stranded DNA virus (2681 nucleotide residues) [Howell, 1984], or a single-stranded RNA bacteriophage, MS2 (3569 nucleotide residues) [Fiers et al., 1976].

3) Viroid levels and symptom expression are exacerbated at elevated temperatures. At temperatures above 20^{o}C and at least up to 35^{o}C, the rate of viroid replication increases and hastens the onset of symptoms (Da Graça and Van Vuuren, 1981; Sänger, 1982; Singh, 1983). This is in contrast to plant viruses where high temperatures

Table 1-1 Viroids that are presently known

a. The disease agent of Burdock stunt has been considered to share some affinities with viroids (Chen et al., 1983). The disease agent of chrysanthemum chlorotic mottle disease (Romaine and Horst, 1975) has often been listed as a viroid but the disease agent has yet to be isolated.

b. CPFV is a sequence variant of HSV since the two viroids share 95% sequence homology (Sano et al., 1984).

c. TASV is also known as tomato bunchy top viroid (Diener, 1979).

Viroid	Abbreviation	Reference
Potato spindle tuber viroid	PSTV	Diener, 1971; Singh and Clark, 1971
Citrus exocortis viroid	CEV	Sänger, 1972;Semancik and Weathers, 1972a
Chrysanthemum stunt viroid	CSV	Diener and Lawson, 1973; Hollings and Stone, 1973
Cucumber pale fruit viroid	CPFV	van Dorst and Peters, 1974
Coconut cadang-cadang viroid	CCCV	Randles, 1975
Hop stunt viroid	HSV	Sasaki and Shikata, 1977
Columnea viroid	CV	Owens et al., 1978
Avocado sunblotch viroid	ASBV	Dale and Allen,1979; Thomas and Mohamed, 1979
Coconut tinangaja viroid	CTiV	Boccardo et al., 1981
Tomato apical stunt viroid	TASV	Semancik and Weathers, 1972b; Walter, 1981
Tomato planta macho viroid	TPMV	Galindo et al., 1982
Citron variable viroid	CVaV	Schlemmer et al., 1985

have been used as a method of curing seeds, bulbs and cuttings of virus.

1-2 The Structure of Viroids

Viroids are unencapsidated, single-stranded RNA of between 246 and 375 nucleotide residues (Sänger, 1982, 1984; Diener, 1983; Riesner and Gross, 1985; Keese and Symons, 1986). Electron microscopy and sequencing has confirmed the covalently closed nature of these circular molecules (Sogo et al., 1973; Sänger et al., 1976; McClements and Kaesberg, 1977; Gross et al., 1978; Palukaitis et al., 1979; Randles and Hatta, 1979; Palukaitis and Symons, 1980; Ohno et al., 1982).

The complete sequence of eight viroid species and more than thirty five sequence variants have been reported. Features shared by most viroids include high G:C content (except ASBV), a polypurine rich sequence of 14-20 nucleotide residues, a similar rod-like secondary structure with a central conserved uridine-bulged helix (except ASBV) and a common partially looped out GAAACC sequence (except HSV).

From extensive physico-chemical and biochemical studies, viroids when purified in solution, appear to adopt a rod-like secondary structure with a series of short helical regions (2-11 base pairs) interspersed by short non-base-paired segments (1-13 nucleotide residues) [Sänger et al., 1976; Gross et al., 1978;

Langowski et al., 1978; Riesner et al., 1979, 1983]. This structure can largely account for the unusual thermodynamic properties of viroids which mimic those of double- stranded RNA, such as, resistance to ribonucleases, behaviour on cellulose columns and high cooperativity upon thermal denaturation.

Although viroids have become one of the best structurally characterised groups of RNA molecules, the correlation of structure to their biology remains poorly understood. This is in part due to the lack of obvious sequence homology with RNA molecules of known function.

1-3 Viroid Replication

Viroid replication proceeds through RNA intermediates. Evidence of this includes the detection of complementary RNA sequences in infected plants of CEV (Grill and Semancik, 1978), PSTV (Branch et al., 1981; Rohde and Sänger, 1981; Owens and Diener, 1982), ASBV (Bruening et al., 1982), HSV (Ishikawa et al., 1984) and CCCV (Hutchins et al., 1985) but an absence, in the case of PSTV, of complementary DNA sequences (Branch and Dickson, 1980; Zaitlin et al., 1980). In addition, both greater-than-unit-length viroid and complementary viroid sequences have also been identified in nucleic acid extracts of infected plants. This, together with circularity of viroids, has led to proposals of rolling circle replication similar to that proposed by Brown and

Martin (1965) [Bruening et al., 1982; Owens and Diener, 1982; Mühlbach et al., 1983; Branch and Robertson, 1984; Ishikawa et al., 1984; Hutchins et al., 1985].

What remains controversial is the polymerase(s) involved in viroid replication. Viroids appear to be too small to code for a polymerase and indeed do not seem to possess any mRNA activity (see Diener, 1983). The most compelling argument for the lack of translation polypeptide products is the absence of any AUG triplets in either the viroid strand or its complement of PSTV (Gross et al., 1978) and CCCV (Haseloff et al., 1982), a necessary prerequisite for translation by eukaryotic ribosomes (Sherman et al., 1980). Consequently host enzymes have been implicated in viroid replication and include DNA-dependent RNA polymerase I (Schumacher et al., 1983) and II (Mülbach and Sänger, 1979; Rackwitz et al., 1981) and an RNA-dependent RNA polymerase (Boege et al., 1982), an enzyme known to exist in healthy plants (Duda, 1976; Ikegami and Fraenkel-Conrat, 1978; Romaine and Zaitlin, 1978). However these studies have relied on indirect measures such as the effect of alpha-amanitin or in vitro studies with purified enzymes. What is lacking is in vivo evidence of a physical association between viroids and a host polymerase.

1-4 Virusoids

Recently a second group of low molecular weight, circular, single-stranded RNA molecules have been found encapsidated together with certain plant viruses (Table 1-2) [Francki et al., 1985]. These RNAs have been termed virusoids by Dr. Adrian Gibbs and first described as such by Haseloff et al., (1982). These RNAs have also been referred to as viroid-like RNAs (see Francki et al., 1985 and references therein).

The first report of this new group of circular RNAs was in 1981 with the discovery of VTMoV (Randles et al., 1981) infecting Nicotiana velutina H. Wheeler, a species endemic to central Australia. When total virion RNA was fractionated by polyacrylamide gel electrophoresis, two RNA components were found, one being a single-stranded linear molecule of molecular weight 1.4×10^6 (RNA 1) and the other a low molecular weight RNA (termed RNA 2 by Randles et al., 1981). The RNA 2 component was subsequently shown to be single-stranded and circular with 366 or 367 nucleotide residues (Randles et al., 1981; Haseloff and Symons, 1982; Kiberstis et al., 1985). Re-investigation of two previously described viruses, SNMV (Gould and Hatta, 1981; Greber, 1981) and LTSV (Tien-Po et al., 1981), showed that each contained a virusoid. The failure to detect virusoids in previous reports of SNMV (Greber, 1978) and LTSV (Forster and Jones, 1979) was probably due to their small size and confusion with breakdown products of the genomic RNA.

Table 1-2 Virusoids that are presently known

a. SNMV RNA 2 is a sequence variant of VTMoV RNA 2 since they share 93% overall sequence homology (Haseloff *et al.*, 1982).

b. Natural isolates of SCMoV contain one or two virusoids (Francki *et al.*, 1983b).

Virusoid	Abbreviation	Reference
Lucerne transient streak virus RNA 2	LTSV RNA 2	Tien-Po *et al.*, 1981
Solanum nodiflorum mottle virus RNA 2	SNMV RNA 2	Greber, 1981; Gould and Hatta, 1981
Subterranean clover mottle virus RNA 2	SCMoV RNA 2	Francki *et al.*, 1983b
Subterranean clover mottle virus RNA 2'	SCMoV RNA 2'	Francki *et al.*, 1983b
Velvet tobacco mottle virus RNA 2	VTMoV RNA 2	Randles *et al.*, 1981

The two most recently reported virusoids were found associated with a single virus, SCMoV, that infects subterranean clover (Trifolium subterranean L.) [Francki et al., 1983b].

All four virusoid-containing viruses share characteristics of the Sobemovirus group (Hull, 1977; Matthews, 1982) of icosahedral plant viruses with an infectious, unipartite, single-stranded RNA genome of molecular weight 1.5 X 10^6 that has a covalently attached small viral protein. Of these four sap transmissible viruses, only LTSV has been reported outside of Australia. However, the place of origin of these viruses is obscured due to the influence of man on their natural host ranges. LTSV and SCMoV infect cultivated crops, SNMV infects the cosmopolitan weed Solanum americanum Miller, (formerly known as Solanum nodiflorum Jacq.) whilst N. velutina, the host of VTMoV, is common in disturbed sites.

In addition to their ability to act as molecular parasites of higher plants, virusoids possess other characteristics similar to viroids. These include single-stranded RNA of similar size range, covalently closed molecules as shown by electron microscopy (Gould and Hatta, 1981; Randles et al., 1981; Tien-Po et al., 1981; Francki et al., 1983b), a secondary structure that exhibits a high degree of cooperativity during thermal denaturation (Gould, 1981; Gould and Hatta, 1981; Tien-

Po et al., 1981) and probable lack of translation polypeptide products (Morris-Krsinich and Forster, 1983). Nevertheless, virusoids differ in two significant aspects from viroids: 1) they are dependent on a helper virus for detectable replication and 2) they are encapsidated.

1-5 Aims

The origin of viroids remains problematical. One group of RNA molecules, the virusoids, have been advanced as showing affinities with viroids. In order to assess the possible functional and evolutionary relationships between viroids and virusoids, two avenues were explored. One approach consisted of ascertaining conserved features and evolutionary relationships within viroids through the derivation of a structural model of viroids. The second approach was to examine the structure and possible replicative strategies of certain virusoids to allow structural and functional comparisons with viroids.

CHAPTER 2

COCONUT TINANGAJA VIROID

INTRODUCTION

Tinangaja disease of coconut palms (Cocos nucifera L.) was first reported in 1917 on Guam, Marianas Islands (Boccardo et al., 1981). Reinking (1961) suggested common aetiology with the cadang-cadang disease of coconut palms in the Philippines (Ocfemia, 1937). Both diseases lead to premature decline and death of the host (Boccardo, 1985) but only the cadang-cadang disease has received intensive study (see Zelazny et al., 1982). Other common features of these two diseases include chlorotic spotting of the leaves, reduced crown, decline in fertility and a prevalence for affecting palms 25 years old or more (Boccardo, 1985). One notable difference is the effect on nut production. Whereas the cadang-cadang disease is associated with smaller, more spherical, scarified nuts, the progression of the tinangaja disease leads to the appearance of small, elongated mummified husks with no kernel present (Reinking, 1961).

Like the cadang-cadang disease, a viroid-like RNA has been detected in nucleic acid preparations of tinangaja infected coconut palms (Boccardo et al.,1981). An RNA component with similar properties to CCCV was noted to possess the same electrophoretic mobility as the smallest, 246 nucleotide residue variant of CCCV, CCCV (246) [Randles,1975; Haseloff et al., 1982]. Furthermore, nucleic acid preparations from diseased

palms hybridised with greater efficiency to tritiated cDNA made to CCCV (246) than the RNA of CCCV (246) itself. Boccardo et al., (1981) thus suggested common identity between the two disease causing agents. The difference in nut symptoms was ascribed to varietal differences between the coconut palm hosts on Guam and in the Philippines.

The RNA species with about the same electrophoretic mobility as CCCV (246) was extracted from leaves of coconut palms bearing the tinangaja disease and sequenced to allow definitive comparisons with CCCV. The isolation and purification of tinangaja associated RNA was done in conjunction with M.E. Keese.

MATERIALS

Source of tinangaja diseased tissue

Coconut leaves from four palms infected with the tinangaja disease were collected by Dr. C. J. Hutchins from Guam, Marianas Islands. Leaves from one palm which had been assayed by Dr.J.W. Randles as being positive for the presence of viroid-like RNA was used as the source material for RNA extractions.

Enzymes

Ribonucleases (RNases) A and T_1 were obtained from the Sigma Chemical Co. RNase U_2 was obtained from

Sankyo. Bacteriophage T_4 polynucleotide kinase was from Boehringer.

Phy M RNase was prepared and kindly provided by Dr. J. Haseloff (Davis-Keller, 1980) from culture supernatants of Physarum polycephalum, the inoculum of which was kindly provided by the School of Biological Sciences, Flinders University of South Australia. The extracellular RNase of Bacillus cereus was prepared as described by Lockard et al. (1978) and was kindly provided by Dr. J. Haseloff.

Radioisotope

Gamma-^{32}P-ATP (2000 Ci/mmol) was obtained from BRESA Pty. Ltd.

METHODS

2-1 Preparation of partially purified nucleic acid extracts

Nucleic acid extracts were prepared from 50-500g of infected leaf material. The leaf tissue was homogenised with 4 volumes of AMES buffer (1.0 M sodium acetate, pH 6.0, 10 mM $MgCl_2$, 20% (v/v) ethanol, 3% (w/v) SDS; Laulhere and Rozier, 1976) and 2 volumes of water-saturated phenol. After the addition of 2 volumes of chloroform and vigorous shaking, the aqueous phase layer was recovered by centrifugation at 6,000 rpm (GS-3 rotor, Sorvall) for 10 min and then re-extracted with an

equal volume of phenol:chloroform (1:1). The nucleic acids in the aqueous phase were precipitated with ethanol and stored in a minimum volume of 0.1 mM EDTA, pH 8.0, necessary to redissolve the pellet.

2-2 Purification of tinangaja associated RNA

A. Polyacrylamide gel electrophoresis of nucleic acids

One volume of formamide-dye mix (95% v/v) formamide, 10 mM EDTA, pH 8.0, 0.02% bromophenol blue and xylene cyanol FF) was added to each sample which was heated at 80°C for 1 minute and loaded onto a 5% polyacrylamide gel (20 x 20 x 0.6 cm or 40 x 20 x 0.05 cm) containing 7 M urea and 90 mM Tris-borate, 2 mM Na_2EDTA, pH 8.3 (TBE).

B. Purification of RNA from acrylamide gels

The RNA was located in polyacrylamide gels by staining with 0.05% (w/v) toluidine blue-O for 10-15 minutes in a 0.6 cm thick gel and 20-60 sec in a 0.05 cm thick gel. RNA was eluted from a 0.6 cm thick gel by electophoresis in TBE at 100 V, 60 mA for 4-6 h after placing the gel slice in a dialysis tubing with about 5-10 volumes of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. RNA was eluted from 0.05 cm thick polyacrylamide gel by soaking in 0.5 M ammonium acetate, 1 mM EDTA, 1% (w/v) SDS at 37°C for 12-24 h. The eluted RNA was extracted with phenol:chloroform (1:1) and twice precipitated with ethanol.

2-3 Isolation and sequence determination of linear RNA fragments

A. Preparation of 5'-^{32}P-RNA linear fragments of tinangaja associated RNA

Procedures used were similar to those described in detail by Haseloff and Symons (1981). Briefly, purified tinangaja associated RNA (2 µg) was dissolved in 20 µl of 600 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5 for RNases T_1 and A digestions or in 600 mM NaCl, 10 mM $MgCl_2$, 20 mM sodium citrate, pH 3.5, for RNase U_2 digestions. Incubation was at 0°C for 60 min with 2,500 unit/ml of RNase T_1, 2 units/ml of RNase U_2, 100 ng of or 1 µg/ml of RNase A. Reactions were terminated by phenol:chloroform (1:1) extraction, ether washing and ethanol precipitation. The dried mixture of RNA fragments was resuspended in 0.1 mM EDTA, pH 8.0, heated at 80°C for 1 min, cooled on ice, and then 5'-labelled with ^{32}P. The reaction mixture (20 µl) for 5'-^{32}P-labelling contained 20 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 5% (v/v) glycerol, 10 mM DTT, 100 µCi of gamma-^{32}P-ATP and 4 units of T_4 polynucleotide kinase. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 20 µl of formamide-dye mix. The RNA fragments were fractionated by gel electrophoresis on a 6-8 % polyacrylamide gels (80 x 20 x 0.05 cm) containing 7 M urea and TBE buffer, at 20-25 mA for 4-6 h. RNA bands were located by autoradiography

for 15-30 min at room temperature, then excised and eluted for 12-24 h. Escherichia coli tRNA (60µg) was added to each tube and the fragments were purified by two ethanol precipitations.

B. Sequencing of 5'-^{32}P-labelled tinangaja associated RNA fragments

Partial enzymic hydrolysis methods were used to sequence the purified 5'-^{32}P-RNA fragments. Partial digestions were carried out with RNase T_1 and alkali (Donis-Keller et al., 1977), RNase U_2 (Krupp and Gross, 1979), RNase Phy M (Donis-Keller, 1980) and Bacillus cereus extracellular RNase (Lockard et al., 1978). The essential details of the sequencing procedure are given below.

Dried aliquots of each fragment were partially digested under each of the following conditions, in a final reaction volume of 10 µl.

i) 20 mM sodium citrate, pH 5.0, 7 M urea, 1 mM EDTA, 10 units RNase T_1.

ii) 20 mM sodium citrate, pH 3.5, 7 M urea, 1mM EDTA, 5 mU RNase U_2.

iii) 50 mM $NaHCO_3/Na_2CO_3$, pH 9.0 (alkali ladder).

iv) 20 mM sodium citrate, pH 5.0, 7 M urea, 1mM EDTA, 1 µl of RNase Phy M extract.

v) 20 mM sodium citrate, pH 5.0, 1 mM EDTA, 1 µl of RNase Bacillus cereus extract.

Enzymic reactions were incubated at 50°C for 20 min, while the alkali ladder was generated by heating at 100°C for 90 sec. Reactions were terminated by the addition of 10 ul of formamide-dye mix (95% [v/v] formamide, 10 mM EDTA, 0.02% [w/v] xylene cyanol FF and bromophenol blue) and then heated at 80°C for 1 min before fractionation on 40 x 20 x 0.05 cm 15% polyacrylamide gels containing 7 M urea and TBE buffer.

RESULTS

2-1 Purification of the viroid-like RNA associated with the tinangaja disease of coconut palms

Boccardo et al. (1981) were able to purify tinangaja associated viroid-like RNA from coconut palms using the same extraction procedure as used for CCCV (Randles, 1975). This method includes precipitation of the viroid with 5% polyethylene glycol (molecular weight about 8000) from crude coconut leaf extracts. When this method was applied to the leaf samples obtained from Guam, too much degradation obscured identification of the RNA migrating with the same mobility as reported by Boccardo et al. (1981) [results not shown]. Instead a more general method was used (adapted from Hutchins et al., 1985) in which total nucleic acid from a crude extract was twice purified by denaturing 5% polyacrylamide gel electrophoresis in 7 M urea. A band corresponding in mobility to the circular form of a 246

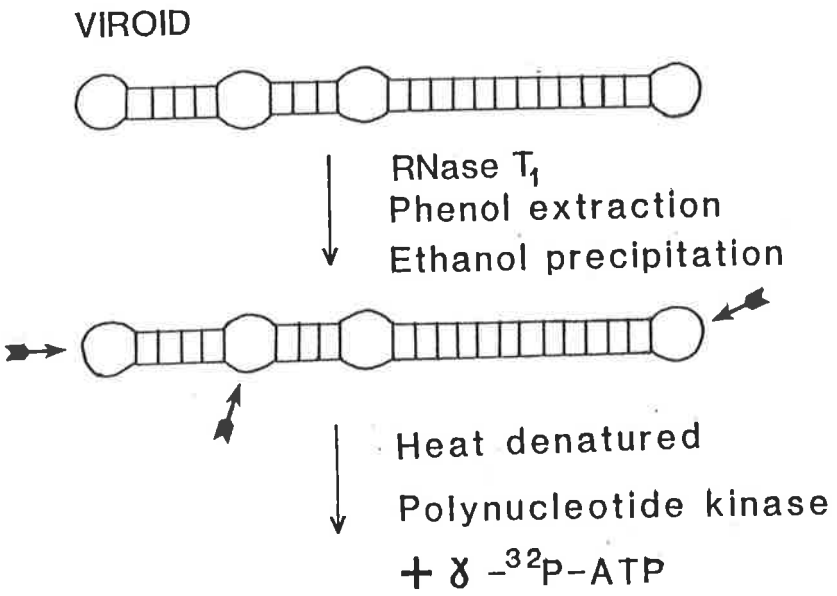
nucleotide residue variant of CCCV was isolated and purified for sequencing by enzymic partial hydrolysis.

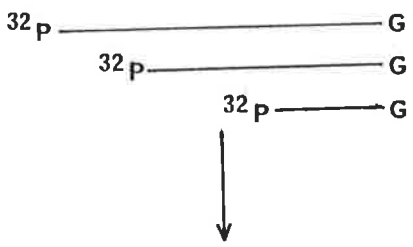
2-2 Sequence determination of the viroid-like RNA associated with tinangaja

Linear RNA fragments were obtained from the tinangaja associated viroid-like RNA by partial hydrolysis under non-denaturing conditions with ribonucleases T_1, U_2 and A (Figure 2-1). These fragments were radiolabelled, fractionated by polyacrylamide gel electrophoresis (Figure 2-2) and initially screened by partial hydrolysis with RNase T_1 to distinguish fragments with different sequences and those which were contaminated with two or more different 5'-terminal sequences. Specifically chosen fragments were then sequenced using the enzymic partial hydrolysis cleavage method (Figure 2-3). The sequences of overlapping fragments were assembled such that they corresponded to a circular molecule with 253 nucleotide residues, presented as a linear sequence in Figure 2-4. Since the RNA migrated with approximately the same mobility as the circular form of a CCCV variant with 246 nucleotide residues on denaturing polyacrlyamide gels, it is presumed that the tinangaja associated RNA represents a circular molecule and not a population of linear molecules with two or more different termini.

Figure 2-1 Strategy for obtaining ^{32}P-labelled linear fragments for sequencing viroids

A set of overlapping fragments is produced by partial enzymic digestion of viroid RNA with RNase T_1 (shown here), U_2 or A since different nucleotide residues are cleaved in different molecules. These fragments are 5'-^{32}P-labelled *in vitro*, fractionated by polyacrlyamide gel electrophoresis and sequenced using base-specific enzymes. See Methods and Results for details.





Fractionation by denaturing
polyacrylamide gel electrophoresis

Purification of
individual fragments

Sequencing of overlapping fragments
using base specific reactions

FIGURE 2-2 5'-^{32}P-labelled fragments from the partial hydrolysis of tinangaja associated RNA with RNases U_2 and A

Tinangaja associated RNA (1 µg) was digested with 0.04 units of RNase U_2, 20 ng of RNase A or 100 units of RNase T_1 (result not shown) under conditions of high salt concentration. 5'-^{32}P-labelled RNA fragments were fractionated on a 6% polyacrylamide gel (80 x 20 x 0.05 cm) containing 7 M urea at 25 mA. Following 20 minute autoradiographic exposure, RNA bands were excised and eluted for sequence determination. XC is the position of the xylene cyanol FF marker dye which corresponds to fragments about 100 nucleotide residues long.

RNase
U_2 A

O-

XC-

FIGURE 2-3 Sequencing gel

Sequencing gel (15% polyacrylamide, 7 M urea, 40 x 20 x 0.05 cm) of two RNase U_2 fragments of tinangaja associated RNA. Partial hydrolysis were with RNase T_1 (G), RNase U_2 (A), alkali (N) to produce a reference ladder, RNase Phy M (A+U) and Bacillus cereus RNase (C+U). A control reaction with no enzyme (-) was also included. A region of band compression is shown in parenthesis. Some cleavage at G can be noted with RNase Phy M. The arrows point to undigested RNA.

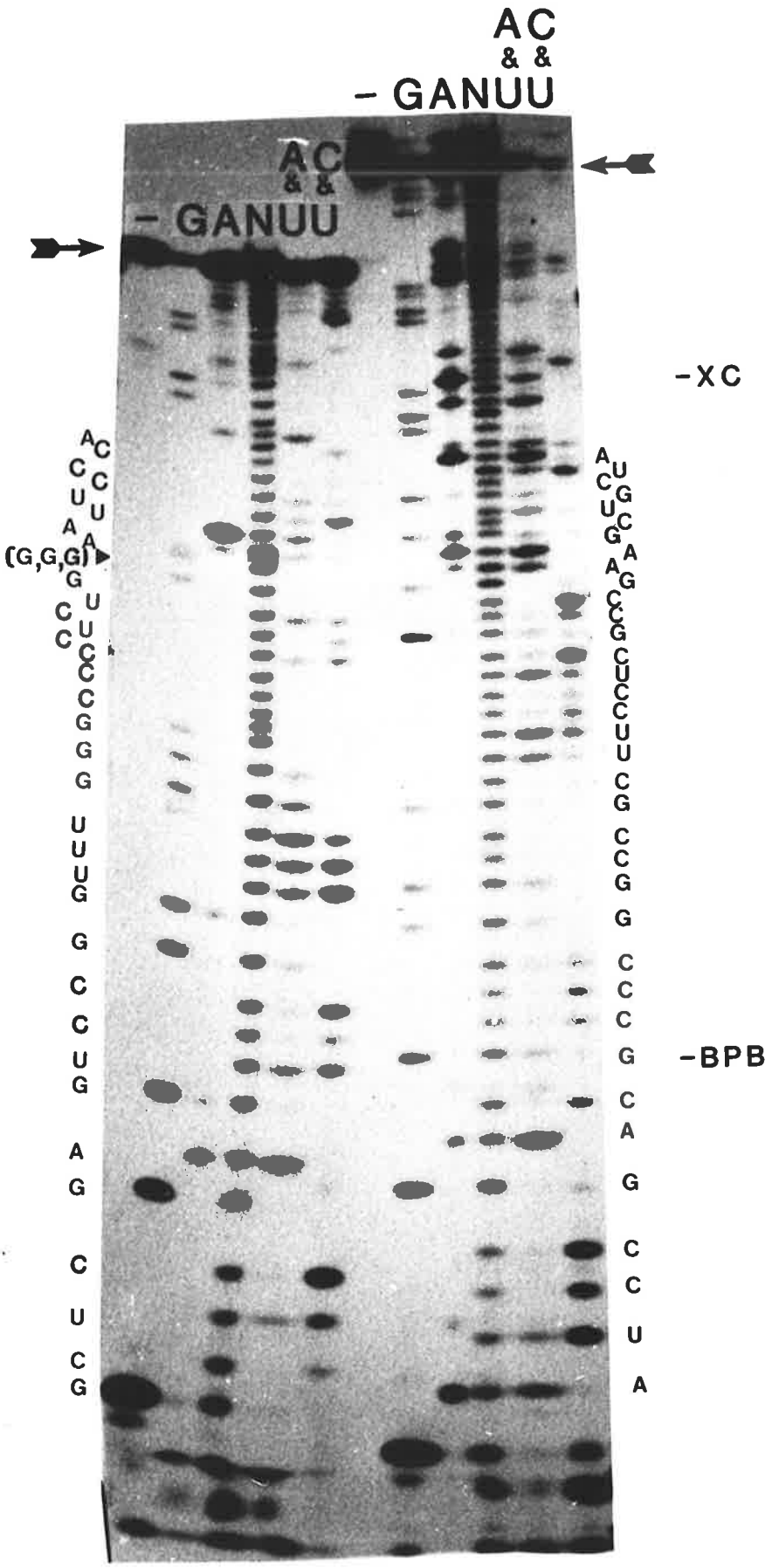

AC
& &
– GANUU
AC
& &
– GANUU
–XC
–BPB

Figure 2-4 Nucleotide residue sequence of tinangaja associated RNA

The circular RNA is presented as a linear sequence with nucleotide residue 1 numbered relative to the position as nucleotide residue 1 of CCCV.

Tinangaja associated RNA

```
1                                                  50
CUGGGGAAUUCCCACGGCAACGGCAAAACAAAGCACAAGAGCGACUGCUA

                                                  100
GAGGGAUCCCCGGGGAAACCCCUAGCAACCGAGGUAGGGAGCGUACCUGG

                                                  150
UGUCGCGAUCGUGCUGGUUGGGCUUCGUGCCCUUCCGAGCUUCGAUCCGA

                                                  200
CGCCCGGCCGCUUCCUCGCCGAAGCUGCUAUGGAGACUACCCGGUGGAUA

                                                  250  253
CAACUCUUUGCAGCGCCCUGUGUAAUAAAAGCUCGAGUCCGGUUUGGGCCCCU
```

2-3 Proposed secondary structure of the viroid-like RNA associated with tinangaja

The secondary structure of the tinangaja associated viroid-like RNA was determined theoretically using parameters described by Steger et al. (1984). The RNA conforms to a helical rod-like structure (Figure 2-5) similar to that of PSTV which has been well characterised structurally (Gross et al.,1978; Langowski et al., 1978; Riesner et al., 1978). The properties of the proposed secondary structure are summarised in Table 2-1 and compared with those of other published viroids. The properties fit most closely those of CCCV and also show a high proportion of G:C base-pairs similar to all other viroids except ASBV. The overall stability when adjusted proportionately to its size, appears to be, together with CCCV, intermediate between ASBV and other viroids.

DISCUSSION

Due to the limited number of investigations into the tinangaja disease and its causal agent the exact biological status of the viroid-like RNA present only in infected palms but not healthy palms (Boccardo et al., 1981) cannot be unambiguously defined. It has not yet been fully established whether the viroid-like RNA is able to replicate independently in healthy coconut palms and whether it is the causal agent of tinangaja disease.

FIGURE 2-5 Proposed secondary structure of tinangaja associated RNA

The intramolecular base-pairing was optimised according to parameters reported by Steger et al. (1984).

Tinangaja associated RNA

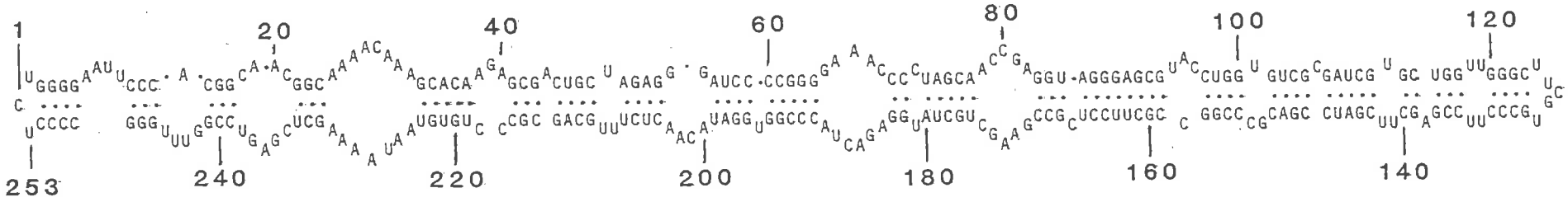


Table 2-1 Properties of proposed secondary structures of viroids

[a] Calculated according to Steger et al. (1984)

[b] Data taken from Steger et al. (1984) and calculated at ionic strength 0.011 M Na^+, pH 6.8.

ND Not determined.

VIROID	NUMBER OF NUCLEOTIDE RESIDUES	BASE PAIRS DEGREE (%)	G:C (%)	A:U (%)	G:U (%)	$\Delta G^a/N$	THERMODYNAMICS[b] T_m °C	$T_{1/2}$ °C	REFERENCE
CTiV	253	62	73	19	8	-1.39	ND	ND	
CCCV	246	66	69	24	8	-1.30	49	1.2	Haseloff et al. (1982)
CEV	371	69	56	28	16	-1.59	51	1.0	Gross et al. (1982) Visvader et al. (1982)
CSV	356	70	52	35	13	-1.52	48	1.1	Haseloff and Symons (1981)
HSV	297	67	64	29	7	ND	ND	ND	Ohno et al. (1983)
TASV	360	73	57	32	11	ND	ND	ND	Kiefer et al. (1983)
TPMV	360	68	60	31	9	ND	ND	ND	Kiefer et al. (1983)
PSTV	359	70	58	29	13	-1.67	51	0.9	Gross et al. (1978)
ASBV	247	67	34	51	14	-1.13	37.5	1.5	Symons (1981)

Improvements in inoculations of coconut palms with CCCV have now achieved 90% efficiency of transmission and allowed detection of the viroid by gel electrophoresis within six months (M.E. Keese, personal communication). Similar methods could be employed with the viroid-like RNA from tinangaja infected palms to establish viroid status and disease aetiology. At present, the nucleotide sequence and structure of the viroid-like RNA and its homology with other viroids add support to its viroid classification. As such the viroid-like RNA is tentatively described as coconut tinangaja viroid (CTiV).

The RNA partial enzymic hydrolysis method used for determining the sequence of CTiV, generated a set of overlapping fragments, supporting the notion of CTiV being a circular RNA. Two problems are normally associated with RNA sequencing using RNases: lack of pyrimidine specificity and band compression. For example, the extracellular RNase from B. cereus does not cleave uniformly at pyrimidines. In particular, strings of cytidylate residues are poorly cleaved. The Phy M RNase does, however, differentiate reliably between pyrimidines by cleaving at the 3'-side of uridylate residues but not at the 3'-side of cytidylate residues. Therefore, the B. cereus enzyme was only used as supporting evidence of the sequence.

Band compression arising from secondary structures that are not denatured in sequencing gels can mask the presence of some nucleotide residues (see Haseloff and Symons, 1981). Four regions of CTiV were affected by partial band compression in sequencing gels (nucleotide residues 4-6, 70-72, 75-77, 130-132). These regions were readily resolved by performing denaturing polyacrylamide gel electrophoresis in 7 M urea at high current and temperature.

Sequence homology between CTiV and CCCV is readily apparent (Figure 2-6) with both molecules showing about 62% overall sequence homology. This extensive but not complete homology between CTiV and CCCV could account for the cross-hybridisation between the two RNAs and the difference in symptom expression of the tinangaja and coconut diseases (Boccardo et al., 1981). Accordingly, the postulated secondary structure of CTiV is also similar to CCCV (246) [Figure 2-7]. The secondary structure homology with CCCV (246) is greater if thermodynamically more stable structures of CCCV (determined according to Steger et al., [1984]) are considered (Figure 2-7). The degree of sequence homology between CTiV and CCCV (246) is not, however, greater than between PSTV, CSV and CEV. Thus CTiV would appear to justify separate nomenclature from that of CCCV.

The secondary structure of CTiV reveals two regions (nucleotide residues 14-40, 217-243, and 87-103, 150-

Figure 2-6 Sequence homology between CTiV and CCCV

The sequence of tinangaja associated RNA was optimally aligned with CCCV. The boxed areas contain 3 or more consecutively homologous nucleotide residues between both RNAs.

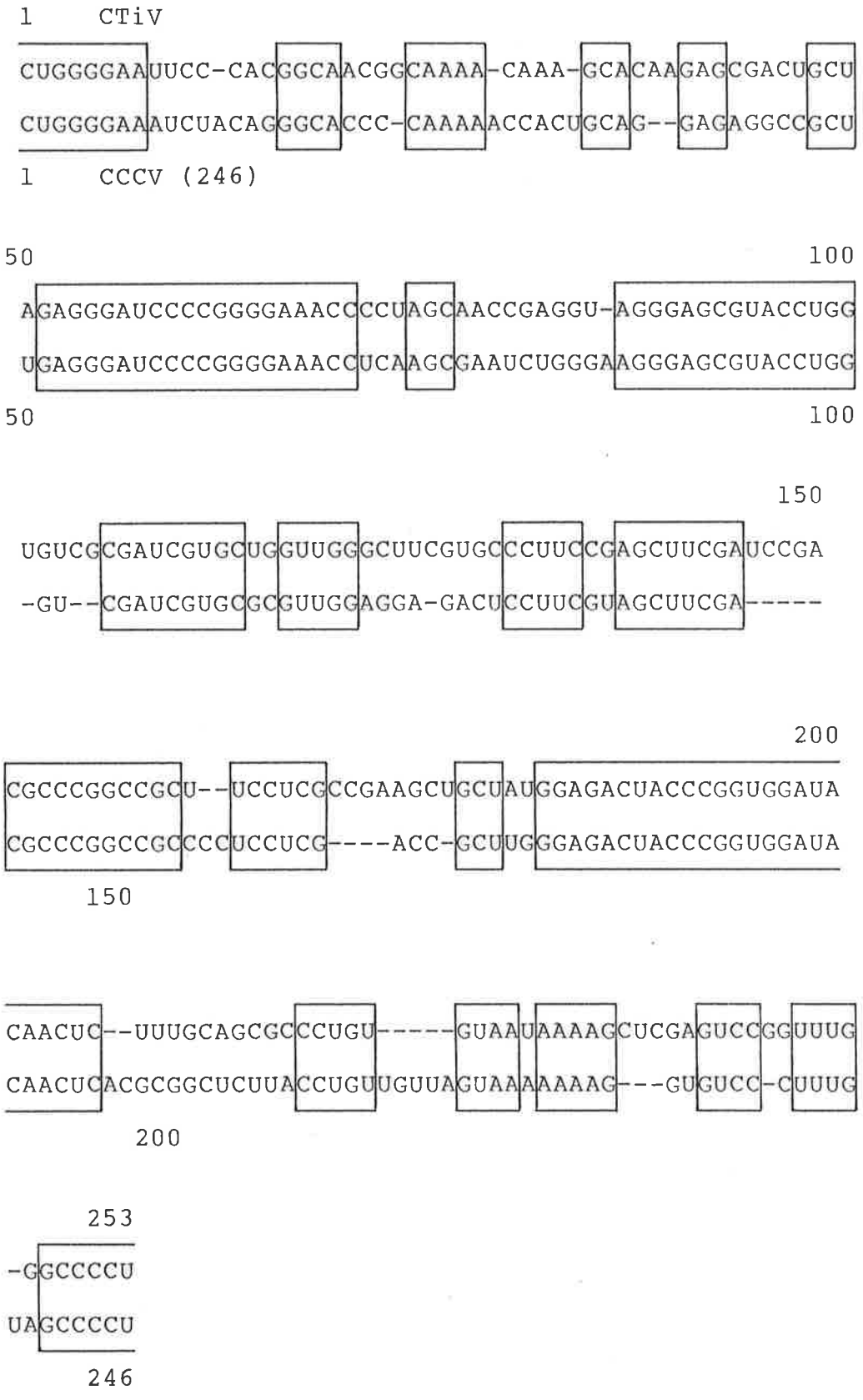
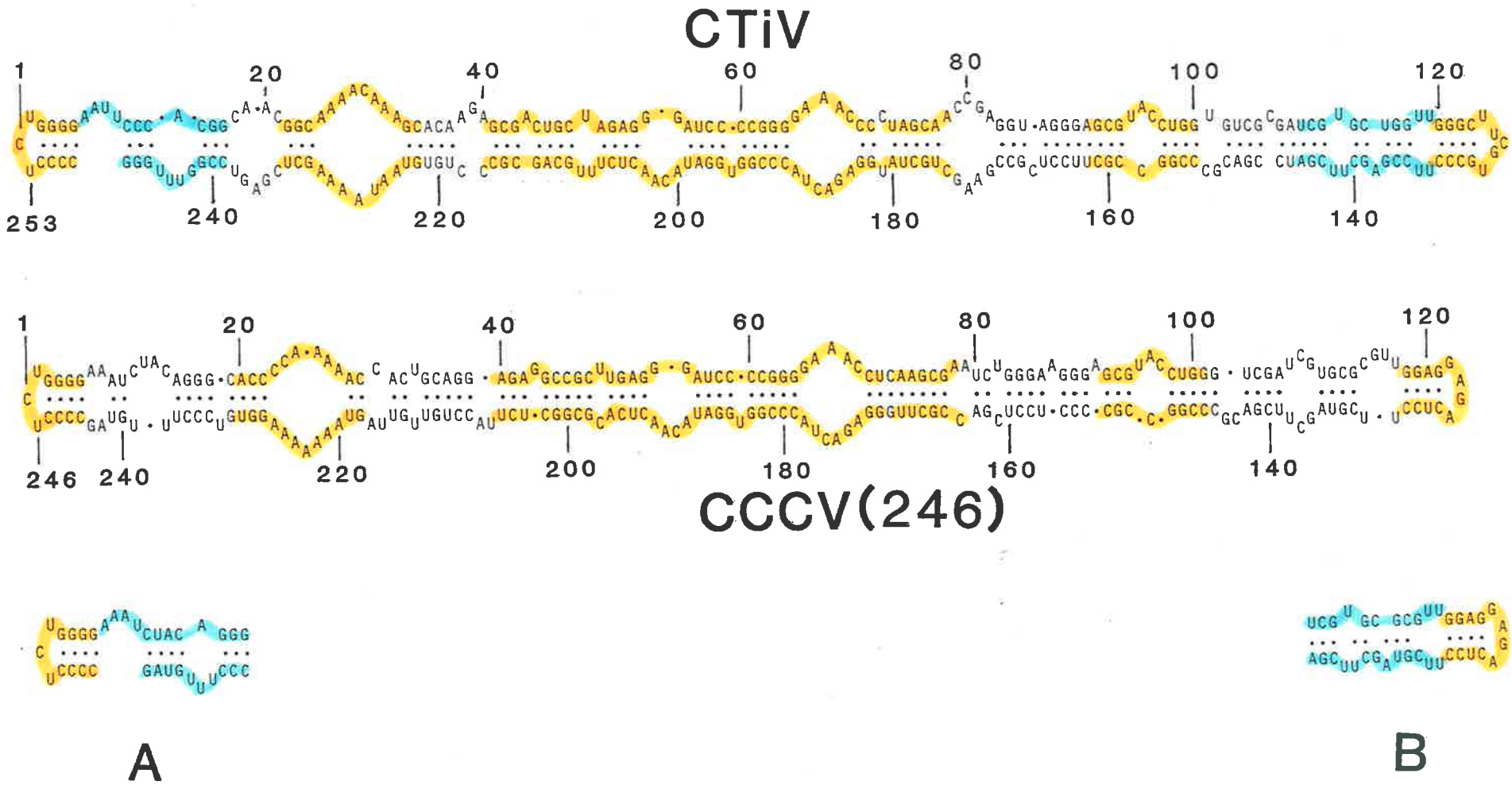

1 CTiV
CUGGGGAAUUCC-CACGGCAACGGCAAAA-CAAA-GCACAAGAGCGACUGCU
CUGGGGAAAUCUACAGGGCACCC-CAAAAACCACUGCAG--GAGAGGCCGCU
1 CCCV (246)
50
100
AGAGGGAUCCCCGGGGAAACCCCUAGCAACCGAGGU-AGGGAGCGUACCUGG
UGAGGGAUCCCCGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGG
50
100
150
UGUCGCGAUCGUGCUGGUUGGGCUUCGUGCCCUUCCGAGCUUCGAUCCGA
-GU--CGAUCGUGCGCGUUGGAGGA-GACUCCUUCGUAGCUUCGA-----
200
CGCCCGGCCGCU--UCCUCGCCGAAGCUGCUAUGGAGACUACCCGGUGGAUA
CGCCCGGCCGCCCCUCCUCG----ACC-GCUUGGGAGACUACCCGGUGGAUA
150
CAACUC--UUUGCAGCGCCCUGU-----GUAAUAAAAGCUCGAGUCCGGUUUG
CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAG---GUGUCC-CUUUG
200
253
-GGCCCCU
UAGCCCCU
246

Figure 2-7 Structural homology between CTiV and CCCV (246)

Regions of structural homology between the proposed secondary structures of CTiV and CCCV (246) [Haseloff et al., 1982] is presented by orange higlight. According to parameters of Steger et al. (1984), thermodynamically more stable structures suggested for CCCV (246) [A, lower ΔG of 5 KJ mol^{-1} and B, lower ΔG of 11 KJ mol^{-1}]) show greater structural homology between both RNAs, coloured blue.


CTiV
CCCV(246)
A
B

165) of sequence homology with CCCV that are distinguishable from that of other viroids. The left hand region contains two opposing adenosine dominated sequences which are highly susceptible to cleavage by the single-strand specific RNase U_2 (results not shown). The equivalent regions in PSTV and CEV-A appear to modulate symptom expression but are characterised by an adenosine-dominated purine sequence complementary to uridine-dominated pyrimidine sequence. One biological difference between the tinangaja and cadang-cadang disease is the effect on the nuts which are reduced to mummified husks in the case of tinangaja, but which retain their meat together with a more spherical appearance in the case of cadang-cadang. It would be interesting to determine if sequence differences between CTiV and CCCV in this region are also responsible for the variation in disease symptoms and not simply due to varietal differences of the coconut hosts.

The second distinct region of homology between CTiV and CCCV includes sequences that border partial duplications of CCCV (see Chapter 4) which give rise to a number of CCCV sequence variants, CCCV (287), CCCV (296 and/or 297) and CCCV (301) [Haseloff et al., 1982]. As yet, no similar larger molecular weight sequence variants of CTiV have been confirmed, however, only a limited number of diseased palms have been examined for the presence of CTiV-related sequences.

Other regions of CTiV exhibit not only sequence homology with CCCV but also with other viroids. These include the central region which has already been noted for its high sequence conservation by Haseloff et al. (1982). The central region of CTiV and CCCV are most closely related to that of TPMV (Figure 2-8). The left hand end loop of CTiV shows discernible sequence homology with all other viroids (except ASBV), in particular HSV (Figure 2-9). The right hand end loop of CTiV has less sequence homology with other viroids. The most notable sequence homology can be found with CCCV, HSV and PSTV (Figure 2-10). The sequence homology includes a CCUUC sequence that occurs in the same relative position of all other viroids except ASBV.

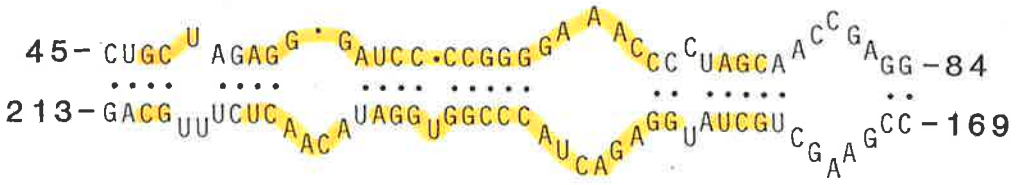
A third region that is poorly conserved between CTiV (nucleotide residues 37-46, 207-221) and CCCV (nucleotide residues 31-46, 196-217), includes a site where a cytidylate residue insertion at position 198 of CCCV occurs during progression of the disease (Imperial et al., 1983). This extra cytidylate residue may not signify a crucial function since no similar sequence is found in the corresponding region of CTiV.

In contrast to the extensive homology that CTiV shares with most other viroids, no significant homology was found between CTiV and either ASBV, virusoids or plant viral satellite RNAs. (see Chapter 5).

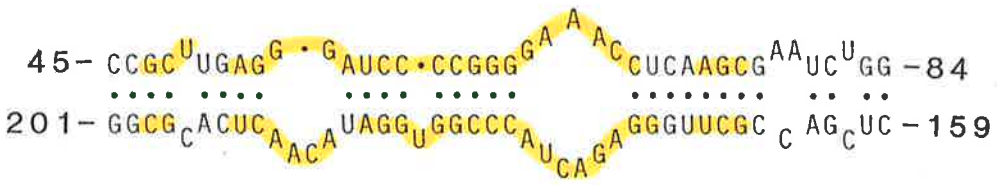
Figure 2-8 Sequence homology between CTiV, CCCV and TPMV

The central region of CTiV shows most sequence homology with CCCV and TPMV; homology is shown as coloured.

CTiV



CCCV



TPMV

Figure 2-9 Sequence homology between CTiV and all other viroids except ASBV

Sequence homology between CTiV and other viroids (underlined) in the corresponding left hand regions of the proposed structures of each respective viroid. ASBV is not included since no significant homology could be found.

CTiV	243 - U U U G G G C C C C U C U G G G G A A U U C C - 12	
HSV	287 - C U U G A G C C C C U C U G G G G A A U U C U - 12	
CCCV	236 - U U G U A G C C C C U C U G G G G A A A U C U - 12	
CEV	361 - A U U G G G U C C C U C G G G A U C U U U C U - 12	
TASV	350 - G A A U G G U C C C U C G G G A U C U U U C G - 12	
TPMV	350 - A A A G G G U C C C U C G G G A U C U U U U C - 12	
CSV	346 - A U U U G U U C C C U C G G G A C U U A C U U - 12	
PSTV	349 - A G U U G G U U C C U C G G A A C U A A A C U - 12	

Figure 2-10 Sequence homology between the CTiV and CCCV, HSV and PSTV

Sequence homology between CTiV and CCCV, HSV and PSTV (underlined) in the corresponding right hand regions of the proposed secondary structures of each respective viroid.

```
CTiV  104 - CGCGAUCGUGCU-GGUUGGGCUUCGUGC-CCUUCCGAGCUUCGAUC - 147
CCCV  102 - GUCGAUCGUGCG-CGUUGGAGGA-GACU-CCUUCGUAGCUUCGACG - 144
HSV   124 - GCCG-CGGUGCUCUGGAGUAGAGGCUCUGCCUUC-GACCAUCGAUC - 171
PSTV  158 - AAUUCCCGCCGA-AACAGGGUUUUCA-C-CCUUCCUUUCUUCGGGU - 200
```

CHAPTER 3

MODEL OF VIROID DOMAINS

3.1 Introduction

The origins of viroids are unknown. One problem is whether viroids are uniphyletic or polyphyletic. At present, viroids have been classified into three groups on the basis of sequence homology: 1) PSTV, CSV, CEV, TASV, TPMV and possibly HSV; 2) CCCV; 3) ASBV (Sänger, 1982; Diener, 1983; Gross, 1985; Randles, 1985; Riesner and Gross, 1985). It would appear, however, that only two natural groupings exist and that these may reflect separate origins for viroids. One group consists solely of ASBV while all other viroids constitute a second group. This second group of PSTV-like viroids share a common structural plan of five domains each of which seem to reflect different functional signals. The model of viroid structure presented below can be readily applied to HSV, CCCV and CTiV and so justify their inclusion amongst other PSTV-like viroids. This model, however, is not applicable to ASBV.

3-2 Model of PSTV-like Viroid Domains

The domains of PSTV-like viroids are depicted schematically in Figure 3-1 and more specifically in Figure 3-2. The general features of each domain are summarised as follows:

C Domain. This conserved central domain is centred around the strictly conserved bulged helix, CC CCGG
GGUGGCC

Figure 3-1 Model of Viroid domains

Model of five viroid domains (T1, P, C, V, T2) was determined from sequence homologies between viroids. The arrows depict an inverted repeat sequence. R, Y: a short oligo-purine, oligo-pyrimidine helix.

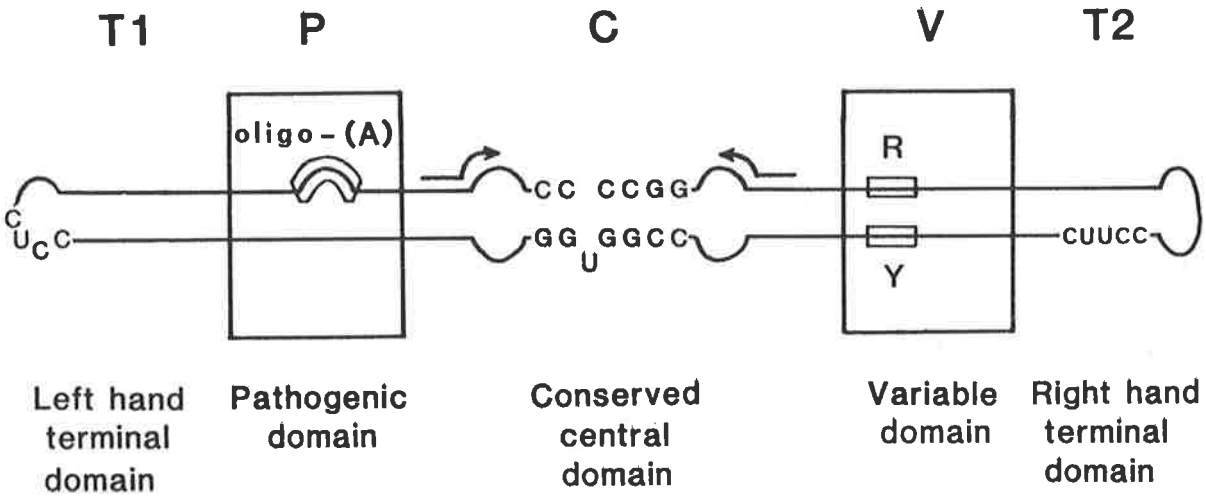
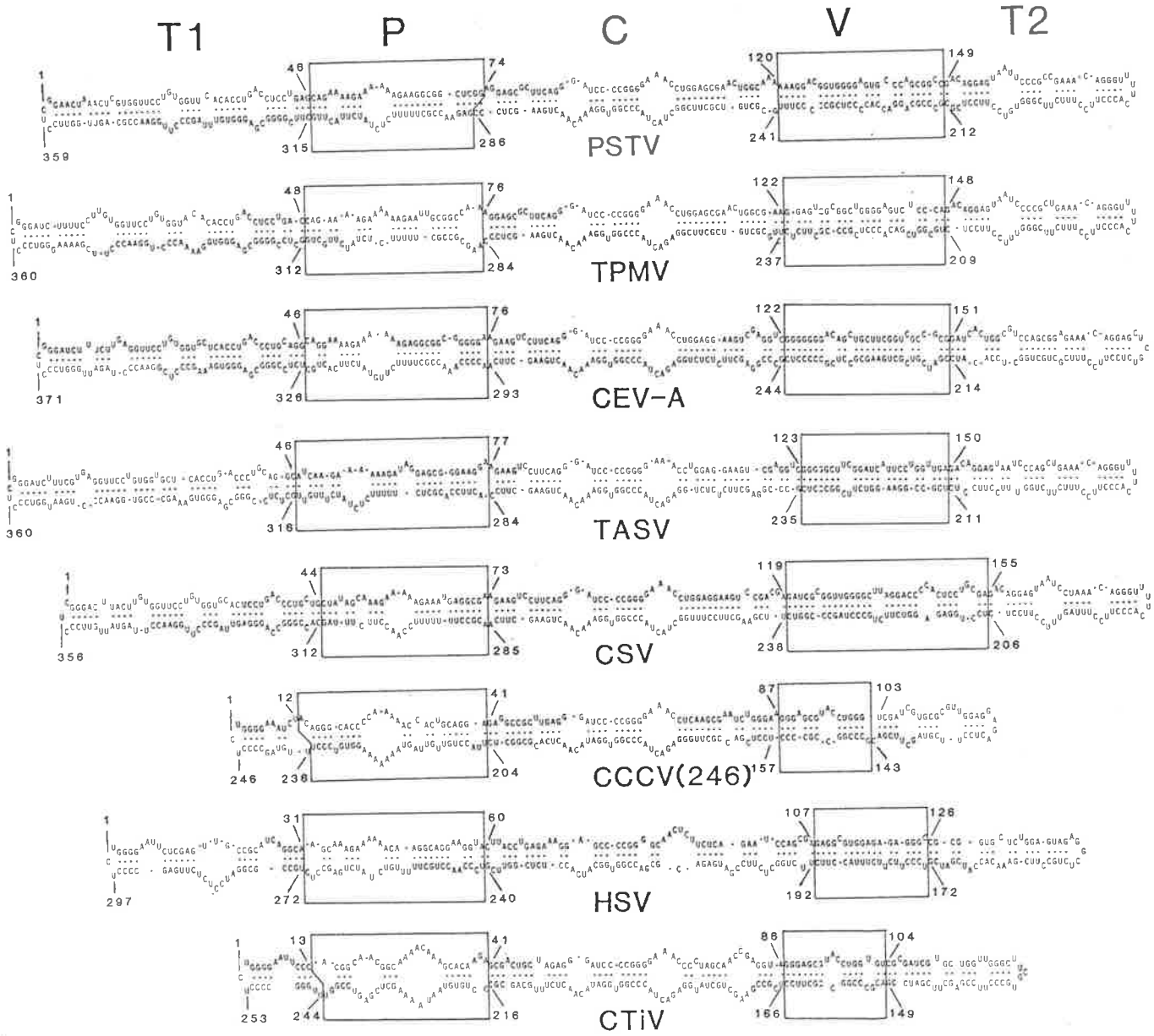

T1
P
C
V
T2
oligo-(A)
R
Y
CC CCGG
GG GGCC
U
CUUCC
Left hand terminal domain
Pathogenic domain
Conserved central domain
Variable domain
Right hand terminal domain

Figure 3-2 Domain boundaries defined for each viroid

In pair-wise sequence comparisons of viroids containing highly homologous C domains (for example, between PSTV, TPMV, CCCV, or between CEV-A, TASV and CSV), the P and V boundaries are defined by the significantly lower sequence homology which starts 5-9 nucleotide residues 5, and 7-15 nucleotide residues 3', of the inverted repeat sequence (Figure 3-1) in the C domain. Similarly, when comparing PSTV, TPMV, TASV and CSV, or CTiV, CCCV and HSV, a change from low sequence homology in the V domain to high homology in the T2 domain defines the boundary for these two domains. The P domain, with a region containing the conserved oligo-adenylate sequence, is flanked by regions with greater variability and has its borders based on homologies between the P region of HSV and other viroids such as PSTV and by certain pair-wise comparisons such as CEV-A and TASV where there is significant change from relatively low sequence homology in the P domain to higher sequence homology in the adjacent T1 and C domains.


T1
P
C
V
T2
PSTV
TPMV
CEV-A
TASV
CSV
CCCV(246)
HSV
CTiV

Haseloff et al. (1982). It may represent an important control region in viroid replication by signalling a functional change through structural alterations.

P Domain. This domain is associated with symptom expression (Schnölzer et al. 1985) and is characterised by an oligo-adenosine sequence.

V Domain. This domain shows the greatest sequence variability between closely related viroids.

T Domains. On the basis of sequence homologies, the terminal regions are considered to have undergone intermolecular RNA exchange between viroids to give rise to new, chimeric viroid species. Although the functional role of these domains is unclear, the evidence for these exchanges suggests a role for RNA rearrangements in the origin and evolution of viroids.

3-3 Control Function of the Highly Conserved C Domain

As originally observed by Haseloff et al.(1982) the two most highly conserved sequences of viroids are base-paired in the centre of each molecule. This central highly conserved domain of viroids can be considered to extend to about 95 nucleotide residues rather than 44 or 56 nucleotide residues previously described (Haseloff et al., 1982; Kiefer et al., 1983). An example of the high degree of sequence conservation is shown by the 99% sequence homology between CEV-A and TASV in the C domain although showing only 73% overall sequence homology

(Table 3-1). The C domain of CCCV (246), which constitutes 40% of the molecule, shows 70% sequence homology with PSTV, a value greater than the 65% sequence homology for this domain between PSTV and two other closely related viroids, CEV and TASV. These comparisons support the association of CCCV with the PSTV-like group of viroids.

Although HSV shares a less closely related C domain (Table 3-1), it has several sequence and structural homologies in common with the C domain of other viroids. These include a common uridine-bulged helix CC CCGG
GGUGGCC
(Haseloff et al., 1982) [Figure 3-2] that is postulated to occur in the native viroid. The top strand of this helix forms part of a larger 16 nucleotide conserved sequence GGANCCCCGGGGNAAC. In addition, an alternative structure that corresponds to stem loop I reported to form during the thermal denaturation of PSTV, CEV, CSV and CCCV (Riesner et al., 1979, 1983) may form in competition to the conserved uridine-bulged helix. By such a scheme, the highly conserved CCCCGGGG sequence would form part of a self complementary loop of a nine base pair stem (Figure 3-3) which gives a structure mutually exclusive to the bulged helix. Despite many sequence differences, the nine base pair stem can be formed for all viroids which in the case of HSV differs only by the presence of a single, non-base paired cytidine (Figure 3-3). The self-complementary loop may

Table 3-1 Sequence homology between domains of different viroids

Sequence homology was determined from the best alignment, allowing for additions and deletions, but constrained by the the requirement of a match consisting of a minimum of three consecutive nucleotide residues.

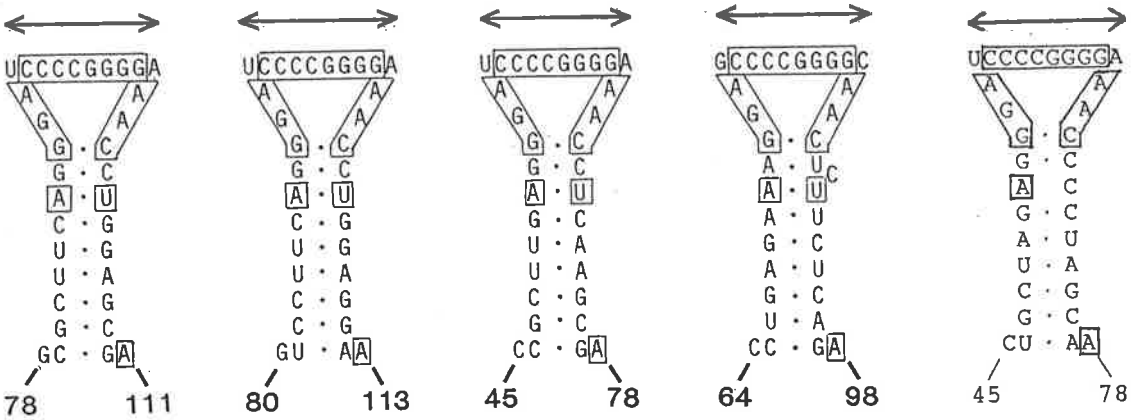
$$\% \text{ Sequence homology} = \frac{\text{number of matching nucleotide residues in both sequences}}{\text{total number of nucleotide residues in both sequences}} \times 100$$

VIROIDS USED FOR PAIRWISE COMPARISON		% SEQUENCE HOMOLOGY					
		DOMAINS					OVERALL
1	2	T1	P	C	V	T2	
PSTV	TPMV	67	73	94	42	95	76
	TASV	67	59	65	30	90	64
	CSV	69	49	71	31	81	61
	CEV-A	62	71	65	31	38	55
	CTiV	28	23	55	36	46	39
	CCCV (246)	25	14	70	37	27	38
	HSV	23	58	35	37	28	35
CEV-A	TASV	91	54	99	49	46	73
	TPMV	80	70	69	29	37	60
	CSV	77	42	82	28	38	59
	CTiV	32	26	62	31	40	41
CCCV (246)	HSV	32	33	42	31	50	39
	CTiV	71	51	61	79	64	62
HSV	CTiV	51	39	41	20	61	53

Figure 3-3 Alternative secondary structure in the C domain of PSTV-like viroids

A similar 9 base pair stem terminated by a loop containing a 10 nucleotide residue self-complementary sequence can be found in the central region of all PSTV-like viroids. The stem has been postulated to form for PSTV (Henco et al., 1979) and CEV, CSV and CCCV (see Riesner et al., 1983) as a transitional structure during melting of the secondary structure with increasing temperature. The CCCGG sequence in the proposed loop corresponds to one strand of the strictly conserved bulged helix (Figure 3-1) which would thus be disrupted by the formation of these hairpin loop structures. Conserved nucleotide residues are boxed.

Deviations from nine base pair stem include HSV with a bulged C residue and CTiV with an A:C pair of nucleotide residues.



PSTV

TPMV

(78–111)

CEV

TASV

(81–114)

CSV

(77–110)

CCCV

HSV

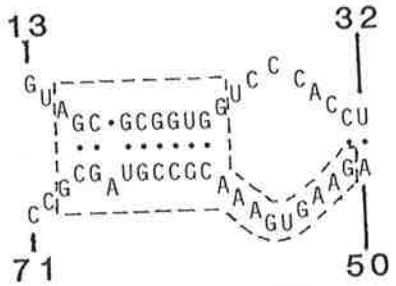
CTiV

aid formation of the stem by base-pairing with another molecule. Such dimer formation may have been detected during non-denaturing polyacrylamide gel electrophoresis of PSTV after heating and then snap cooling (Riesner et al., 1979). Upon staining, two bands were detected, the slower migrating band of which may correspond to a dimeric form of PSTV.

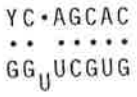
It is proposed that both mutually exclusive but highly conserved structures (Figures 3-3 and 3-4) are important in viroid function and that structural switching from the native form to the nine base pair stem structure controls a switching in function for this region. For example, one function postulated for the C domain is a role in processing of viroid RNA replicative intermediates (Meshi et al., 1985; Robertson et al., 1985; Visvader et al., 1985; Diener, 1986). This possibility is supported by infectivity studies. cDNA clones and in vitro-synthesised RNA transcripts from cDNA clones of PSTV, CEV and HSV have been shown to be infectious (Cress et al., 1983; Visvader et al., 1985; Meshi et al., 1985). Infectivity of less-than-dimeric length viroid clones (Tabler and Sänger, 1984; Visvader et al., 1985; Meshi et al., 1985) has been correlated with a partial duplication of at least 11 nucleotide residues (underlined) of the central conserved 16 nucleotide residue sequence, GGANCCCCGGGGNAAC. It has been postulated that in vivo processing occurs within

Figure 3-4 Structural homology in the C domain of PSTV-like viroids with a protein binding site

Structural homology is indicated between the ribosomal protein L18 binding site on 5S RNA of E. coli (Peattie et al., 1981) [dashed box] and the central conserved bulged helix of viroids (solid base in HSV and PSTV). The central conserved bulge helix of the 5S RNAs of higher plants is given (Peattie et al., 1981); V represents a pyrimidine.

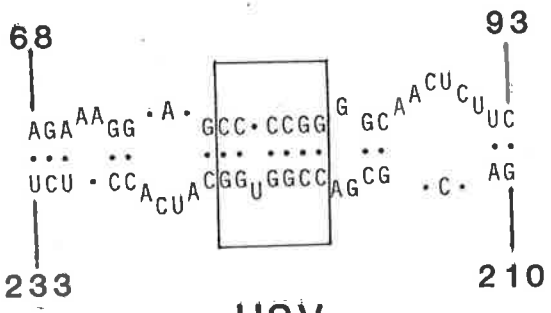


E.coli 5S RNA

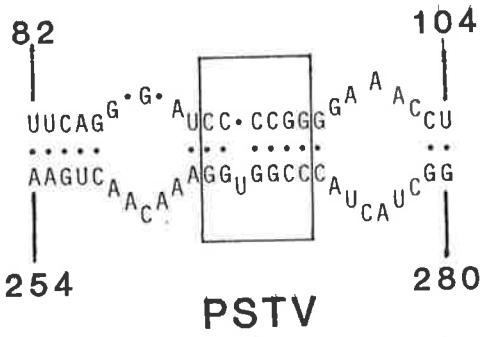


higher plants

Conserved central helix
of plant 5S RNAs



HSV



PSTV

Conserved central helix
of PSTV-like viroids

the 11-nucleotide residue repeat to generate a unit-length monomeric viroid free of any vector sequences (Visvader et al., 1985).

An alternative function for this region which may require a structural signal different from that involved in processing, is based on the similarity of the bulged helix with several protein binding sites (Peattie et al., 1981), in particular the ribosomal protein L18 binding site of 5S RNAs (Figure 3-4). It has been proposed that the single unpaired nucleoside (uridine in plant 5S RNAs) and the adjacent guanosine of 5S RNA are crucial to the interaction with L18 (Peattie et al., 1981; Christiansen et al., 1985). This analogous region in viroids may, for example be a site for RNA polymerase binding and/or the initiation of synthesis of the viroid or complementary viroid RNA strand. Therefore, structural switching in the C domain could control switching between two phases of the replication cycle, namely, transcription and processing.

3-4 Association of pathogenicity with the P domain

Many sequence variants of PSTV and CEV have now been characterised. Six variants of PSTV have been shown to differ in both sequence and severity of symptoms when propagated in tomato (Dickson et al., 1979; Gross et al., 1981; Schnölzer et al., 1985). The nucleotide differences are confined to the P and V domains (Table

3-2) but only sequence changes in the P domain are correlated with variation in symptom expression (Gross et al., 1981; Schnölzer et al., 1985); this region has been defined by Sänger (1984) as a virulence modulating domain.

Interestingly, 17 sequence variants of CEV which differ by up to 29 nucleotide residues between any two variants (Visvader and Symons, 1983, 1985) have most of these sequence differences located in the P and V domains (Table 3-2), in the same relative positions as the PSTV variants. The CEV variants form two classes of sequence which differ by a minimum of 23 nucleotide residues in a total of 370-375 nucleotide residues (Visvader and Symons, 1985). These two classes correlate with two biologically distinct groups when propagated in tomato plants where one produces severe symptoms (leaf epinasty and stunting) and the other mild symptoms (no detectable morphological changes).

Infectious CEV chimeras have since been constructed from two CEV clones such that the P domain of a 'mild' variant was joined through the central conserved region to the V domain of a 'severe' variant (Visvader and Symons, 1986). Infection with the resulting chimera induced mild symptoms. The reverse construction induced severe symptoms, indicating that the P domain is the primary region responsible for modulating symptom expression.

Table 3-2 Location of sequence differences between 17 sequence variants of CEV and 6 sequence variants of PSTV

Information for CEV was obtained from Gross et al. (1982); Visvader et al. (1982); Visvader and Symons (1983, 1985). Information for PSTV was obtained from Gross et al. (1978); Schnölzer et al. (1985).

[a] Number of nucleotide residues in each domain (Figure 3-2) is given in parenthesis.

VIROID	NUMBER OF NUCLEOTIDE RESIDUES WHICH VARY IN VIROID DOMAIN				
	T1	P	C	V	T2
CEV	2(92)[a]	16(61)	5(97)	20(57)	0(64)
PSTV	0(91)	12(55)	0(93)	2(50)	0(64)

Several features in common between the P domains of PSTV, CSV, CEV, TASV, and TPMV are also found in HSV. HSV shows high sequence homology with PSTV in this domain of 58% compared to an overall sequence homology of 35% with PSTV (Table 3-1). These include an adenine-dominated oligopurine sequence of 15-17 nucleotide residues in one strand and an oligouridylate (4-7 nucleotide residues) sequence in the opposite strand. These features may represent common recognition signals that interact and possibly interfere with homologous host components, resulting in symptom expression.

A further possibility is that the P domain is also involved in determining the host range of each viroid. For example, the conserved features and sequence homologies within the P domain of PSTV, CEV, CSV, HSV, TASV and TPMV (Table 3-1) may reflect their overlapping host range of dicotyledonous plants, for example, tomato, potato, chrysanthemum and cucumber (Runia and Peters, 1980; Singh, 1983) which is quite distinct from the monocotyledonous palm family host range of CCCV (Imperial et al., 1985) and CTiV (Boccardo et al., 1985). The P domains of both CCCV and CTiV, although related to each other, show less sequence homology with the other viroids (Table 3-1). Instead they are notable for the presence of an oligoadenylate sequence in both strands of the P domain.

3-5 Sequence variability of the V domain

This is the most variable domain. Except between CCCV and CTiV, there is less than 50% sequence homology between the V domains of otherwise closely related viroids, such as between TASV and CEV-A, or TPMV and PSTV (Table 3-1). Similarly, different variants of CEV show considerable variation in this region (Visvader and Symons, 1985). It is the V domain, rather than the more highly conserved T2 domain (Table 3-1) that is responsible for the low sequence conservation reported for the right half portions of PSTV, CEV, CSV, TASV and TPMV (Sänger, 1982, 1984; Riesner and Gross, 1985). The only significant relationship between viroids in the V domain appears to be the presence of an oligo-purine:oligo-pyrimidine helix, usually with a minimum of three G:C pairs.

3-6 RNA rearrangements of the terminal T domains

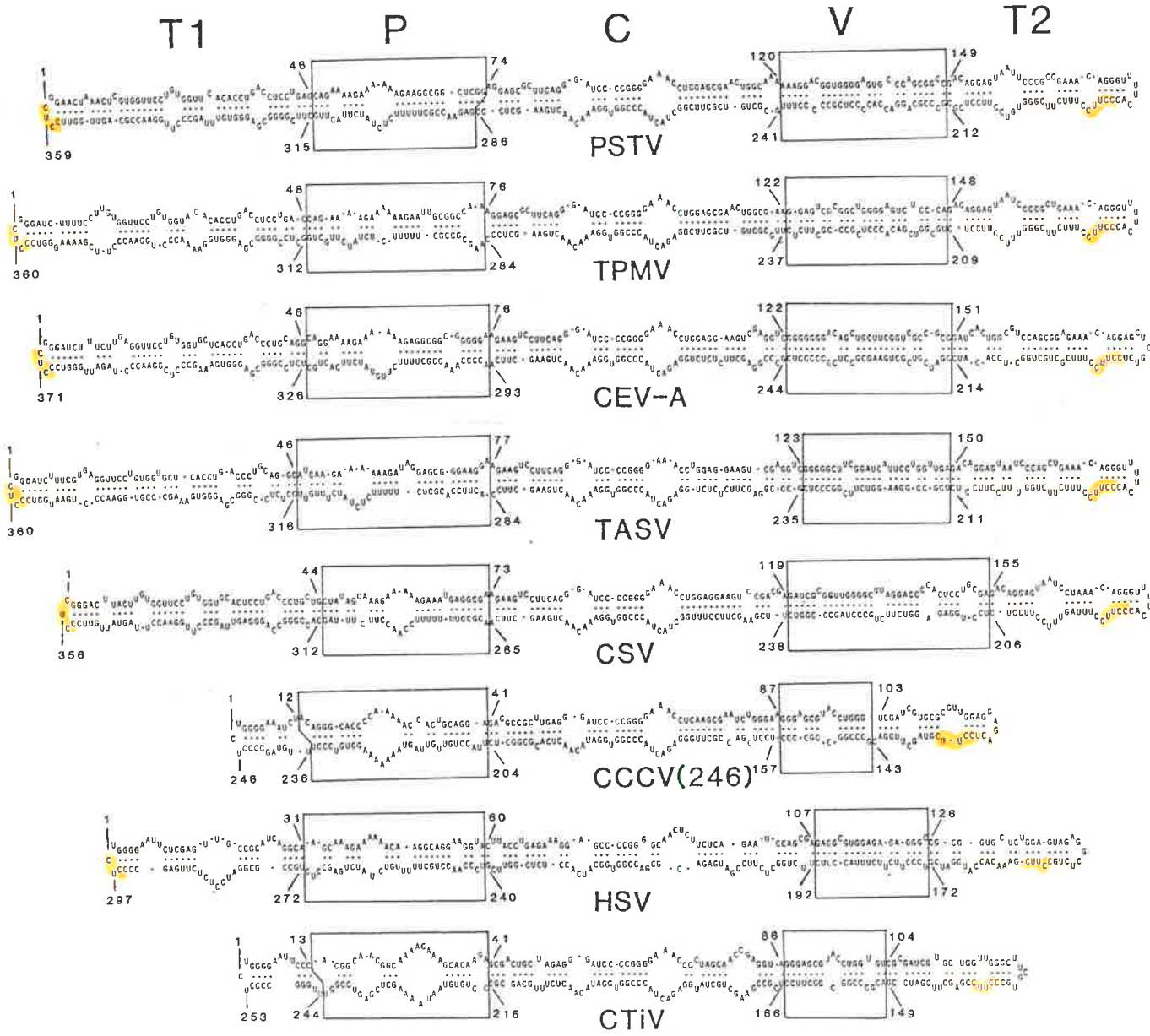
The functional role of these domains remains controversial. The termini of some PSTV linears with 2':3'-cyclic phosphates have been located in both the T1 and T2 domains (Kikuchi et al., 1982; Palukaitis and Zaitlin, 1983) suggesting an association with the processing site of viroid replicative intermediates. However, these linears could also have arisen by the nicking of circles in highly susceptible regions during isolation and purification. The T domains are also the

in-vitro preferential binding sites for purified tomato DNA-dependent RNA polymerase II (Goodman *et al.*, 1984), but the RNA replicase responsible for the replication of viroids *in vivo* as well as the site of initiation of RNA synthesis are unknown. Other roles for the T domains may also exist. For example, viroids are presumably transported across membranes to account for their systemic spread and site of accumulation which at least for PSTV, occurs in the nucleolus (Schumacher *et al.*, 1983). This movement of rod-like molecules may be achieved by structural signals and orientation of the end loops. These terminal hairpin loops show prominent sequence homology amongst all the PSTV-like viroids (Figure 3-2). Conserved sequences found for all PSTV-like viroids, including HSV, CCCV and CTiV include a CCUC in the T1 domain end-loop and a CCUUC sequence near the end-loop of the T2 domain (Figure 3-5).

In addition, sequence data show that CTiV, HSV, TASV and CCCV exhibit unusual relationships with respect to their terminal hairpin loop sequences. For example, TASV shares 73% overall sequence homology with CEV-A (Jane Visvader first noted the close sequence homology between these two viroids) but the T2 domains are only 46% homologous (Table 3-1). In contrast, TASV shares less overall sequence homology with PSTV (64%) but the T2 domains are highly homologous (90%). Therefore, TASV appears to be a recombinant between the T2 domains of a

Figure 3-5 Conserved sequences in the T1 and T2 domain of viroids

The strictly conserved sequences, CCUC and CCUUC, are coloured and occur in the same relative position of the T1 and T2 domains respectively.


T1
P
C
V
T2
PSTV
TPMV
CEV-A
TASV
CSV
CCCV(246)
HSV
CTiV

PSTV-ancestral viroid and all but the T2 domain of a CEV-ancestral viroid (Figure 3-6). CCCV (246) shares 70% sequence homology with the C domain of PSTV but only 23% sequence homology with respect to the T1 domains. In contrast, CCCV (246) shares low homology with the C domain of HSV (42%) but the T1 domains are more homologous (58%) since the T1 domain of CCCV (246) is almost identical to the left half of the HSV T1 domain (Figure 3-2). Therefore, CCCV appears to be a recombinant between a viroid with PSTV lineage and a T1 domain of an HSV-ancestral viroid. Similarly, the C and T2 domains of CTiV are more homologous to PSTV than to HSV but its T1 domain shares greater identity with HSV (see Chapter 2).

3-7 Sequence comparisons between ASBV and PSTV-like viroids

With respect to the conserved features of PSTV-like viroids, ASBV (Figure 3-7) can be distinguished in several ways from other viroids.

1) Low overall sequence homology. Whereas, all other viroids share 35% or greater overall sequence homology with PSTV, ASBV possesses only 20% overall sequence homology with PSTV when calculated as in Table 3-1. Previously, ASBV sequence homology with PSTV was reported to be 18% (Symons, 1981) while CCCV was considered to be less homologous to PSTV, reported by

Figure 3-6 Sequence homology between TASV and CEV-A: TASV and PSTV

Sequence homology between TASV and either CEV-A or PSTV is indicated by arrows. Sequence homology for the Tl, P and C domains is combined. The sequences highlighted in orange show high sequence homology between TASV and CEV-A and those highlighted in blue show high sequence homology between TASV and PSTV. The V domain shows low sequence homology between TASV and both CEV-A and PSTV.

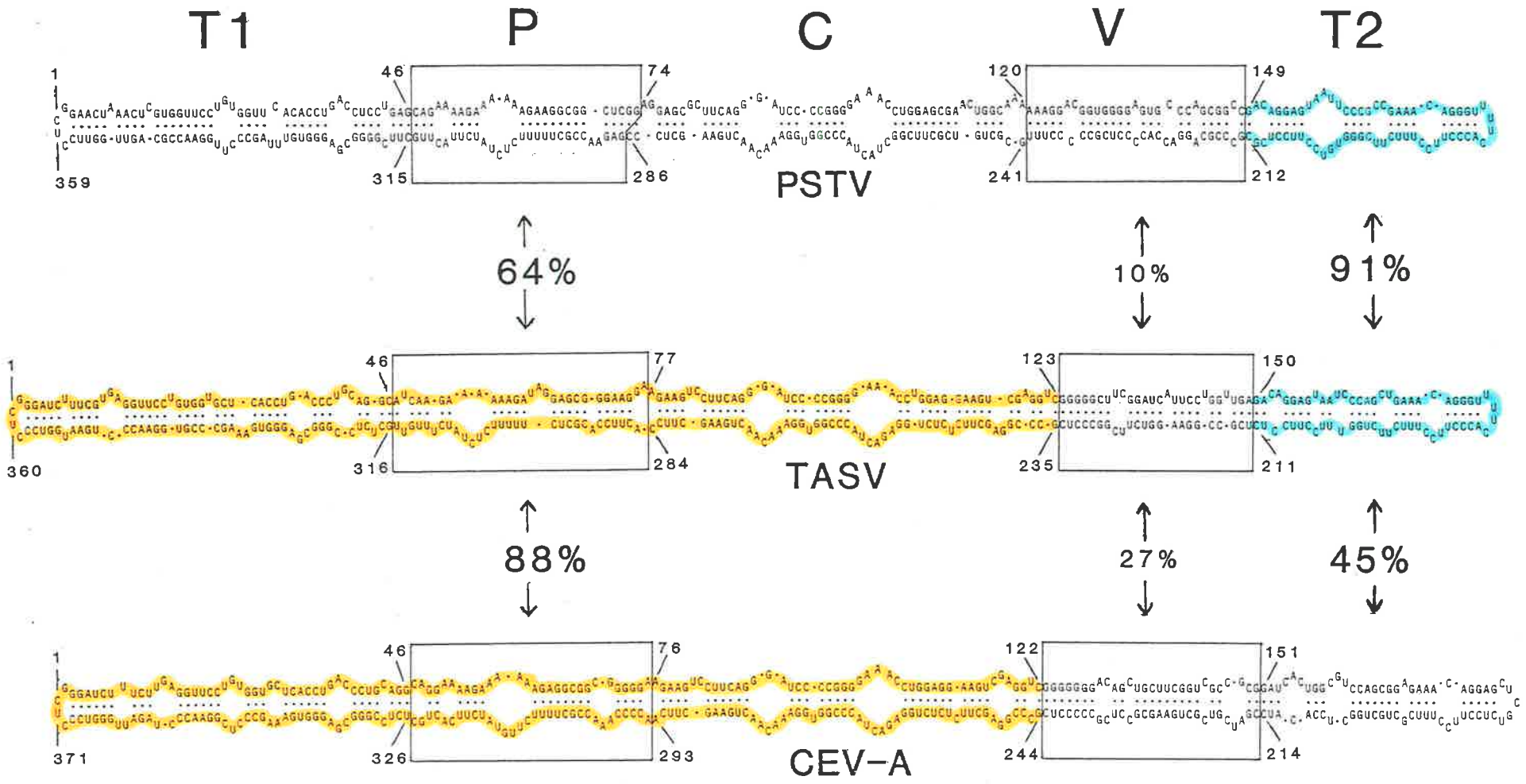
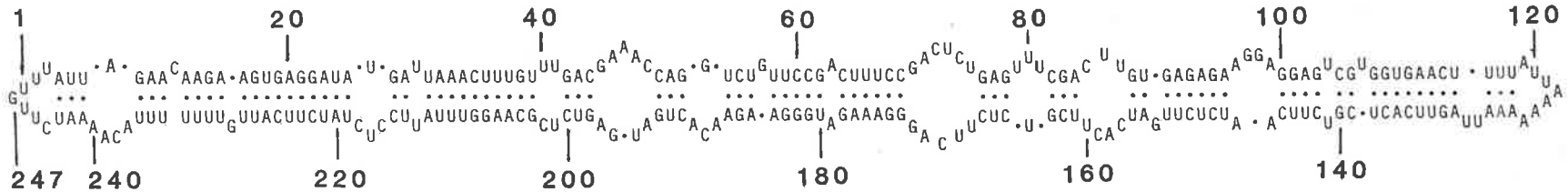

T1
P
C
V
T2
PSTV
TASV
CEV-A
64%
10%
91%
88%
27%
45%

Figure 3-7 Sequence and proposed secondary structure of ASBV (Symons, 1981).



ASBV

Haseloff et al. (1982) as showing 11% sequence homology. However, the sequence homology between CCCV and other viroids only included the central conserved region of viroids (Haseloff et al., 1982).

2) Lack of C domain conserved sequences and structures. No CC CCGG helix is present in the GGUGGCC postulated native structure of ASBV (Figure 3-6) [Symons, 1981] and there is a lack of any potential nine base-pair stem loop with a self complementary loop. Also lacking is the GGANCCCCGGGGNAAC sequence. The only conserved sequence reported to be also present in ASBV is GAAACC (Symons, 1981). This sequence corresponds to part of the above 16 nucleotide residue conserved sequence.

3) Lack of homology with T domain conserved sequences. The CCUUC sequence that occurs in the T2 domain of all PSTV-like viroids is absent in ASBV. The CCUC sequence found in the end loop of the T1 domain of PSTV-like viroids does not occur in either end loop of ASBV (Symons, 1981). The only CCUC sequence present (nucleotide residues 215-218, Figure 3-6) is not followed by at least two guanosines as in other viroids.

4) Differences in thermodynamic stability and cooperativity. The secondary structure of ASBV is less thermodynamically stable and shows less cooperativity than PSTV-like viroids and may be bifurcated (Steger et

al., 1984). ASBV has a higher ΔG/number of nucleotide residues and a lower T_m, with a larger half-width of structural transition (Steger et al., 1984). This may, in part, be explained by the A,U rich nature of ASBV compared to all other viroids which are G,C rich. The high A,U content of ASBV would be expected to have a pronounced effect on the secondary structure due to the large difference in stability of A:U base pairs compared to G:C base pairs (Kallenbach, 1968; Tinoco jr. et al., 1971, 1973; Steger et al., 1985).

The only other prominent sequence homology between ASBV and and the PSTV-like viroids is the presence of several adenine-dominated oligopurine sequences (such as nucleotide residues 7-25; 122-128; 175-188; or 237-242) analogous to those in the P domain of PSTV-like viroids.

3-8 Discussion

One of the notable features of viroid sequence homologies is that they are most readily correlated with the native rod-like secondary structure of the purified viroid as it behaves experimentally in solution or determined theoretically (Gross et al., 1978; Riesner et al., 1979, 1983; Steger et al., 1984). For example, the conserved sequences of PSTV-like viroids in the C domain as well as the more variable sequences in the P and V domains occur as complementary sequences in the secondary structures depicted in Figure 3-2. In

addition, conserved sequences occur at the same relative positions of each molecule such as the centrally placed GGANCCCCGGGGNAAC sequence that occurs in the centre of each molecule or the CCUC and CCUUC sequences that are located near the left-hand and right-hand end loops (Figure 3-2). This suggests that the postulated *in vitro* secondary structures are also the primary determinants of function *in vivo*.

Furthermore, the proposal here of five structural domains indicates that, despite the small size and the apparent lack of protein coding capacity, viroids may be multigenic with distinct functions corresponding to different regions of the molecule.

PSTV, CSV, CEV, TASV, and TPMV are closely related viroids (Haseloff and Symons, 1981; Gross *et al.*, 1982; Visvader *et al.*, 1982; Keifer *et al.*, 1983). Many of the sequence and structural similarities between these viroids extend to HSV, CCCV and CTiV. Since these similarities encompass most regions of each molecule, common ancestry appears likely for all PSTV-like viroids. The same structural plan, however, is not apparent for ASBV. Therefore, the few sequence and structural homologies that ASBV does share with other viroids may be due to chance homologies or reflect convergence.

Finally, there is the possibility that the evolution of some viroids such as TASV and CCCV may be

explained by recombination between different ancestral viroids. Intramolecular RNA rearrangements have been shown to naturally occur with other pathogenic RNAs. The defective interfering particles of animal viruses provide examples of both recombination between different viral segments such as the defective interfering RNA of Sindbis virus and a cellular tRNA (Monroe and Schlesinger, 1983). More recently a deletion mutant of the RNA 3 segment of the tripartite plant virus, brome mosaic virus, near the 3'-terminus (that is conserved in terms of sequence amongst all three segments of the virus) was shown to recombine with the homologous sequences of either RNA 1 or 2 during infection of individual plants (Bujarski and Kaesberg, 1986).

Other RNA rearrangements have been postulated for viruses on the basis of sequence homology between non-structural proteins of different plant and animal RNA viruses (Haseloff et al., 1984). Further examples of possible chimeric molecules include the virusoids of SCMoV (Haseloff, 1983; Symons et al., 1985) which show 95% sequence homology for the left hand 218 nucletide residues of each molecule and only 25% sequence homology for the remainder of each molecule. It was originally postulated that this was due to minimal functional constraints of the right hand regions of the virusoids of SCMoV (Haseloff, 1983) but may be due to recombination. Finally, bovine leukaemia virus, a

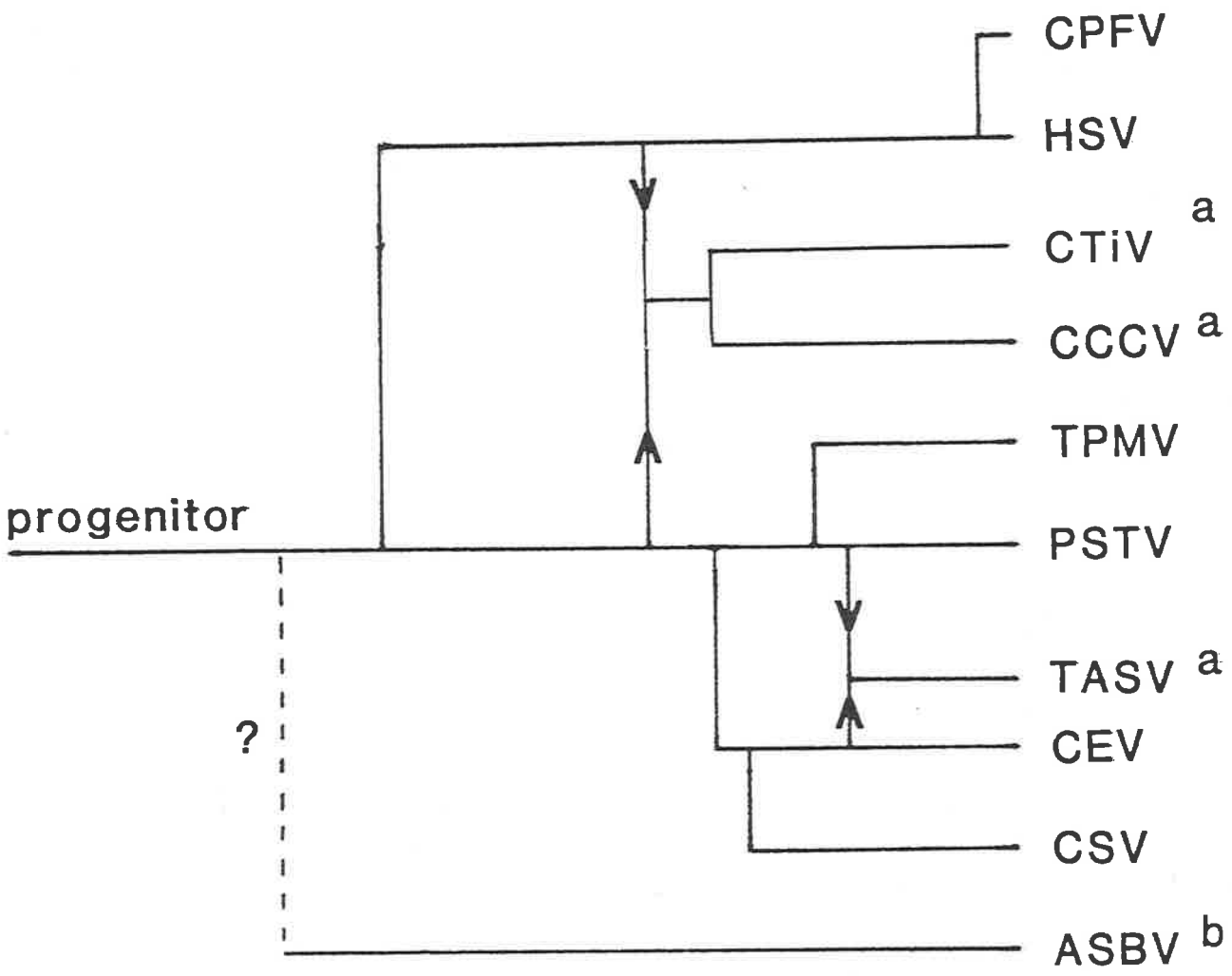
retrovirus, shows high amino acid sequence homology of its pol gene product with the pol gene product of Rous sarcoma virus but greater amino acid sequence homology of its env gene product with the env product of Moloney murine leukaemia virus (Sagata et al., 1985). Consequently, intermolecular RNA rearrangements may be a significant factor in the evolution of viroids as well as other pathogenic RNAs. Such RNA rearrangements between viroids would present an unusual evolutionary tree with transmission of genetic material both horizontally (between viroid 'species') as well as vertically (descent from a common ancestor) [Figure 3-8].

Contrary to the above viewpoint, Gross (1985) has suggested that even closely related viroids such as PSTV, CSV and CEV do not share a common viroid or non-viroid ancestor. The argument is based on the recent recognition this century of viroid diseases (Diener, 1979) and the apparent low mutation rate exhibited by geographic variants of the same viroid (Sänger, 1982). However, the natural hosts of viroids could well be symptomless non-cultivated plants such as Columnea erythrophae (the host of CV) [Owens et al., 1978]. It is presumably the intensive cultivation practices of the 20th century that has allowed the rapid spread and invasion of new hosts by viroids, giving a misleading impression of a recent origin for viroids. In addition,

Figure 3-8 A possible evolutionary tree for viroids based on sequence homology between viroids

a TASV and the common ancestor of CCCV and CTiV are considered to have arisen by RNA exchange between two separate viroid 'species' as indicated by arrows.

b ASBV shows low sequence homology with other viroids and may indicate a polyphyletic origin.


CPFV
HSV
CTiV a
CCCV a
TPMV
PSTV
TASV a
CEV
CSV
ASBV b
progenitor
?

there is no certainty that viroids have a low mutation rate. One isolate of CEV (CEV-J), when propagated in tomato, revealed nine sequence variants out of eleven full-length cDNA clones examined, with up to 26 nucleotide residue differences between sequence variants (Visvader and Symons, 1985). Also, the high mutation rates of RNA genomes during transcription (Holland et al., 1982) as well as the possibility of recombination between viroids as described above, indicate that rapid sequence changes amongst viroids may have occurred.

CHAPTER 4

A VARIANT OF COCONUT CADANG-CADANG VIROID

INTRODUCTION

Cadang-cadang (meaning death or dying) remains a serious and uncontrolled disease of coconut palms in the Philippines (Zelazny *et al.*, 1982). The first reliable report of the disease was in 1931 on San Miguel Island (Ocfemia, 1937) but may have been present earlier except for the difficulty in distinguishing the disease from other maladies of coconut palms. Cadang-cadang infects more than 200,000 palms a year over an area of greater than 40,000 square kilometres, thus posing a serious threat to the coconut industry. In the very early stage of the disease the palm is symptomless but the viroid can already be detected. The first symptom to appear is the rounding of newly developing nuts with equatorial scarification. Later on, chlorotic leaf spots appear and the inflorescences are stunted. Eventually, spathe, inflorescence and nut production declines and ultimately ceases while the leaf spots become more numerous and enlarged. In the last stage the fronds decline in size and number, and eventually the palm dies. The average duration of the disease symptoms in naturally infected palms ranges from 7-16 years and is normally first detected only in mature palms.

Viroid aetiology was implicated by the isolation of a low molecular weight RNA from infected but not from healthy coconut palms (Randles, 1975). Further structural studies of this RNA supported its viroid

designation (Randles et al., 1976; Randles and Hatta, 1979; Haseloff et al., 1982). In addition both partially and highly purified samples of the viroid-like RNA could be mechanically transmitted to healthy coconut palms (Randles et al., 1977). Although the involvement of a helper virus or pathogen has yet to be rigorously excluded, the ability to transmit disease using partially purified coconut cadang-cadang viroid (CCCV) to a number of other palm species (Imperial et al., 1985) as well as other coconut varieties from other countries (Anonymous, 1982) suggests true viroid status for CCCV.

Studies of CCCV has been complicated by the presence of a number of other sequence variants present in infected palms. These include a single nucleotide addition at position 198 (CCCV 246/247) [Haseloff et al., 1982], partial duplications of 41, 50 or 55 nucleotide residues (CCCV 287, CCCV 296/297, CCCV 301) and dimers of all forms (Haseloff et al., 1982; Mohamed et al., 1982). It has been proposed that the partially duplicated forms arise during the progression of the disease (Imperial et al., 1981; Haseloff et al., 1982; Mohamed et al., 1982). Recently, another electrophoretic variant was observed by J.S. Imperial and M.J.B. Rodriguez (unpublished results) in two palms in the most advanced stages of the cadang-cadang disease. This RNA migrated between CCCV 297 and a dimer of CCCV (CCCV

492). This variant was sequenced to confirm its identity and to further elucidate the role of these variants with the progression of the cadang-cadang disease.

MATERIALS

Source of a variant of CCCV

A partially purified nucleic acid extract of coconut leaves infected with an unusual electrophoretic variant of CCCV was generously provided by J.S. Imperial, Philippine Coconut Authority, Albay Research Centre, Philippines. This extract was further purified by two-cycle polyacrlyamide gel electrophoresis method described by Palukaitis and Symons (1980) and kindly provided by J.L. Cassady and Dr. J.E. Visvader.

Enzymes

Restriction endonucleases were obtained from New England Biolabs. Moloney murine leukaemia virus reverse transcriptase and RNase H were obtained from Bethesda Research Laboratories. Bacteriophage T_4 DNA ligase, *E. coli* DNA polymerase I and the Klenow fragment thereof were obtained from BRESA Pty. Ltd. Calf intestinal phosphatase was obtained from Sigma Chemical Co. and purified free of nucleases by Dr. R.H. Symons and Dr. A. Rezaian.

Radioisotopes

Alpha-^{32}P-dATP and Alpha-^{32}P-dCTP (both 1700 Ci/mmol) were obtained from BRESA Pty. Ltd.

Synthetic oligodeoxyribonucleotides

The CCCV-specific oligodeoxynucleotide primers were kindly synthesised by Stephen Rogers and Roger Smyth. The M13-specific 17-nucleotide residue primer was obtained from New England Biolabs.

Bacterial strains and cloning vectors

E. coli JM101 and the vectors M13mp18 and M13mp19 were generously provided by Dr. A. Robins.

Growth media for E. coli JM101

a) Minimal salts media: 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g sodium citrate per litre, supplemented after autoclaving with 0.8 µl of 1 M $MgSO_4$, 0.5 µl of 1% thiamine-HCl and 10 ml of 20% (w/v) glucose. Minimal agar contains minimal media with 1.5% (w/v) bacto-agar (Difco).

b) YT broth : 8 g bacto-tryptone (Difco), 5 g yeast extract (Difco) and 5 g NaCl per litre.

c) 2 X YT broth : 16 g bacto-tryptone, 10 g yeast extract and 5 g NaCl per litre.

Chemicals

Deoxyribonucleotide and dideoxyribonucleotide triphosphates and isopropylthiogalactoside (IPTG) were obtained from Sigma Chemical Co. 5-bromo-4-chloro-3-

indolyl-galactoside (BCIG) was from Bethesda Research Laboratories.

METHODS

4-1 Sequencing of CCCV by partial RNA enzymic hydrolysis

As for Chapter 2 except that partial enzymic hydrolysis with only RNase T_1 (2,500 units/ml) was used for generating linear overlapping fragments.

4-2 Cloning of CCCV cDNA

A. Synthesis of double-stranded cDNA

For first strand cDNA synthesis, 1 µg of RNA from a sequence variant of CCCV and 0.4 µg of DNA primer 5'-d(GTTTCCCCGGGGATCC)-3'or 5'-d(GATGGGCCACCT[A/T]TGTTG)-3' were heated at 100°C for 1 min, snap cooled on ice, and then inoculated with 200 units of reverse transcriptase in a 25 µl reaction mixture (essentially as described in Bethesda Research laboratories catalogue) containing 50 mM Tris-HCl pH 7.4, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dCTP, dGTP, and dTTP, 0.1 mM dATP, and 0.0012 mM alpha-^{32}P-dATP (1700 Ci/mmol). After incubation at 37°C for 30 min, 1 µl of 10 mM dATP was added and the reaction was continued for a further 30 min. The reaction was terminated by extraction with phenol:chloroform (1:1), followed by ethanol precipitation. The products were resuspended in 10 mM

Tris-HCl, 0.1 mM EDTA, pH 8.0, and precipitated with 2 mM spermine at 0°C for 15 min (Hoopes and McClure, 1981). The products were then pelleted by centrifuging at 10,000 rpm for 10 min at 4°C. The pellet was washed with 0.3 M sodium acetate pH 5, 0.01 M magnesium acetate, 1 mM EDTA, 75% (v/v) ethanol for 60 min at 0°C, washed briefly with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 0.1 mM EDTA pH 8.0.

The reaction mixture (50 µl) for second strand cDNA synthesis (Gubler and Hoffman, 1983) contained 20 mM Tris-HCl pH 7.4, 100 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.03 mg/ml BSA (nuclease free), 1 mM DTT, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 20 units of *E. coli* DNA polymerasae I, 1 unit of RNase H and 1.25 units of T_4 DNA ligase.

After sequential incubation at 12°C for 60 min and 22°C for 60 min, the reaction was terminated by phenol:chloroform (1:1) extraction, followed by ethanol precipitation.

B. Restriction endonuclease cleavage and isolation of cDNA fragments

Double-stranded cDNA was digested with HaeIII and the resulting fragments were fractionated by electrophoresis on a 6% polyacrylamide gel containing 2 M urea and TBE buffer. The cDNA fragments were excised, eluted and ethanol precipitated.

C. Ligation of double-stranded cDNA restriction fragments

Purified fragments were ligated into M13mp18 or M13mp19 (linearised with restriction endonuclease SmaI and then dephosphorylated) in a 10 µl reaction mix containing 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP and 2.5 units of T_4 DNA ligase at 14°C for 5-8 h.

D. Transformation of E. coli JM101

An overnight culture of E. coli JM 101 in minimal media was subcultured (1/100 dilution) into 50 ml of 2 X YT broth and grown to an OD_{600} of 0.4-0.8. The cells were then pelleted by centrifugation (3,000 rpm, HB-4 rotor, Sorvall, 5 min, 4°C) and resuspended in 5 ml of cold 50 mM $CaCl_2$. Cells were left at 0°C for at least 1 h prior to transformation.

The competent cells (200 µl) were mixed with 5 µl of ligated DNA and left at 0°C for 40 min. The cells were then heat-shocked at 42°C for 2 min before the addition of 3 ml of 0.7% YT agar containing 20 µl of 20 mg/ml BCIG and 10 µl of 100 mM IPTG. The mixtures were plated directly onto minimal agar plates and grown overnight at 37°C.

4-3 Preparation of phage DNA for sequence analysis

Recombinant plaques (white) were toothpicked into 1.8 ml of YT broth containing JM101 cells (1/100 dilution of fresh overnight culture) and incubated at

37°C for 6-7 h. Bacteria were removed by two centrifugations and bacteriophage were precipitated from the supernatant by the addition of 0.1 volume of 40% (w/v) PEG 8000 (saturated with NaCl) and standing at room temperature for 20 min. Bacteriophage were collected by centrifugation for 5 min and resuspended in 100 µl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. The bacteriophage protein coats were removed by extraction with 50 µl of phenol saturated with 10 mM Tris-HCl pH 8.0. Bacteriophage DNA was ethanol precipitated, washed with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 30 µl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 .

4-4 Sequence determination of CCCV cDNA clones

Recombinant M13 bacteriophage DNA was sequenced by the dideoxynucleotide chain termination method of Sanger *et al.* (1980) using alpha-^{32}P-dATP and the M13-specific 17 nucleotide residue primer. After fixing the sequencing gel with 10% (v/v) acetic acid, the gel was washed with 20% (v/v) ethanol prior to drying on the glass plate for autoradiography.

4-5 Containment facilities

All manipulations involving recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

RESULTS

4-1 Sequence analysis of a sequence variant of CCCV by partial enzymic hydrolysis.

Sequencing was performed with a cadang-cadang specific RNA species whose mobility on denaturing polyacrylamide gel electrophoresis was intermediate to the circular forms of the virusoids of LTSV (324 nucleotide residues) and VTMoV (366 nucleotide residues) [Figure 4-1]. The same method as in Chapter 2, Methods 3-2, was initially used to determine the sequence, except that only a RNase T_1 partial hydrolysate was employed to generate linear, overlapping fragments. This method indicated that the sequence was essentially the same as CCCV (296) but with added partial duplications.

4-2 Synthesis and cloning of CCCV double-stranded cDNA restriction fragments.

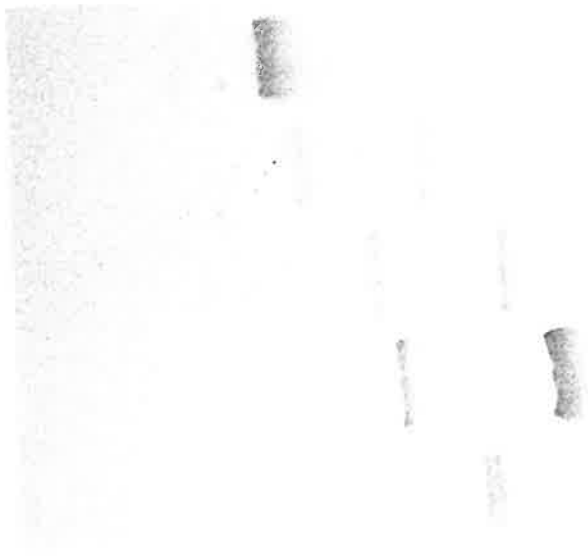
As a more rapid means of establishing the sequence, it was decided to sequence the RNA indirectly by cloning double-stranded cDNA into the replicative form of bacteriophage M13.

The scheme used for obtaining double-strand cDNA is depicted in Figure 4-2. Complementary DNA was synthesised with Moloney murine leukaemia virus reverse transcriptase after hybridising one of two oligodeoxyribonucleotide primers to the RNA. The cDNA

Figure 4-1 Mobility of an electrophoretic variant of CCCV.[a]

The mobility of an electrophoretic variant of CCCV[a] observed by J.S. Imperial (personal communication) was compared with CCCV (246 and 296 nucleotide residues, kindly purified and provided by Dr R.H. Symons), and the virusoids of VTMoV and LTSV-NZ on a 5% polyacrylamide gel containing 7 M urea and TBE run at 20 mA and stained with toluidine blue-O.

In each track the slower migrating band corresponds to circular RNA forms and the faster migrating band to full length linear RNA forms.



CCCV (246)

CCCV (296)

CCCV[a]

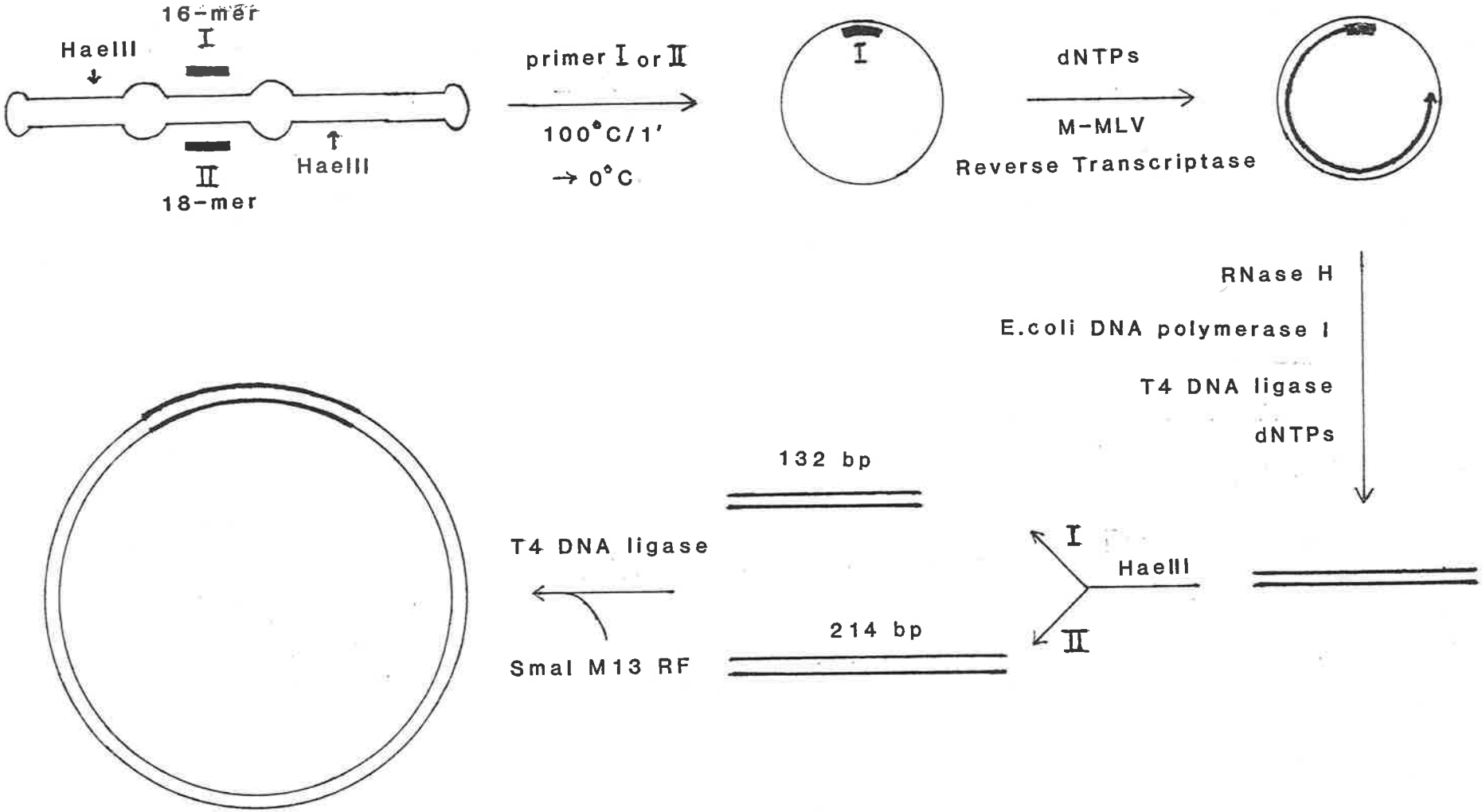
VTMoV virusoid

LTSV virusoid

O

Figure 4-2 Strategy for the construction of cDNA clones to a variant of CCCV

Full details of this procedure are given in Methods and Results. Primer I is 5'-GTTTCCCCGGGGATCC-3' and Primer II is 5'-GATGGGCCACCT(A or T)TGTTG-3'.


16-mer
I
HaeIII
II
18-mer
HaeIII
primer I or II
100°C/1'
→ 0°C
I
dNTPs
M-MLV
Reverse Transcriptase
RNase H
E.coli DNA polymerase I
T4 DNA ligase
dNTPs
132 bp
I
HaeIII
II
214 bp
T4 DNA ligase
SmaI M13 RF

was converted to double-stranded DNA and then digested at two potential sites with HaeIII restriction endonuclease to obtain two adjoining fragments. One HaeIII fragment (132 base pairs) was generated when using primer I (Figure 4-2, 4-3). A second HaeIII fragment (214 base-pairs) was generated when using primer II (data not shown). A third anomalous HaeIII fragment (92 base-pairs) seen in Figure 4-3 corresponds to restriction endonuclease cleavage at nucleotide residues 43-46 and a possible cryptic HaeIII site $\begin{matrix} CG & CG \\ GC & GC \end{matrix}$ at nucleotide residues 289-292. These three HaeIII fragments were ligated into the SmaI site of the replicative form of either M13mp18 or M13mp19.

4-3 Sequence determination of a sequence variant of CCCV cDNA recombinant clones

Sequencing of cloned HaeIII restriction fragments was by the dideoxynucleotide chain termination method (Sanger *et al.*, 1980) [Figure 4-4].

In accordance with the size of the RNA and by homology with the other sequence variants of CCCV, the two largest HaeIII fragments (214 and 132 base-pairs) are presumed to correspond to adjoining sites of a circular molecule of 346 nucleotide residues. The sequence, together with its postulated secondary structure is depicted in Figure 4-5. Except for an uridylate residue at position 31, the RNA has the same

Figure 4-3 Restriction endonuclease cleavage of CCCV double-stranded cDNA

Double-stranded cDNA synthesised using primer I as in Figure 4-2 was digested with HaeIII restriction endonuclease and then electrophoresed on a 40 cm, 5% polyacrylamide gel in the presence of 2 M urea. Two bands of 132 and 92 base pairs were isolated from the HaeIII digest. The smallest fragment arises from presumed restriction endonuclease cleavage at a site within the larger fragment.

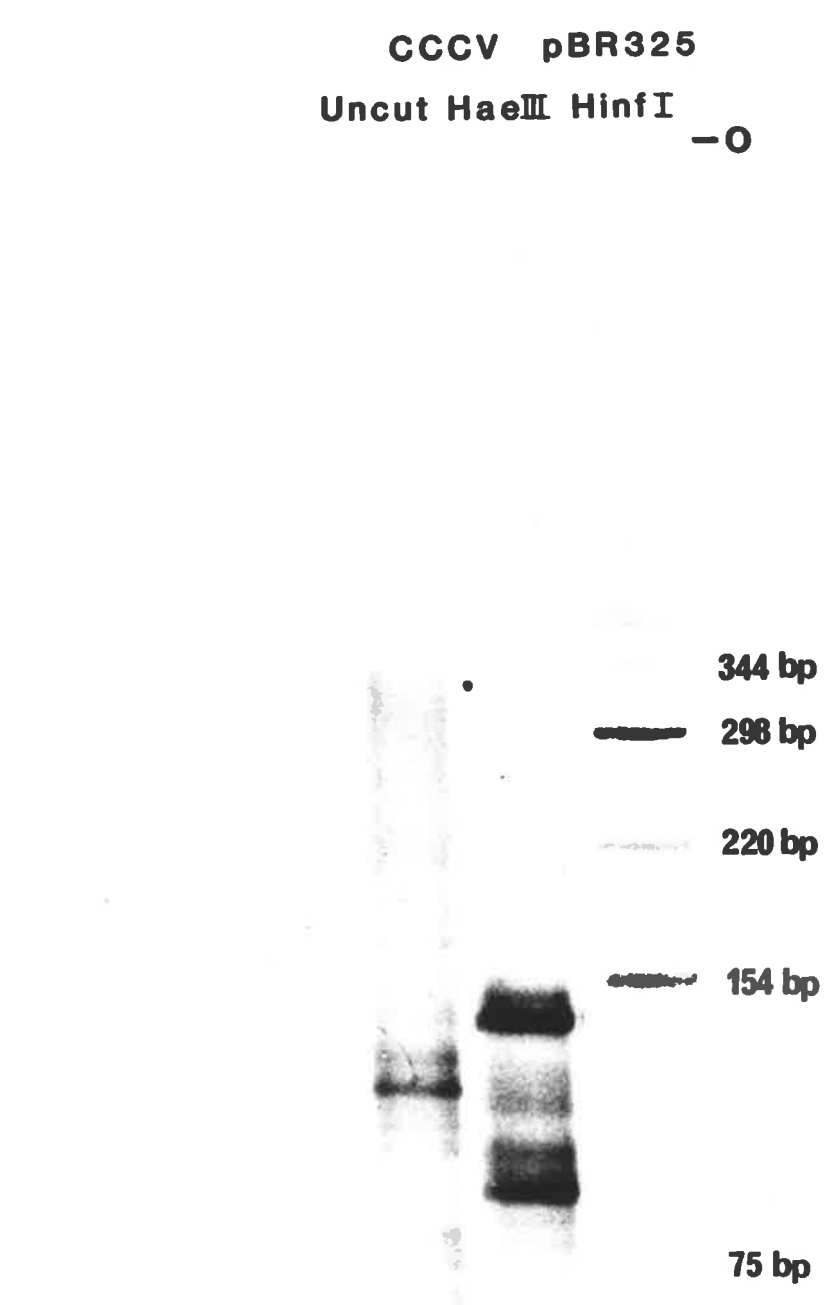

CCCV pBR325
Uncut HaeIII HinfI
344 bp
298 bp
220 bp
154 bp
75 bp

Figure 4-4 Sequence determination of cDNA clones of a sequence variant of CCCV

Recombinant phage M13mp93 was sequenced by the dideoxynucleotide chain termination technique using the 17-nucleotide residue M13-specific primer. The reaction mixtures were electrophoresed on a 40 x 20 x 0.025 cm, 6% polyacrylamide/7 M urea/TBE gel at 1100 volts. The sequence corresponding to a fragment generated from primer I in M13mp18 (Figure 4-2, 4-3) is complementary to the viroid, while the sequence corresponding to a fragment generated from primer II in M13mp19 (Figure 4-2) is of the same orientation as the viroid.

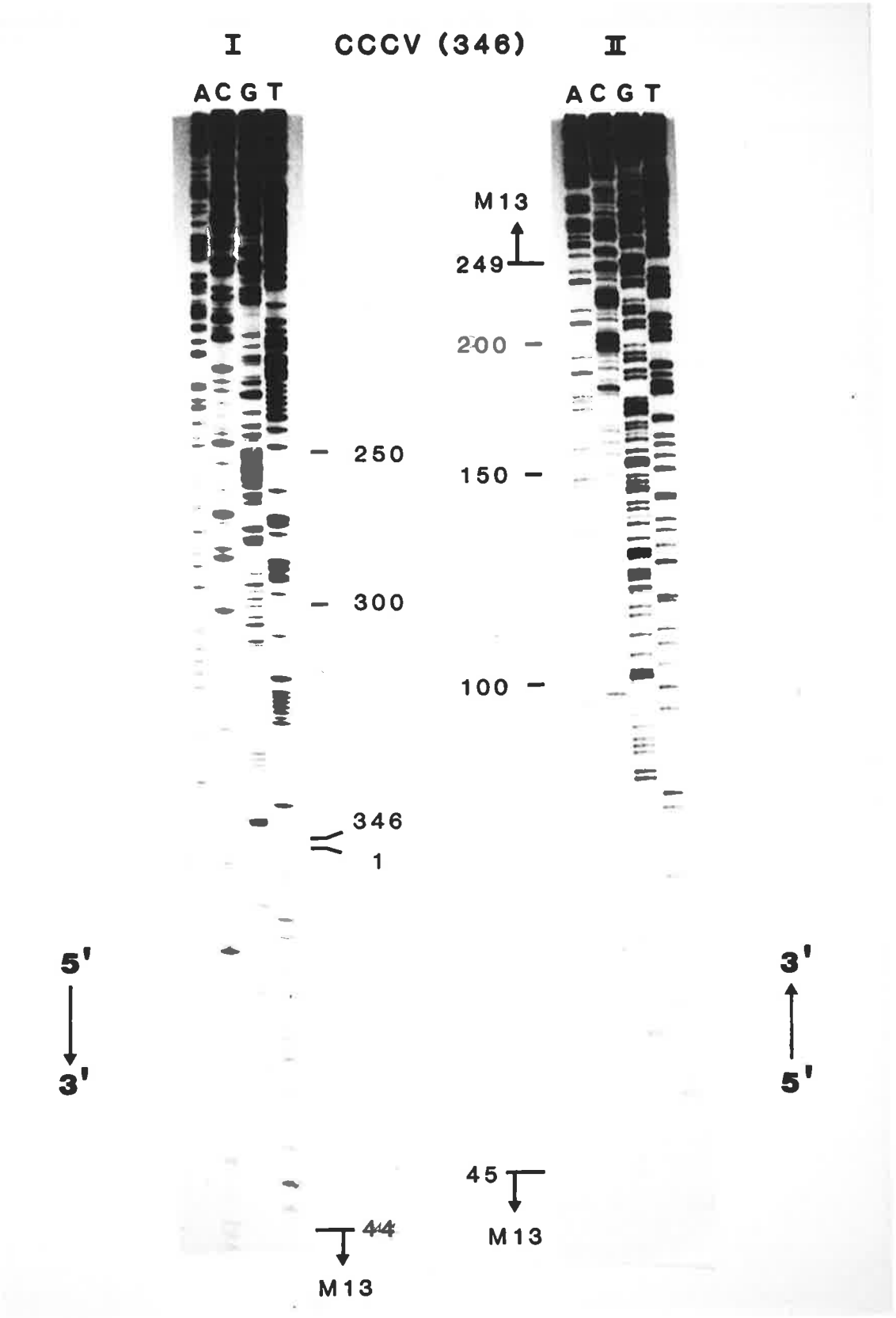

I
CCCV (346)
II
A C G T
A C G T
M13
249
200
150
100
250
300
346
1
5'
3'
3'
5'
45
M13
444
M13

Figure 4-5 Sequence and proposed secondary structure of CCCV (346)

CCCV (346)

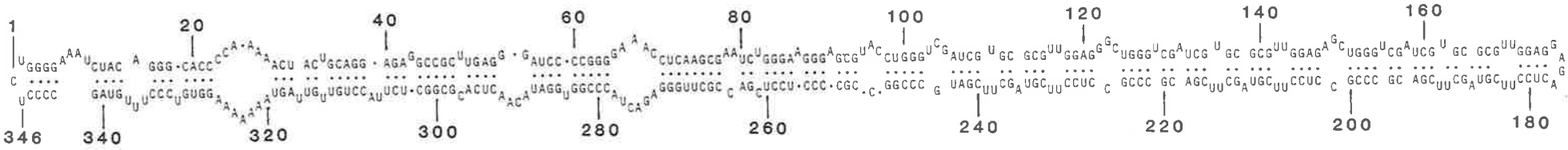
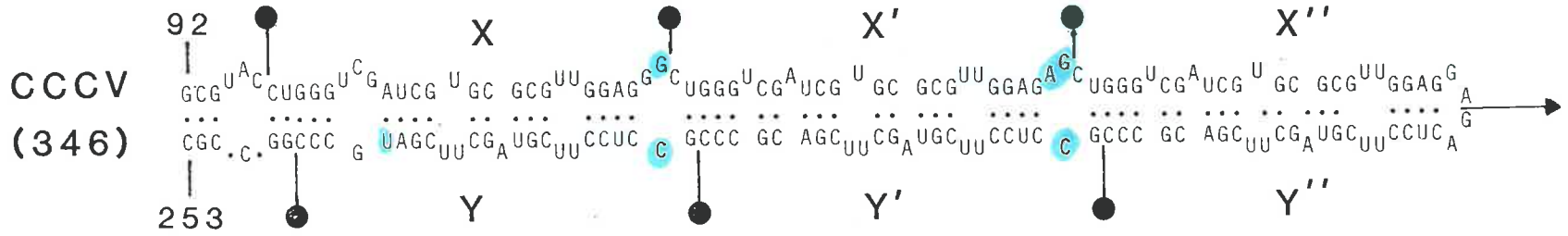

1
20
40
60
80
100
120
140
160
346
340
320
300
280
260
240
220
200
180

Figure 4-6 Partial sequence duplications within CCCV (346)

The partial duplications of the 346 nucletide variant of CCCV. X, X' and X'' are sequence related as are Y, Y' and Y''. Coloured nucleotide residues are non-homologous with CCCV (246). Filled in circles piont to approximate sites of recombination.


CCCV
(346)
92
253
X
Y
X'
Y'
X''
Y''

sequences present as reported for CCCV (246) by Haseloff et al. (1982) [CCCV (246) shows a cytidylate residue at position 31]. The presence of an uridylate residue at position 31 in CCCV (246) is supported by the occurrence of a thymidylate residue in the corresponding position of a cDNA clone of the 246 nucleotide residue variant of CCCV (J.E. Visvader, personal communication).

DISCUSSION

RNA sequencing using partial enzymic hydrolysis indicated that CCCV (346) had essentially the same sequence complexity as CCCV (246) but included duplications in the same region as those reported for CCCV (287), CCCV (296/297) and CCCV (301) [Haseloff et al., 1982]. Confirmation of the sequence was achieved by dideoxynucleotide chain termination sequencing of cDNA clones in M13. Two synthetic oligodeoxynucleotides complementary to opposite regions of the molecule were utilised to prime cDNA synthesis of the entire molecule.

CCCV (346) consists of four adjacent partial duplications totalling 100 nucleotide residues (Figure 4-6). The partial duplications are so arranged that the postulated secondary structure of the purified molecule retains the rod-like form of the smallest CCCV sequence variant CCCV (246) [Haseloff et al., 1982]. This provides added support for the view that the rod-like

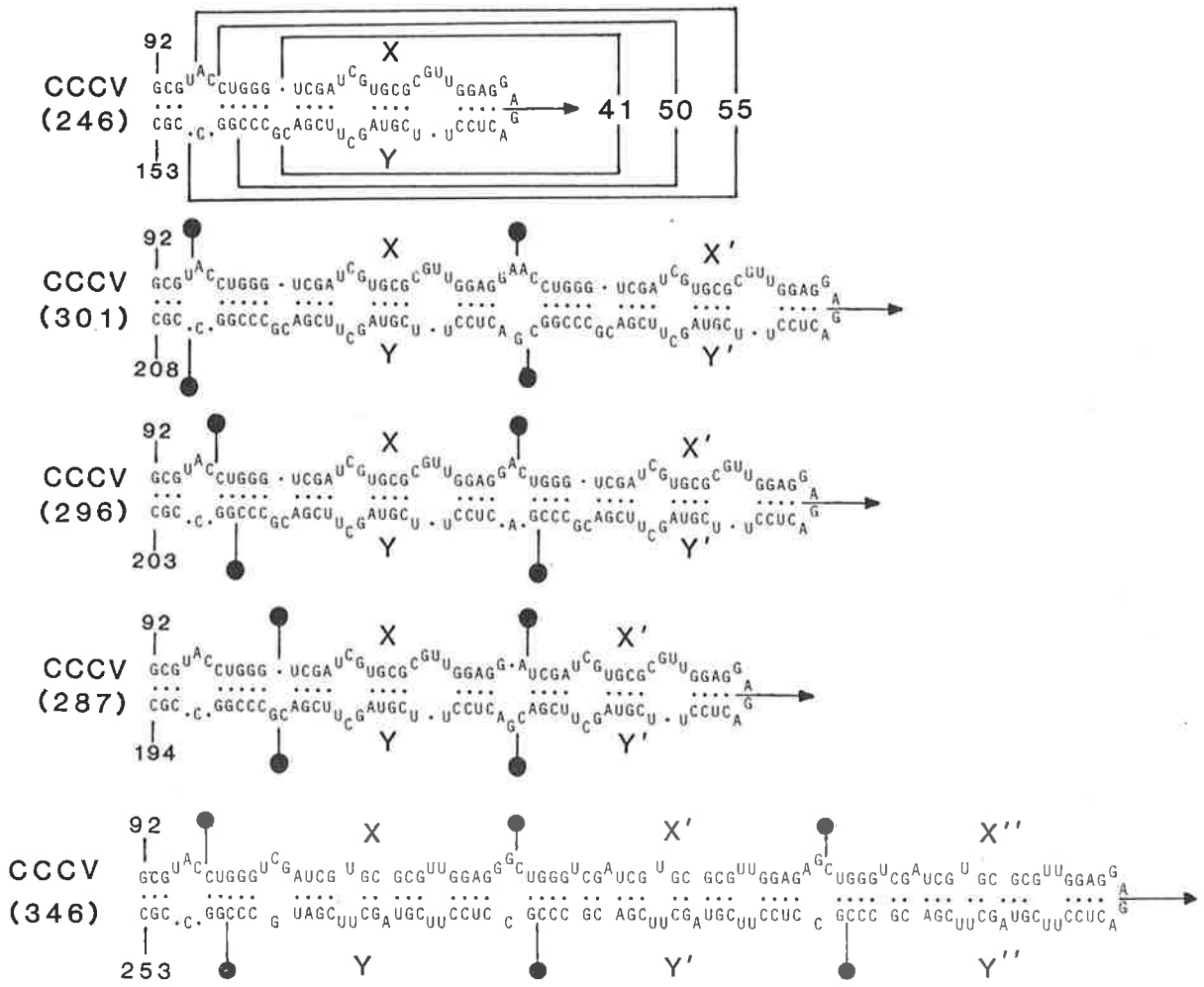
structure first predicted for PSTV by Langowski *et al.* (1978) is biologically significant *in vivo*.

Unlike CCCV (287), CCCV (296/297) and CCCV (301) whose partial duplications are strictly homologous to the corresponding sequences in CCCV (246) there is sequence heterogeneity at the junctions of the four partial duplications of CCCV (346) (Figure 4-6).

The means of CCCV transmission from palm to palm remains unknown. Therefore, it is uncertain as to when and how these different sized variants of CCCV (Figure 4-7) arise. Following the progression of the disease in the field over seven years allowed observation of changes within individual palms from the CCCV (246 and/or 247) sequence variants to a 20% larger variant (Anonymous, 1982). In addition, screening of individual fronds from a single palm showed changes from the CCCV (246 and/or 247) sequence variant in older fronds to a larger sequence variant in newly developing fronds (Imperial *et al.*, 1981). Therefore the larger variant appeared only in older palms after the appearance of the CCCV (246 and/or 247) sequence variant since there was no apparent migration of the viroid from younger fronds to older fronds. Consequently, it has been postulated that the CCCV (287), CCCV (296 and/or 297) and CCCV (301) sequence variants arose *de novo* independently in each palm from the small CCCV (246 and/or 247) sequence variant that is the only variant found early in

Figure 4-7 Partial sequence duplications of CCCV (246)

Partial sequence duplications of the 246 nucleotide sequence variant of CCCV are shown. Two adjacent sequences (X,Y) of variable size are repeated, leading to a double duplication of either 41, 50 or 55 nucleotide residues (CCCV [287]; CCCV [296]; CCCV [301]) or a quadruple duplication of 100 nucleotide residues (CCCV [346]). The arrow depicts the boundary of the X and Y sequences. The filled in circles mark the boundaries of the duplicated sequences.


CCCV
(246)
92
153
X
Y
41
50
55
CCCV
(301)
92
208
X
Y
X'
Y'
CCCV
(296)
92
203
X
Y
X'
Y'
CCCV
(287)
92
194
X
Y
X'
Y'
CCCV
(346)
92
253
X
Y
X'
Y'
X''
Y''

infection (Imperial et al., 1981; Haseloff et al., 1982; Mohamed et al., 1982). Recent evidence supporting this claim is provided by mechanical inoculations of the primary shoot with partially purified CCCV (246 and/or 247) that resulted in the appearance of these small sequence variants six months after inoculation and CCCV (296 and/or 297) after ten months (M. E. Keese, personal communication).

In accordance with the above view it is suggested that CCCV (346) also arose during infection of individual palms by a smaller sequence variant of CCCV. CCCV (346) was only observed in the last dying stages of the cadang-cadang disease (J.S. Imperial personal communication). It has yet to be excluded that the different size variants of CCCV cause different symptoms in coconuts. However, mechanical inoculations with the smallest CCCV (246) sequence variant is able to accelerate symptom development and induce late stage symptoms such as stunting and prevention of flower and nut development (Anonymous, 1982).

The sequence of events on those palms with CCCV (346) is predicted to be

healthy----> CCCV (246)----> CCCV (296)----> CCCV (346).

If this chain of sequence changes has occurred, then at least questions need to be addressed:

1) Why is CCCV (246 and/or 247) the only sequence variant found early in infection? Simple answers may be

that the larger sequence variants are poorly infectious or revert to the smaller variant at the initial stage of infection (see Zelazny et al., 1982). Alternatively, it may reflect the means of transmission, such as by pollen whereby the stigma is used as a site of entry. Consistent with this hypothesis is a) the high levels of CCCV present in pollen (J.S. Imperial, personal communication); b) only palms that have reached the bearing stage are normally susceptible to the disease; c) when the larger variants of CCCV appear, pollen production is minimal or has ceased (Zelazny et al., 1982). Infection of immature palms and other naturally infected palms such as buri palm (Corypha elata Roxb.) which does not flower after contracting cadang-cadang (Randles et al., 1980) may be rare cases of infected pollen entering other sites such as wounds. A corollary of the hypothesis that transmission of the cadang-cadang disease is by pollen is that inbreeding varieties of coconuts should limit spread of the disease in the field.

2) Why do the larger sequence variants of CCCV appear to have a selective advantage over the CCCV (246 and/or 247) sequence variant? Once a larger variant of CCCV becomes detectable it eventually becomes the dominant variant of CCCV to persist during infection. This may be due in some manner to the loss of cellular integrity with the progression of the disease.

Two points worthy of consideration however, are that the levels of larger sequence variants from infected tissue appear to be no greater than the CCCV (246) sequence variants and that the duplications retain the same structural arrangement found in CCCV (246). The only result, in effect, is to provide two T2 domains in the cases of CCCV (287), CCCV (296/297) and CCCV (301) and three T2 domains in the case of CCCV (346) [Figure 4-7]. Rather than a means to match in size the right hand arm of PSTV, CSV and CEV as suggested by Haseloff et al. (1982) the T2 domain may bind a host component essential for its replication (for example, purified DNA-dependent RNA polymerase II from tomato was shown to bind to the T1 and T2 domains of PSTV [Goodman et al., 1984]). Therefore, through a titration effect the larger sequence variants of CCCV will have a selective advantage over the CCCV (246) form. The CCCV (346) sequence variant having three T2 domains would potentially bestow a selective advantage over the CCCV (287), CCCV (296 and/or 297) and CCCV (301) sequence variants.

3) What is the mechanism whereby the larger sequence variants of CCCV arise? Presumably, the same mechanism is involved in all cases, either by recombination involving strand scission and ligation or by some form of non-processive transcription whereby extension of transcription switches to a different

template or to a non-adjacent nucleotide residue on the same template (variously referred to as template switching, copy choice, jumping or leaping polymerisation, or discontinuous transcription; see Perrault [1981] and Campbell et al. [1984]). Examples of the former cleavage and ligation mechanism include intron splicing (see Cech [1983]) or reversibility of self-cleaving RNAs such as the satellite RNA of tobacco ringspot virus, STobRV (Prody et al., 1986; Buzayan et al., 1986). The indications in these mechanisms are that precise sequence and structural signals may operate. However, the RNA rearrangements of the CCCV partial duplications occur at a number of neighbouring sites and in the case of CCCV (346) involve sequence heterogeneity at the junctions of recombination. In contrast, models of non-processive transcription are not limited by such requirements and have been variously proposed as the main mechanism for the generation of defective interfering particles (see Perrault, 1981), the leader sequences of influenza RNAs (see Krug, 1981) and trypanosome variant surface antigen genes (Campbell et al., 1984). Although there is lack of convincing experimental evidence however for non-processive transcription, the partial double duplications of CCCV (287), CCCV (296/297) and CCCV (301) could all arise as a single event by the same non-specific means of non-processive transcription.

By analogy with the influenza ribonucleoprotein complex, which is postulated as the structure for generating influenza defective interfering particles (Jennings et al., 1983), CCCV RNA may be coiled around host binding proteins or temporarily around a replicase complex unit. This would bring into juxtaposition sites of RNA rearrangements. Two non-processive transcription events during the generation of a single RNA transcript from a CCCV (246) template could give rise to one of the larger CCCV sequence variants (Figure 4-8). In an identical manner the CCCV (296) sequence variant could be the template for further double partial duplications to give rise to CCCV (346) with its quadruple partial duplications. Interestingly the sequence heterogeneity at the junctions of the duplication mirrors the heterogeneity of 4 or 5 nucleotide residues reported to occur at the junctions of RNA rearrangements with defective interfering RNAs of vesicular stomatitis virus (Keene et al., 1981) and influenza (Fields and Winter, 1981; Jennings et al., 1983). Thus the sequence heterogeneity in CCCV (346) may have arisen during non-processive transcription as postulated for vesicular stomatitis virus and influenza virus defective interfering RNAs and not as mutations after the duplications arose.

A single non-processive event would give rise to a single sequence duplication. This may be non-viable in

Figure 4-8 Proposed mechanism for the generation of RNA rearrangements by non-processive transcription

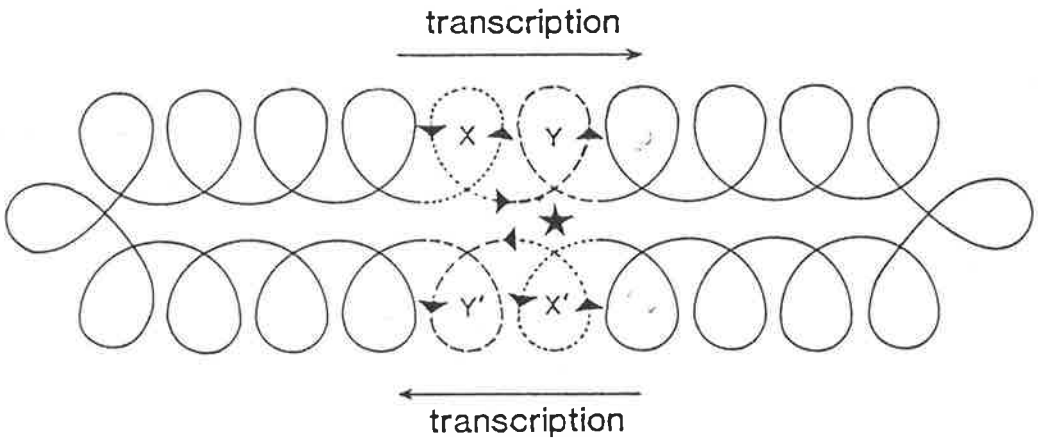
CCCV is represented here as a ribonucleoprotein replicating complex (adapted from Jennings et al., 1983) in which transcription occurs on a dimeric RNA template coiled around a protein core. Boundaries of RNA exchange are juxtaposed in this ribonucleoprotein structure (asterisk) and allow for the possibility of non-processive transcription. Consequently, two 'jumping' events by an RNA polymerase can generate the double partial duplications shown by CCCV (287), CCCV (296) or CCCV (301) [Figure 4-7]. For example:

$$\text{transcription} \xrightarrow{\quad} X \xrightarrow{1^{st}\ \text{jump}} X\ X' \xrightarrow{\quad} X\ X'Y' \xrightarrow{2^{nd}\ \text{jump}} X\ X'Y'Y.$$

Similarly CCCV (346) could arise by a further two non-processive transcriptional events, such that transcription would result in the following:

$$\text{transcription} \xrightarrow{\quad} X\ X' \xrightarrow{1^{st}\ \text{jump}} X\ X'X'' \xrightarrow{\quad} X\ X'X''Y' \xrightarrow{2^{nd}\ \text{jump}} X\ X'X''Y''Y'Y.$$

A similar scheme can be applied to a monomeric template.

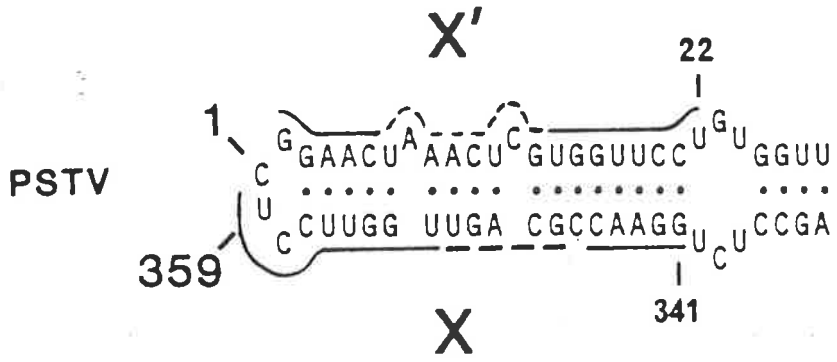

transcription
X
Y
Y'
X'
transcription

the case of CCCV but may have occurred in other viroids, for example, PSTV. The T1 domain of PSTV is the least sequence homologous amongst the viroids PSTV, TPMV, CSV, CEV and TASV (Table 3-1). In addition, the T1 domain of TPMV is more closely aligned with the T1 domains of other viroids, such as CEV-A (80% sequence homology), than to the T1 domain of PSTV (67% sequence homology) despite the high overall sequence homology between TPMV and PSTV (76%) (Table 3-1). This is due to a distinctive region of PSTV from nucleotide residues 341-22 in which nucleotide residues 341-359 are homologous to nucleotide residues 2-22 (Figure 4-9). Pairs of homologous sequences in the same relative position of other viroids are not present.

Similarly, the intermolecular rearrangements postulated for the T domains of TASV, CCCV and CTiV may also occur by a similar mechanism of non-processive transcription during co-infection by two viroids in a common host.

Figure 4-9 Possible partial duplication within PSTV

Part of the T1 domain of PSTV has a sequence in which 13 nucleotide residues (continuous line) out of 19 in the region denoted by X (nucleotide residues 341-359) are repeated in the adjacent X' sequence (nucleotide residues 1-22). Dashed lines indicate non-homologous nucleotide residues. A possible partial duplication of PSTV in this region may account for the relatively low sequence homology that the T1 domain of PSTV shows with the T1 domains of other closely related viroids such as CSV, CEV-A, TASV or in particular TPMV (Table 3-1).


X'
22
1
PSTV
359
341
X

CHAPTER 5

LUCERNE TRANSIENT STREAK VIRUS

INTRODUCTION

Lucerne transient streak virus (LTSV) is a small isometric plant virus (about 30 nm in diameter) [Blackstock, 1974, 1978; Forster and Jones, 1979] and was originally isolated from lucerne crops (*Medicago sativa* L.) in Australia (LTSV-Aus) [Blackstock, 1974, 1978] and New Zealand (LTSV-NZ) [Forster and Jones,1979; Jones *et al.*, 1983]. More recently a third serologically related strain has been detected in lucerne crops from Canada (LTSV-Ca) [Paliwal, 1983].

In lucerne, LTSV causes chlorotic streaking along the lateral veins and sometimes distortion of the leaves. Blackstock (1978) reported a loss of 18% in dry matter yield from a field trial of infected lucerne. LTSV is sap transmissible to species from at least four plant families, although each strain differs somewhat in host range and symptomatology. No vectors have yet been reported (Blackstock, 1978; Forster and Jones, 1979; Paliwal, 1984a). Low seed transmission has been observed only for the Canadian strain of LTSV (Paliwal,1983).

The properties of LTSV that align it with the Sobemovirus group include, a sedimentation coefficient of 112-114S, a major coat protein of molecular weight about 32,000 , 18% single-stranded ribonucleic acid content and a covalently linked protein moiety essential for the infectivity of the RNA (Blackstock, 1978; Forster and Jones, 1979; Paliwal, 1983, 1984a). Each

strain of LTSV has been noted to also encapsidate a single low molecular weight RNA component that has circular and linear forms (Tien-Po et al., 1981); Paliwal, 1983). This latter characteristic is shared by three other related viruses, VTMoV (Randles et al., 1981), SNMV (Gould and Hatta, 1981) and SCMoV (Francki et al., 1983b). Francki et al., (1983b) were able to demonstrate a weak unidirectional serological reaction between LTSV antisera and SCMoV..

Viroid-like structural features of these low molecular weight components was first noted for the virusoids of VTMoV and SNMV, in particular, circularity and a high degree of structural cooperativity upon heating, as shown by the narrow temperature range of melting (Randles et al., 1981; Gould, 1981; Gould and Hatta, 1981). These features have since been shown to apply to the virusoids of LTSV (Tien-Po et al., 1981) and SCMoV (Francki et al., 1983b). The sequences of the virusoids from VTMoV, SNMV and SCMoV have since been obtained (Haseloff and Symons, 1982; Haseloff, 1983).

Preliminary RNA enzymic sequence data of the virusoids from the Australian and New Zealand strains of LTSV indicated high sequence homology (Keese, 1981). The RNA was transcribed into DNA, cloned into M13 and the sequence determined to establish the relationship between these two virusoids and other virusoids, as well as, viroids and other pathogenic RNAs.

MATERIALS

Strains of LTSV and host plants

Two strains of LTSV, LTSV-Aus and LTSV-NZ, were generously provided together with glasshouse facilities, by Dr. R.I.B. Francki (The University of Adelaide, Waite Institute, Glen Osmond, South Australia). Chenopodium quinoa Wild. used for passaging LTSV-Aus and Nicotiana clevelandii L. for passaging LTSV-NZ were kindly propagated and provided by D.W. Talfourd.

Enzymes

Calf-intestinal phosphatase was obtained from Sigma Chemical Co. and purified free of nucleases by Dr. R.H. Symons. Avian myeloblastosis virus reverse transcriptase was from Molecular Genetics Resources Inc. E. coli poly(A) polymerase, purified according to Sippel (1973), was kindly provided by J.L. Cassady.

Radioisotope

Alpha-^{32}P-dCTP (400-500 Ci/mmol) was kindly provided by Dr. R.H. Symons.

Bacteriophage cloning vectors

M13mp73, M13mp8 and M13mp93 were generously provided by Dr. A. Robins.

Synthetic oligonucleotides

The primer $(dT)_{10}$ was obtained from P.L. Biochemicals. The M13-specific 14-nucleotide residue sequencing primer was obtained from New England Biolabs.

METHODS

5-1 Purification of RNA from LTSV-Aus and LTSV-NZ

A. Extraction of virus from infected leaf tissue

Leaves from plants infected with LTSV-Aus and LTSV-NZ were extracted essentially as described by Tien-Po *et al.* (1981). Infected leaves were homogenised with 2 volumes of extraction buffer containing 70 mM sodium phosphate, 1 mM EDTA, pH 7.2, and 0.1% thioglycollic acid for 2 min in a Waring blendor. The resultant slurry was mixed with 2 volumes of $CHCl_3$:CCl_4 (1:1) at 4°C for 30 min, and then clarified by centrifugation at 10,000 rpm in a JA-14 rotor, Beckman, for 15 min at 4°C. The aqueous phase was then centrifuged at 48,000 rpm in a Ti-50 rotor, Beckman, for 90 min at 4°C. The pellet was resuspended in 70 mM sodium phosphate, 1 mM EDTA, pH 7.2 and centrifuged at 10,000 rpm in a JA-21 rotor, Beckman, for 15 min at 4°C. The supernatant was gently layered onto a 2 ml 20% (w/v) sucrose cushion and centrifuged at 48,000 rpm in a Ti-50 rotor, Beckman, for 75 min at 4°C. The subsequent pellet was resuspended in 20 mM Tris-HCl, 1 mM EDTA, pH 7.4 and 10.0 mM NaCl and microcentrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was then used for viral RNA extraction.

B. Purification of viral RNA

EDTA and SDS were added to the viral suspension to 10 mM and 1% (w/v) respectively. The viral RNA was then twice extracted with phenol:chloroform (1:1), ethanol precipitated and stored, resuspended in 0.1 mM EDTA pH 8.0.

C. Fractionation of Viral RNA

The viral RNA was fractionated on a 4-5% polyacrylamide gel with 7 M urea, stained with toluidine blue-O, and the virusoid (circular and linear bands) excised, eluted and ethanol precipitated.

5-2 Preparation and cloning of double-stranded virusoid cDNA restriction endonuclease fragments

A. Phosphatasing of virusoid RNA

A 2 µg mixture of circular and linear virusoid RNAs were heated at 100°C for 2 min in 10 mM Tris-HCl pH 9.0, snap cooled on ice and incubated with 0.1 units of phosphatase at 37°C for 20 min. The reaction was terminated by extraction with phenol:chloroform (1:1), followed by ethanol precipitation.

B. Polyadenylation of RNA

Phosphatase-treated RNA was resuspended in 47 µl of H_2O, heated at 80°C for 1 min and snap cooled on ice. The following solutions were then added: 3 µl of 10 mM ATP, 200 µl of 5X *E. coli* poly(A) polymerase buffer (comprising 105 µl H_2O, 50 µl of 1 M Tris-HCl pH 7.9, 25

µl of 0.1 M $MnCl_2$, 10 µl of 1 M $MgCl_2$ and 10 µl of 0.1 M DTT) and 30 µl of E. coli poly(A) polymerase extract. After incubation at 37°C for 45 min, 10 µl of E. coli poly(A) polymerase extract and 1 µl 10 mM ATP were added and was further incubated at 37°C for 15 min. The reaction was terminated by phenol:chloroform (1:1) extraction and ether washing; the RNA was then ethanol precipitated and dried in vacuo.

C. Synthesis of double-stranded cDNA

The reaction mixture (20 µl) for first strand synthesis (D.J. Kemp, personal communication) contained polyadenylated virusoid RNA of LTSV, 0.5 µg oligo$(dT)_{10}$, 50 mM Tris-HCl pH 8.3, 10 mM DTT, 10mM $MgCl_2$, 1 mM each of dATP, dTTP, and dGTP, 0.2 mM dCTP in the presence of 0.002 mM alpha-^{32}P-dCTP and 22 units of reverse transcriptase. Transcription was carried out at 42°C for 30 min, dCTP was added to a final concentration of 1 mM and incubated with a further 11 units of reverse transcriptase for 30 min, then terminated by boiling for 2 min and then cooled on ice.

The reaction mixture (50 µl) for second strand cDNA synthesis contained 30 mM Tris-HCl pH 8.3, 6 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of dATP, dTTP, dGTP and dCTP and 55 units of reverse transcriptase. After incubation for 6 h at 37°C, the reaction was terminated by phenol:chloroform (1:1) extraction, followed by ether washing and ethanol precipitation.

D. Restriction endonuclease cleavage and isolation of cDNA fragments

Double-stranded cDNA of LTSV-NZ virusoid was digested with the restriction endonuclease HaeIII and LTSV-Aus virusoid cDNA with restriction endonucleases HaeIII, TaqI or MspI and the resulting fragments were fractionated by electrophoresis on a 6% polyacrylamide gel containing 2 M urea and TBE buffer. The cDNA fragments were excised, eluted, ethanol precipitated and finally spermine precipitated.

E. Ligation and transformation

Purified fragments from the HaeIII digests were ligated in to the SmaI site of M13mp93 while the TaqI and MspI digests were ligated into the AccI site of M13mp73 (Messing and Vieira, 1982) using T_4 DNA ligase. The ligation mix was then used to transform E. coli JM101 competent cells.

5-3 Sequence determination of cDNA clones

Recombinant bacteriophage DNA was purified and sequenced by the dideoxynucleotide chain termination technique of Sanger et al. (1980) as described, Chapter 4.

RESULTS

5-1 Synthesis and cloning of double-stranded cDNA restriction fragments of the virusoids of LTSV

The linear forms of the virusoids of LTSV present in viral RNA preparations are composed of molecules with different 5'-termini, as indicated by sequence heterogeneity of 5'-terminal radiolabelled RNA (data not shown). Mixtures of these permuted linear RNA forms together with circular RNA forms of the virusoids of LTSV-Aus and LTSV-NZ were heated at 100°C for 2 min in 10 mM Tris-HCl pH 9.0 (calculated at 22°C), prior to polyadenylation. This heating step may cause additional cleavages due to the presence of trace divalent cations. Complementary DNA synthesis of polyadenylated RNA was performed with avian myeloblastosis virus reverse trancriptase, utilising oligo(dT_{10}) as a primer. After conversion to double-stranded DNA, the virusoids of LTSV-Aus cDNA were cleaved with either restriction endonucleases HaeIII, MspI or TaqI and the virusoid of LTSV-NZ cDNA with restriction endonuclease HaeIII (Figure 5-1). The appropriate restriction fragments were ligated into either the SmaI site of M13mp8 or M13mp93 or the AccI site of M13mp73.

5-2 Sequence of the virusoids of LTSV-Aus and LTSV-NZ

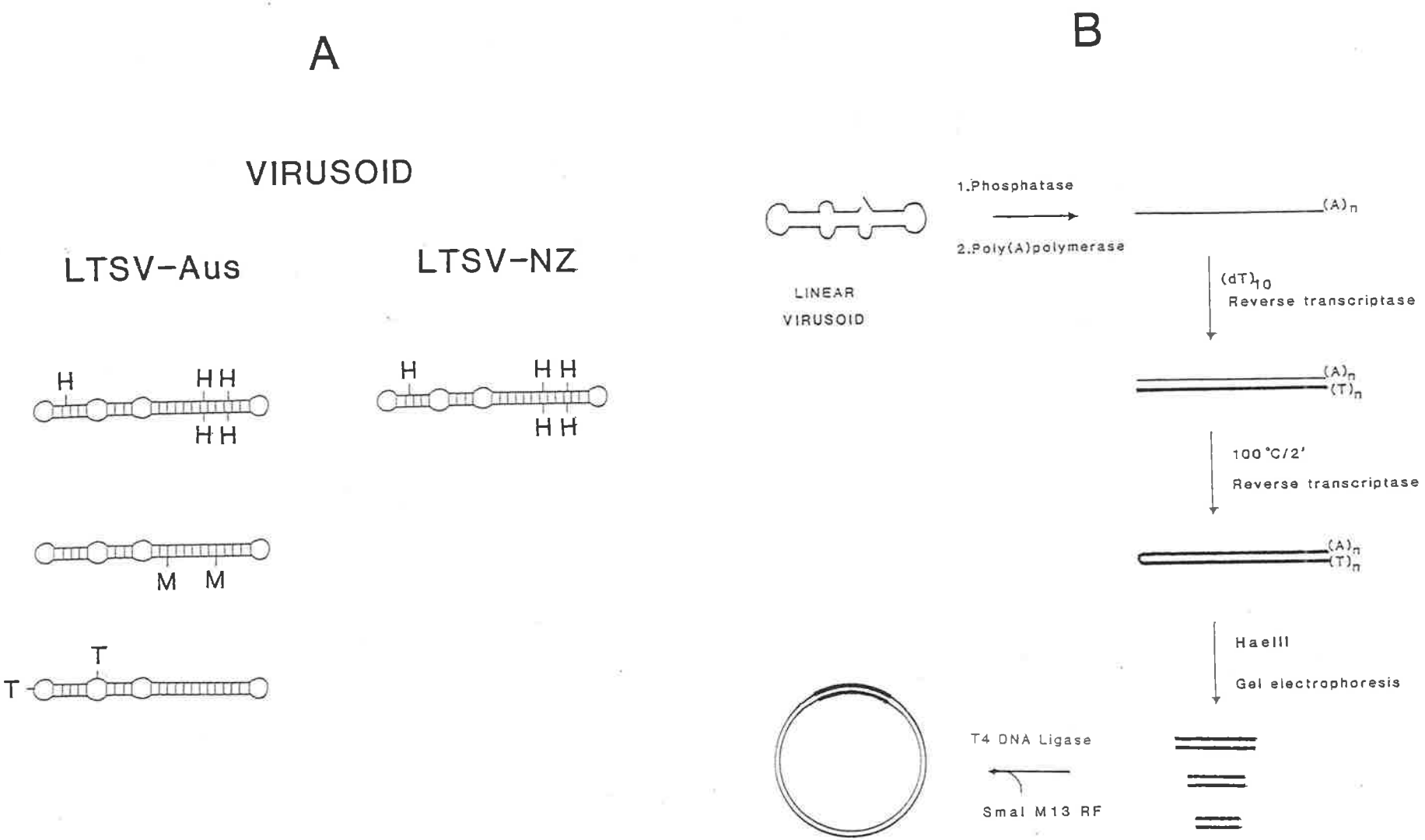
The M13 clones with cDNA to the virusoids of LTSV-Aus and LTSV-NZ were sequenced by the dideoxynucleotide chain termination method used in Chapter 4, Methods 4-4. Overlapping clones to the virusoid of LTSV-Aus allowed independent sequence data of the entire molecule to be obtained. From the sequencing of these clones in both

Figure 5-1 Strategy for cloning double-stranded restriction endonuclease fragments of cDNA to the virusoids of LTSV.

Full details of this procedure are given in Methods and Results.

A HaeIII (H), MspI (M) and TaqI (T) restriction endonuclease recognition sequences relative to the virusoid of LTSV-Aus and LTSV-NZ.

B Schematic outline for the generation of double-stranded cDNA HaeIII restriction endonuclease fragments, from virusoids, for ligating a SmaI digested M13.RF vector.


A
VIRUSOID
LTSV-Aus
LTSV-NZ
H
H H
H H
M M
T
T
B
LINEAR
VIRUSOID
1.Phosphatase
2.Poly(A)polymerase
(A)n
(dT)10
Reverse transcriptase
(A)n
(T)n
100°C/2'
Reverse transcriptase
(A)n
(T)n
HaeIII
Gel electrophoresis
T4 DNA Ligase
SmaI M13 RF

orientations the RNA sequence of the virusoid was inferred to be circular with 324 nucleotide residues.

These neighboring HaeIII fragments of the virusoid of LTSV-NZ double-stranded cDNA allowed the determination of 312 nucleotides residues. Sequence data obtained by RNA partial enzymic hydrolysis (Keese, 1981) allowed confirmation of sequences overlapping these HaeIII recognition sequences and revealed two short additional sequences. The entire molecule (324 nucleotide residues) indicates the presence of five HaeIII recognition sequences, two pairs of which are ten nucleotide residues apart. These were not detected after fractionation of cDNA fragments arising from cleavage with HaeIII restriction endonuclease.

The sequences of the virusoids of LTSV-Aus and LTSV-NZ are depicted in linear form in Figure 5-2. They reveal 98% overall sequence homology. Most nucleotide residue differences are located in two regions of the molecule. Sequence data of one of these regions is shown in Figure 5-3.

5-3 Proposed secondary structures of the virusoids of LTSV-Aus and LTSV-NZ

Secondary structure models for the virusoids of LTSV-Aus and LTSV-NZ were constructed as described by Tinoco *et al.* (1971) and are shown in Figure 5-4. Optimisation of the theoretically most stable structures used parameters determined by Steger *et al.* (1984). Both

Figure 5-2 Nucleotide residue sequences of the virusoids of LTSV-Aus and LTSV-NZ.

The circular virusoid of LTSV-Aus and LTSV-NZ are presented in a linear form and are aligned for maximum sequence homology. Nucleotide residue 1 corresponds to the left hand end of the proposed secondary structure as depicted in Figure 5-4. The sequence differences between the two RNAs are boxed.

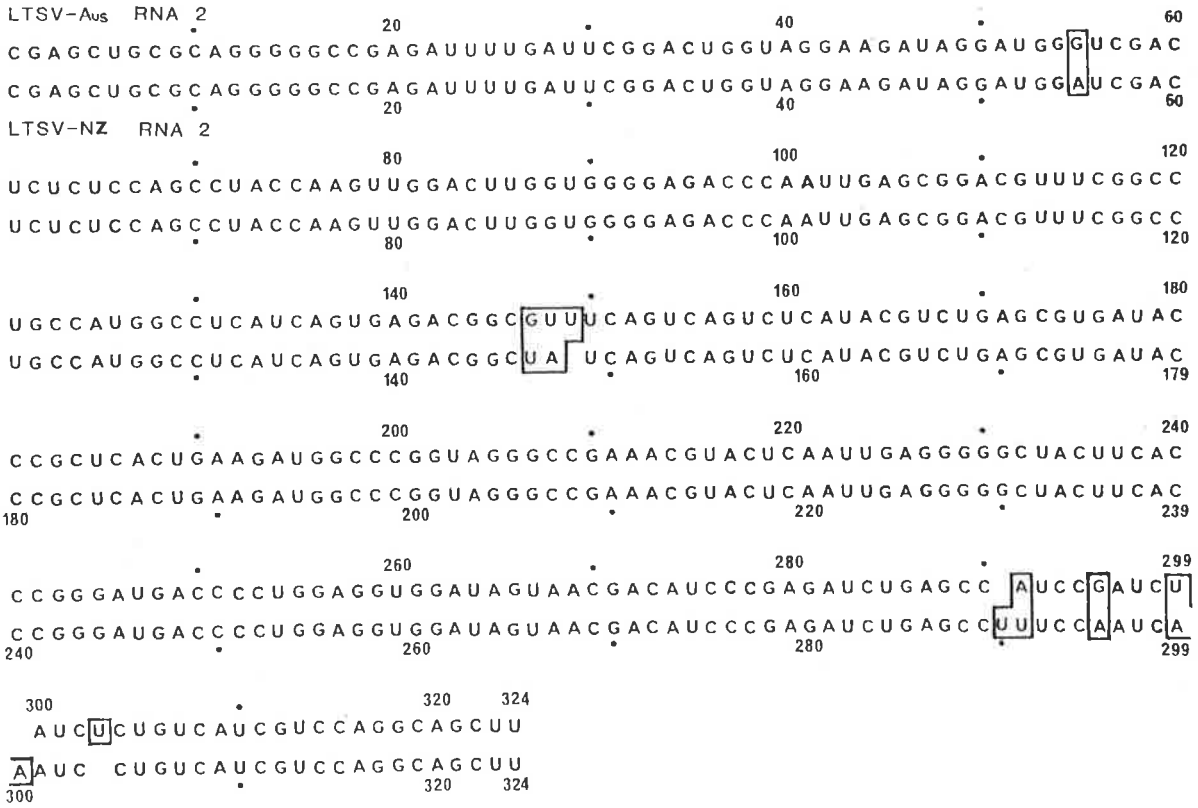

LTSV-Aus RNA 2
CGAGCUGCGCAGGGGGCCGAGAUUUUGAUUCGGACUGGUAGGAAGAUAGGAUGGGUCGAC
CGAGCUGCGCAGGGGGCCGAGAUUUUGAUUCGGACUGGUAGGAAGAUAGGAUGGAUCGAC
LTSV-NZ RNA 2
UCUCUCCAGCCUACCAAGUUGGACUUGGUGGGGAGACCCAAUUGAGCGGACGUUUCGGCC
UCUCUCCAGCCUACCAAGUUGGACUUGGUGGGGAGACCCAAUUGAGCGGACGUUUCGGCC
UGCCAUGGCCUCAUCAGUGAGACGGCGUUUCAGUCAGUCUCAUACGUCUGAGCGUGAUAC
UGCCAUGGCCUCAUCAGUGAGACGGCUA UCAGUCAGUCUCAUACGUCUGAGCGUGAUAC
CCGCUCACUGAAGAUGGCCCGGUAGGGCCGAAACGUACUCAAUUGAGGGGGCUACUUCAC
CCGCUCACUGAAGAUGGCCCGGUAGGGCCGAAACGUACUCAAUUGAGGGGGCUACUUCAC
CCGGGAUGACCCCUGGAGGUGGAUAGUAACGACAUCCCGAGAUCUGAGCC AUCCGAUCU
CCGGGAUGACCCCUGGAGGUGGAUAGUAACGACAUCCCGAGAUCUGAGCCUUUCCAAUCA
AUCUCUGUCAUCGUCCAGGCAGCUU
AAUC CUGUCAUCGUCCAGGCAGCUU

Figure 5-3 Sequence determination of cloned double-stranded cDNA restriction endonuclease fragments of the virusoids of LTSV

Recombinant phage M13mp73 and M13mp93 DNA with a MspI restriction endonuclease fragment of the virusoid of LTSV-Aus double-stranded cDNA and a HaeIII fragment of the virusoid of LTSV-NZ double-stranded cDNA were sequenced by the dideoxynucleotide chain termination technique using the 17-nucleotide residue M13-specific primer. The reaction mixtures were electrophoresed on 6% polyacrylamide/7 M urea/TBE gels (40 x 20x 0.025 cm for the MspI insert and 80 x 20 x 0.05 cm for the HaeIII insert). The sequence diferences between the two virusoids are coloured.

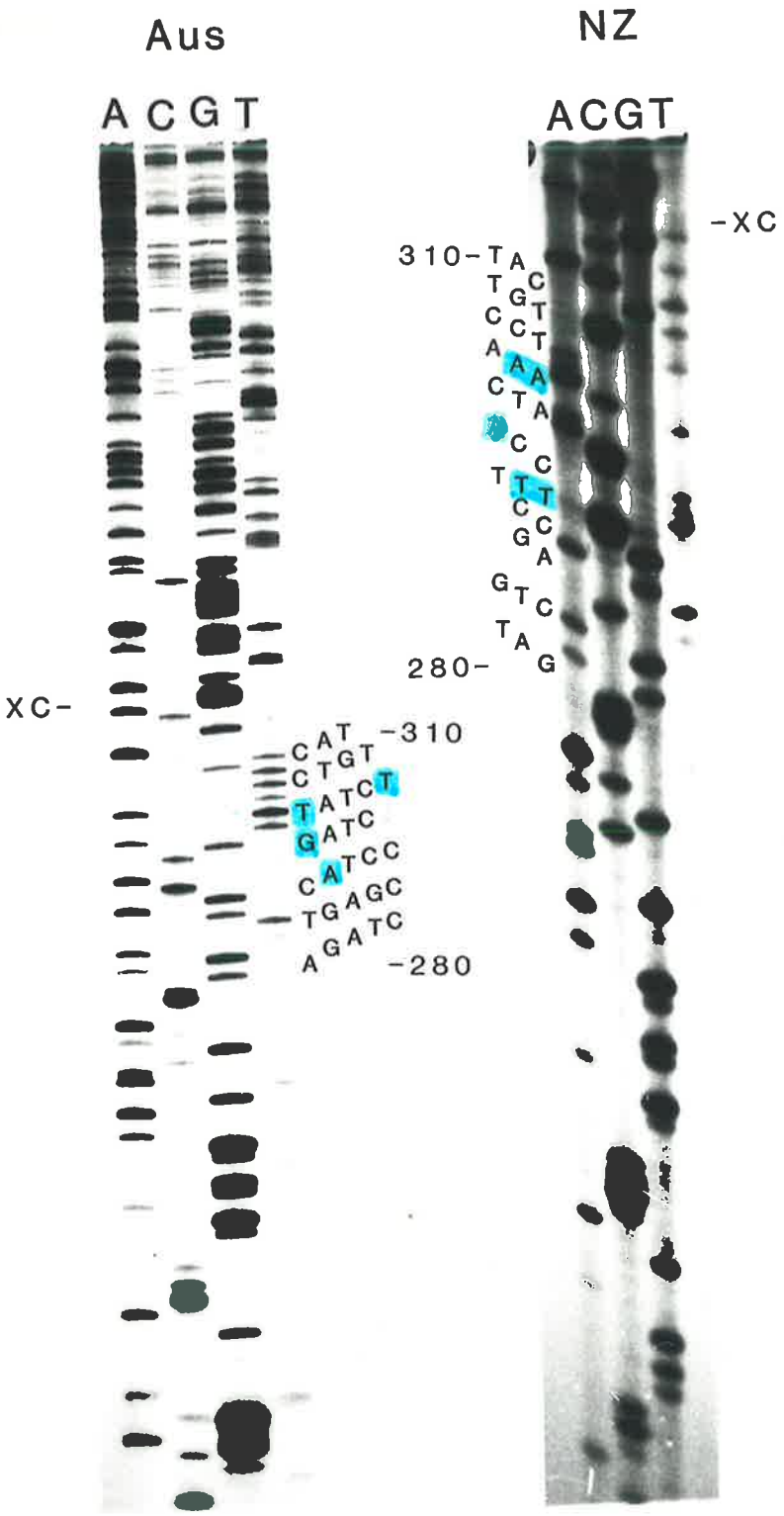
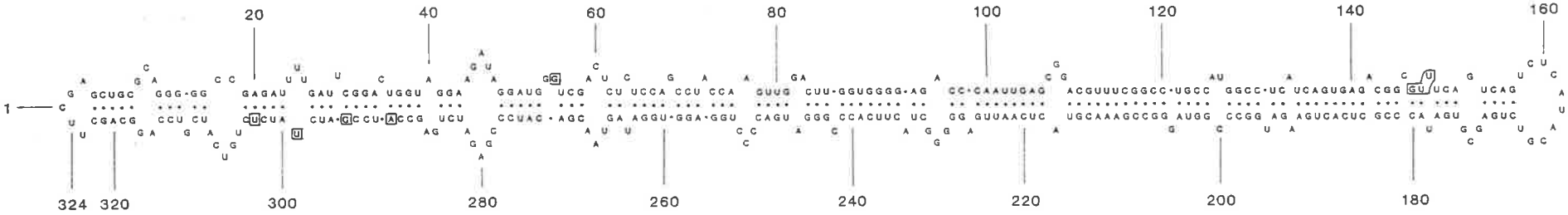

Aus
NZ
A C G T
A C G T
XC-
-XC
310-
280-
-310
-280

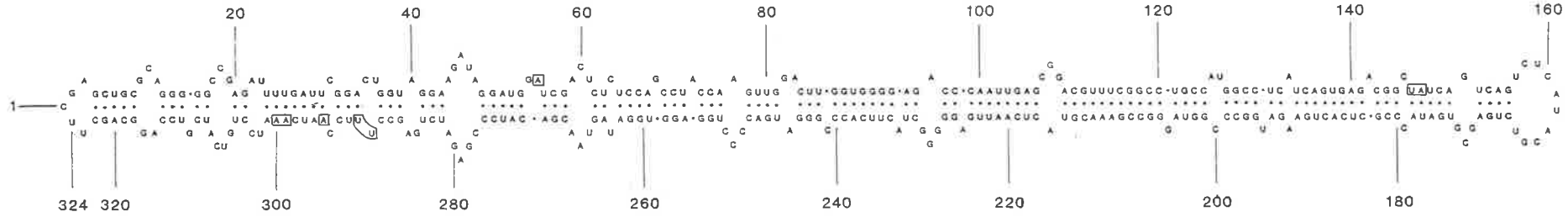
Figure 5-4 Proposed secondary structures of the virusoids of LTSV-Aus and LTSV-NZ

The sequence differences between the virusoids of LTSV-Aus and LTSV are boxed.

LTSV-AusRNA 2



LTSV-NZ RNA 2



RNAs form extensively base-paired rod-like structures. Any possible bifurcations (Figure 5-5) similar to those proposed for the virusoids of VTMoV and SNMV (Steger et al., 1984) lower the stability of the predicted secondary structures.

The properties of the proposed secondary structures are summarised in Table 5-1 and compared to the virusoids of VTMoV and SNMV, and to five viroids, PSTV, ASBV, CSV, CEV and CCCV. All RNAs, except ASBV, contain a similar proportion of G:C base-pairs while the percentage of nucleotide residues base-paired varies in the range from 66-73%. Circular RNA molecules of random sequence and similar size to viroids were calculated to contain about 55% of their nucleotide residues base-paired (Riesner et al., 1979).

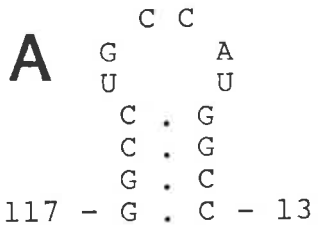
5-4 Possible polypeptide translation products from the virusoids of LTSV-Aus and LTSV-NZ and their complements.

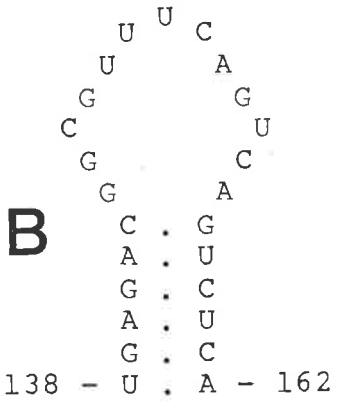
Although eukaryotic ribosomes do not interact with circular RNAs (Kozak, 1979), sub-genomic linear fragments derived from either the infectious plus strand or its complement could act as mRNAs. Thus seven potential polypeptides are encoded by RNA 2 and its complement for each of the isolates of LTSV (Figure 5-6). All possible translation products are less than 75 amino acids long; the gene coding for the coat protein (about 300 amino acids [Blackstock, 1978; Forster and Jones, 1979]) must therefore reside in the RNA 1

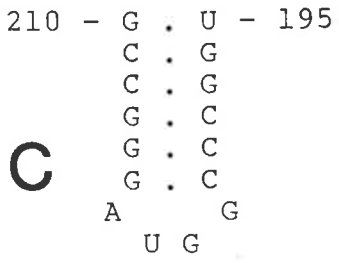
Figure 5-5 Alternative secondary structures of the virusoid of LTSV-Aus

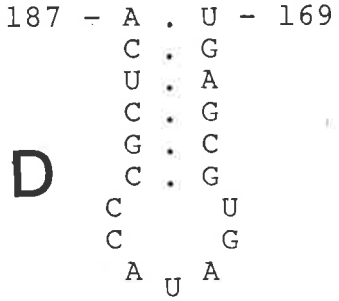
A, B, C, and D ; alternative secondary structures that introduce bifurcations into the postulated rod-like secondary structure of the virusoid of LTSV-Aus. A bifurcation is proposed for the secondary structures of the virusoids of VTMoV and SNMV (Steger et al., 1984) but bifurcations, such as A-D, in the virusoid of LTSV-Aus give theoretically less stable secondary structures when calculated according to Steger et al. (1984).

E ; an alternative secondary structure that has a similar predicted stability at the right hand end loop of the virusoid of LTSV-Aus (Figure 5-4).


A
C C
G A
U U
C . G
C . G
G . C
117 - G . C - 13


B
U U C
U A
G G
C U
G C
G A
C . G
A . U
G . C
A . U
G . C
138 - U . A - 162


210 - G . U - 195
C . G
C . G
G . C
G . C
G . C
A G
U G
C


187 - A . U - 169
C . G
U . A
C . G
G . C
C . G
C U
C G
A U A
D

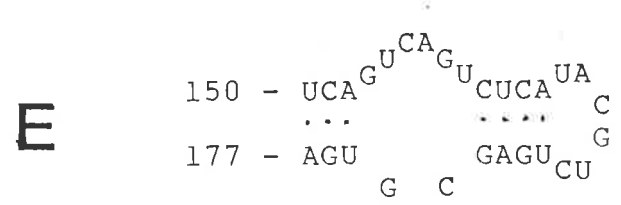

E
150 - UCA G U CA G U CUCA UA C
... . G
177 - AGU GAGU CU
G C

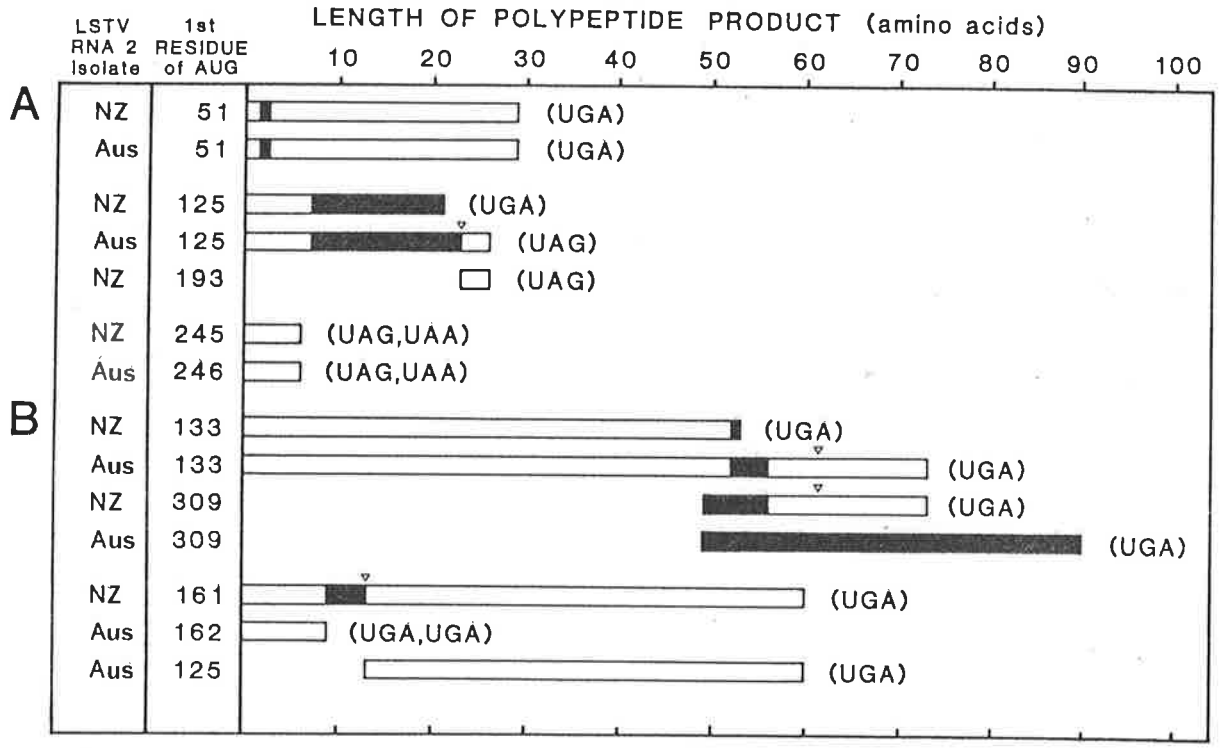
Table 5-1 Properties of proposed secondary structures of the virusoids of LTSV-Aus and LTSV-NZ and other virusoids and viroids

[a] Calculated according to Steger et al. (1984)

RNA	NUMBER OF NUCLEOTIDE RESIDUES	BASE PAIRS DEGREE (%)	G:C (%)	A:U (%)	G:U (%)	$\Delta G^a/N$	REFERENCE
LTSV-Aus RNA-2	324	72	56	34	10	-1.36	Keese et al. (1983)
LTSV-NZ RNA-2	324	71	53	37	10	-1.37	Keese et al. (1983)
VTMoV RNA-2	366	68	58	31	11	-0.96	Haseloff and Symons (1982)
SNMV RNA-2	377	73	55	30	15	-1.21	Haseloff and Symons (1982)
ASBV	247	67	34	51	14	-1.13	Symons (1981)
PSTV	359	70	58	29	13	-1.70	Gross et al. (1978)
CSV	356	70	52	35	13	-1.52	Haseloff and Symons (1982)
CEV	371	69	56	28	16	-1.59	Gross et al. (1982)
CCCV	246	66	69	24	8	-1.30	Haseloff et al. (1982)

Figure 5-6 Possible polypeptide products of the virusoids of LTSV-Aus and LTSV-NZ (A) and their putative complementary RNAs (B)

Each possible translation product is given in schematic form with the nucleotide residue number of the first nucleotide residue of the AUG initiation codon and termination codon(s) in parenthesis. For the complementary sequences, the same nucleotide numbers are retained and therefore run in the 3'-to-5' direction. The clear areas represent regions of amino acid sequence homology and the black areas of non-homology for each group of polypeptides. Inverted triangles indicate sites of internal methionine residues.


LSTV RNA 2 Isolate
1st RESIDUE of AUG
LENGTH OF POLYPEPTIDE PRODUCT (amino acids)
10
20
30
40
50
60
70
80
90
100
A
NZ 51 (UGA)
Aus 51 (UGA)
NZ 125 (UGA)
Aus 125 (UAG)
NZ 193 (UAG)
NZ 245 (UAG,UAA)
Aus 246 (UAG,UAA)
B
NZ 133 (UGA)
Aus 133 (UGA)
NZ 309 (UGA)
Aus 309 (UGA)
NZ 161 (UGA)
Aus 162 (UGA,UGA)
Aus 125 (UGA)

species. Despite the considerable sequence homology between the two virusoids of LTSV only two of these polypeptides, of 6 and 29 amino acids (Figure 5-6), are shared between the RNAs. This limited conservation of possible translation products between the highly conserved virusoids of the two LTSV strains suggests that they may lack functional mRNA activity *in vivo*. The virusoid of LTSV-NZ does not appear to be translated in either the rabbit reticulocyte lysate or wheat germ extract translation systems (Morris-Krsinich and Forster, 1983). Lack of mRNA activity is characteristic of other similar RNAs such as viroids (see Diener, 1983; Keese and Symons, 1986) or the satellite RNA of tobacco ringspot virus (STobRV) [Owens and Schneider, 1977].

DISCUSSION

Restriction endonuclease digestion of cDNA to the virusoid of LTSV-Aus with TaqI, MspI and HaeIII allowed the generation of overlapping clones in M13 that included all sequences of the virusoid. Dideoxynucleotide chain termination sequencing of these clones allowed complete sequencing in both orientations of the virusoid cDNA. This sequence was able to confirm direct RNA sequencing data obtained by partial enzymic hydrolysis.

Complementary DNA clones of the virusoid of LTSV-NZ were able to allow DNA sequencing of all but 12

nucleotide residues (corresponding to nucleotide residues 121-126 and 200-205). These latter residues were confirmed by partial RNA enzymic hydrolysis (Keese, 1981). The ability to derive overlapping cDNA transcripts of the virusoid of LTSV-Aus supports the electonmicrographic data of Tien-Po et al. (1981) that the virusoids of LTSV contain covalently closed circular molecules.

In overall structure the virusoids of LTSV resemble both viroids and the virusoids of SNMV, VTMoV (Haseloff and Symons,1982) and SCMoV (Haseloff, 1983) in consisting of low molecular weight single-stranded covalently closed RNA molecules. The secondary structure predicted to occur when purified in solution gives a similar rod-like form to other virusoids with extensive base-paired regions interspersed with short single-stranded regions. Although the predicted free energy of the virusoids of LTSV is similar to the virusoids of VTMoV and SNMV (Table 5-1) the lower ratio of free energy to size (Table 5-1), the higher T_m (70°C, the virusoid of LTSV-Aus [Tien-Po et al., 1981]; 57°C, the virusoid of VTMoV [Gould,1981]; 64°C, the virusoid of SNMV [Gould and Hatta,1981]; all determined in 0.15 M NaCl, 0.015 M trisoidium citrate, pH 7.0), and the greater resistance to ribonuclease (Table 5-2), indicate that they are more stable than the virusoids of VTMoV and SNMV. The virusoids of LTSV may therefore show

Table 5-2 Ribonuclease sensitivity of the virusoids of LTSV-Aus and LTSV-NZ relative to other virusoids and viroids

Concentration of RNases T_1, U_2 and A under high salt conditions at $0^{o}C$ for 60 min to obtain partial hydrolysis of the virusoids of LTSV-Aus and LTSV-NZ (Keese, 1981) are compared to the virusoids of VTMoV and SNMV (Haseloff and Symons, 1982); the virusoids of SCMoV (Haseloff, 1983); CTiV (Chapter 2) and CSV (Haseloff and Symons, 1982).

ND Not done.

RNA	RNase		
	T_1 U/ml	U_2 U/ml	A µg/ml
virusoid of LTSV-Aus or LTSV-NZ	5,000	2.5	0.8
virusoid of VTMoV	150	0.25	ND
virusoid of SNMV	300	0.25	ND
virusoids of SCMoV	150	0.25	0.1
CTiV	2,500	2	1
CSV	3,750	2	2

hydrodynamic properties more akin to viroids such as PSTV and CCCV which demonstrate greater stiffness in solution than the virusoids of VTMoV and SNMV (Riesner et al., 1982).

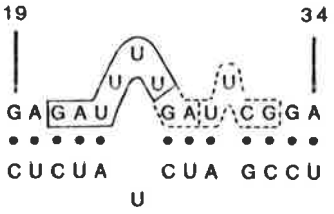
The virusoids of LTSV-Aus and LTSV-NZ share 98% overall sequence homology but these virusoids exhibit only about 35% overall sequence homology with the virusoid of VTMoV or either virusoid of SCMoV (Haseloff, 1983; in Keese and Symons, 1986). Despite the low level of overall sequence homology with other virusoids, two specific regions appear to have notable sequence homology. These include a GAUUUU sequence found in all known virusoids (Figure 5-7) but which is absent from viroids and satellite RNAs. In addition, this virusoid-specific sequence occurs in the same relative position of the postulated secondary structures (beginning 19-21 nucleotide residues from one end of the rod-like structures) and the oligo-uridylate sequence appears to be mostly non-base-paired.

The second region of sequence homology between virusoids extends to about 50 nucleotides residues (Figure 5-8) and includes a GAAAC sequence which was reported by J. Haseloff to occur in viroids, including ASBV, as well as the virusoids of VTMoV and SNMV. This region, from nucleotide residues 165-215 of the virusoids of LTSV, shows not only homology with the virusoids of VTMoV and SNMV but also homology with

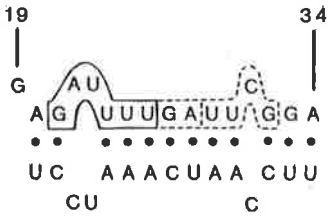
Figure 5-7 Virusoid specific sequence homology

The virusoids of LTSV-Aus, LTSV-NZ, VTMoV and SNMV, (Haseloff and Symons, 1982) and SCMoV (Haseloff, 1983) share a common GAUUUU sequence (solid box) in a homologous position relative to the proposed structures. Broken boxes indicate sequence homology between at least two RNAs. Nucleotide residues are numbered from the left hand end of the predicted secondary structures for each RNA. The virusoids of VTMoV and SNMV show 93% overall sequence homology (Haseloff and Symons, 1982). The two virusoids of SCMoV have identical sequence in this region (Haseloff, 1983).

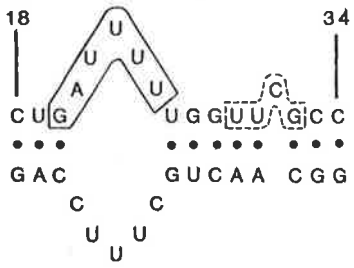
LTSV-Aus RNA 2



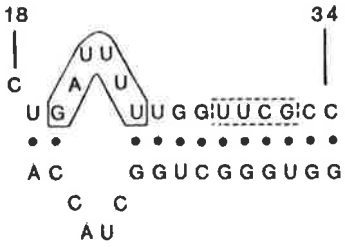
LTSV-NZ RNA 2



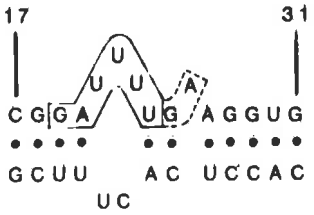
VTMoV RNA 2



SNMV RNA 2



SCMoV RNA 2

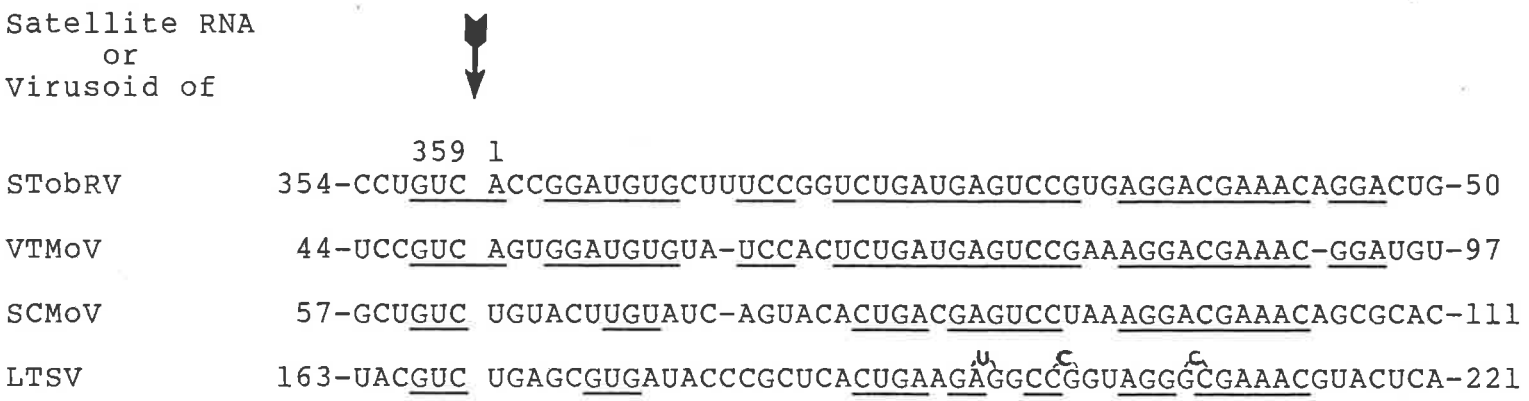


STobRV (Schneider, 1969). STobRV is of similar size to virusoids (359 nucleotide residues, Buzayan et al., 1986b). Circular forms have been isolated from infected tissue as well as the predominant linear form that is encapsidated (Sogo and Schneider, 1982; Linthorst and Kaper, 1984). In contrast to virusoids which are supported by members of the Sobemovirus group, STobRV is supported by a virus which belongs to the Nepovirus. The strongest sequence homology, surprisingly, is between the virusoids of VTMoV (that of SNMV) and STobRV (Figure 5-8). After this initial alignment similar sequences were discernible in the virusoids of SCMoV (J. Haseloff, 1983) and LTSV.

J. Haseloff (1983, and in Kiberstis et al., 1985; Symons et al., 1985) proposed that this region was a recognition site for processing of replicative intermediates of rolling circle replication (see Chapter 7). This was later supported experimentally by the findings of non-enzymic specific cleavage of STobRV (Prody et al., 1986) and RNA transcripts of the virusoids of LTSV (A.C. Forster, personal communication) and SCMoV (J. Haseloff, personal communication). In addition, the virusoids of SNMV and VTMoV possess a 2'-phosphomonoester, 3'-5' phosphodiester bond at the homologous cleavage site, indicating enzymic ligation (Kiberstis et al., 1985).

Figure 5-8 Sequence homology between virusoids and STobRV

Virusoids show noteworthy sequence homology between themselves and with the terminal sequences of STobRV. The virusoids of LTSV-Aus and LTSV-NZ have the same sequence in this region. The virusoid of SNMV is the same as that of VTMoV except for an A residue at position 79. The 388 nucleotide residue virusoid of SCMoV is the same as the 332 nucleotide residue virusoid of SCMoV depicted here except for an A residue at position 70. The highest sequence homology is between virusoid of VTMoV and STobRV and as such have been aligned for maximum sequence homology (underlined). The virusoids of SCMoV has been noted to share common sequences with those conserved above (underlined) [Haseloff, 1983] as well as the virusoids of LTSV-Aus and LTSV-NZ. The arrow points to a postulated site of processing (Haseloff, 1983; Symons, 1985).


Satellite RNA
or
Virusoid of
359 1
STobRV 354-CCUGUC ACCGGAUGUGCUUUCCGGUCUGAUGAGUCCGUGAGGACGAAACAGGACUG-50
VTMoV 44-UCCGUC AGUGGAUGUGUA-UCCACUCUGAUGAGUCCGAAAGGACGAAAC-GGAUGU-97
SCMoV 57-GCUGUC UGUACUUGUAUC-AGUACACUGACGAGUCCUAAAGGACGAAACAGCGCAC-111
LTSV 163-UACGUC UGAGCGUGAUACCCGCUCACUGAAGAGGCCCGGUAGGGCGAAACGUACUCA-221
U
C
C

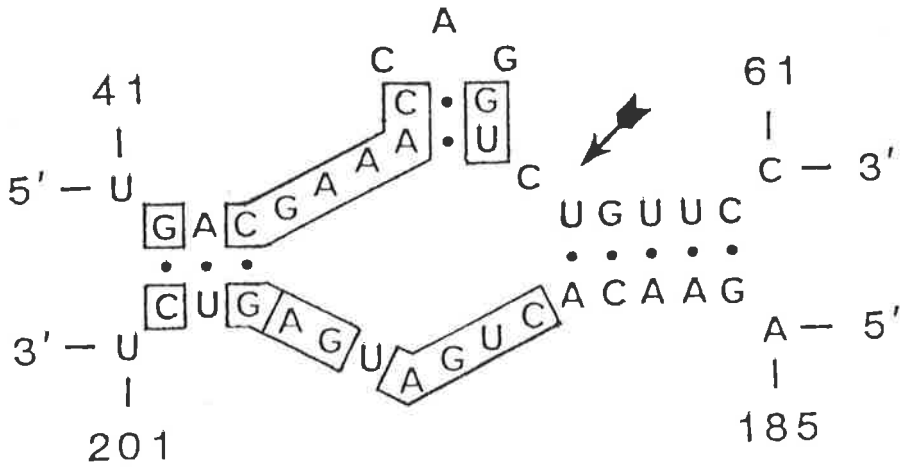
It has also been shown that dimeric RNA transcripts of ASBV specifically cleave to generate a unit length form (Hutchins et al., 1986). Sequences adjacent to the cleavage site appear to be homologous to the corresponding sequences in virusoids and STobRV (Hutchins et al., 1986), Figure 5-9.

The RNA transcripts complementary to the virusoid of LTSV-Aus are also capable of specific non-enzymic cleavage (A.C. Forster, personal communication). Sequences adjacent to the cleavage site are homologous with the conserved sequences depicted in Figure 5-8, in particular to the virusoid of LTSV-Aus (Figure 5-10). This may be a consequence of the high degree of self-complementarity within virusoids, but no equivalent sequences can be found in the virusoids of VTMoV, SNMV, and SCMoV. This raises the intriguing possibility that the evolution of the virusoids of LTSV has involved recombination with its complementary strand so that the processing-specific sequences have been duplicated but in opposite orientations.

The sequence homology described here between virusoids, STobRV and ASBV suggests that all of these RNAs may share a common ancestor. These sequences appear in all cases to signal the same RNA specific function of processing. If this is the case then the virusoids of VTMoV and SNMV may share a more recent ancestor with STobRV than with other virusoids. Therefore the absence

Figure 5-9 Sequences adjacent the cleavage site of ASBV

Hutchins et al. (1986) have proposed a secondary structure for signalling self-cleavage of ASBV in which the adjacent sequences are homologous (boxed) to those found near the postulated self-cleavage sites of virusoids and STobRV (A.C. Forster, personal communication; J. Haseloff, personal communication; Prody et al., 1986).

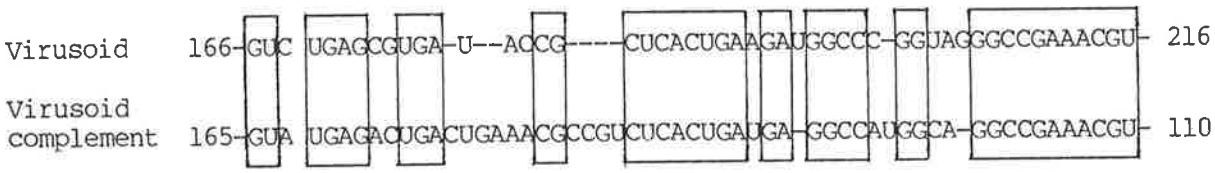

A
C
G
41
61
C
G
U
5′ — U
C — 3′
G A C G A A A
C
U G U U C
A C A A G
C U G A G U A G U C
3′ — U
A — 5′
201
185

ASBV

Figure 5-10 Self-complementary sequences of the virusoids of LTSV-Aus

The complementary strand of the virusoid of LTSV-Aus from nucleotide residues 162-110 is homologous in function (signalling self-cleavage; A.C. Forster, personal communication) and sequence (boxed).

LTSV-Aus


Virusoid
166-GUC UGAGCGUGA-U--ACCG----CUCACUGAAGAUGGCCC-GGUAGGGCCGAAACGU- 216
Virusoid
complement
165-GUA UGAGACUGACUGAAACGCCGUCUCACUGAUGA-GGCCAUGGCA-GGCCGAAACGU- 110

of the GAUUUU sequence in STobRV and ASBV would indicate that this sequence is neither a virusoid RNA-specific functional signal (such as autocatalytic processing) nor a host-specific interaction (such as modulating symptom expression). Instead the GAUUUU sequence may signify an interaction with the helper virus, since virusoids are supported by members of the Sobemovirus group and not by a nepovirus such as TobRV, which supports STobRV (for example, encapsidation or template recognition by a viral-encoded polymerase). However, it has yet to be tested experimentally that members of the Sobemovirus group are indeed unable to support STobRV.

CHAPTER 6

SATELLITE NATURE OF VIRUSOIDS

INTRODUCTION

A plant virus satellite usually refers to a virus or nucleic acid that is unable to multiply detectably in cells without the assistance of a helper virus, is not necessary for the multiplication of the helper virus, and has no appreciable sequence homology with the genome of the helper virus or that of the host plant (Schneider, 1977; Murant and Mayo, 1982; Kaper and Tousignant, 1984; Francki, 1985; Francki et al., 1985). Virusoids appear to comply with some, if not all, of these characteristics. For example the virusoid of SNMV is unable to replicate independently to detectable levels in the same hosts that the intact virus succesfully infects (Gould et al., 1981; Jones and Mayo, 1983, 1984) and it lacks significant sequence homology with SNMV RNA 1 or with host DNA or RNA (Gould and Hatta, 1981). An initial report indicated that the virusoid of SNMV exists as a component of a bipartite genome since infectivity was only obtained by co-inoculation with both the purified virusoid and RNA components (Gould et al., 1981). It has subsequently been shown that SNMV RNA 1 is able to multiply independently of the virusoid (Jones and Mayo, 1984) and would thus appear to act as a helper RNA for the virusoid.

One problem with attempting infectivity studies of separate viral components is the elimination of any

traces of virusoid from preparations of the helper viral RNA. This difficulty has been reported for several satellite RNAs such as those associated with cucumber mosaic virus (Kaper and Tousignant, 1977; Mossop and Francki, 1979) and tobacco ringspot virus (Rezaian, 1980; Gerlach et al., 1986). In addition, attempts to eliminate the virusoid of LTSV by separation of RNA components with polyacrylamide gel electrophoresis has proved unproductive (Jones et al., 1983; unpublished observations). In the case of SNMV, succesful isolation of the helper RNA was achieved through amplication of single lesions that developed in Nicotiana debneyi Domin. The inoculum used was partially purified SNMV RNA 1. This method was derived from that succesfully employed with partialy purified LTSV RNA 1 inoculated into the local lesion hosts Chenopodium amaranticolor Coste et Reyn. and C. quinoa (Jones et al., 1983). On these two hosts, necrotic lesions were correlated with the presence of the LTSV virusoid, while chlorotic lesions were correlated with the absence of the virusoid. The isolation of virus particles from chlorotic lesions containing only LTSV RNA 1 implied that the virusoid is not an essential component of the virus genome. The virusoid of LTSV, however, was able to alter symptom expression which was not observed for the virusoid of SNMV (Jones and Mayo, 1984).

Virusoids have also been reported to multiply succesfully in heterologous combinations amongst serologically unrelated viruses. These include the virusoid of SNMV and LTSV RNA 1 (Jones and Mayo, 1983), and the virusoids of LTSV and either southern bean mosaic virus [Paliwal, 1984b] or sowbane mosaic virus [Francki et al., 1983a].

While passaging LTSV in a glasshouse within the vicinity of a number of other virus stocks, contamination was observed to occur, once with VTMoV RNA 1 and once with the large virusoid (388 nucleotide residues) of SCMoV. These chance events were used for further infectivity studies into the satellite nature of virusoids.

MATERIALS

Viruses

An isolate of LTSV-NZ that replicates without the presence of the virusoid was generously provided by Dr. R.L.S. Forster (Plant Disease Division, Department of Scientific and Industrial Research, Private Bag, Auckland, New Zealand). Viral RNA extracted from virions of VTMoV, SNMV and SCMoV was generously provided by Dr. J. Haseloff.

Recombinant clone

A recombinant clone of Sau3AI restriction endonuclease fragment of SNMV virusoid double-stranded cDNA (corresponding to nucleotide residues 131-216) in bacteriophage M13mp8 was generously provided by Dr. J. Haseloff.

Chemicals

The nylon based membrane filter, GeneScreen, was from New England Nuclear. Deoxyribonuclease treated salmon sperm DNA was generously provided by T.W. Marriott.

METHODS

6-1 Viral purification and RNA extraction

LTSV-NZ and VTMoV in 50 mM sodium phosphate, 1mM EDTA, pH 7.0, were inoculated onto carborundum dusted N. clevelandii. Infected leaves were harvested 7-15 days after inoculation. The virus was purified and the RNA extracted as described in Chapter 5, Methods 5-1.

6-2 Preparation of radiolabelled probes

Modified from Bruening et al. (1982), recombinant M13mp73 bacteriophage DNA (5-10 µg) with either a TaqI or an MspI insert of the virusoid of LTSV-Aus double-stranded cDNA (corresponding to nucleotide residues 56-324 and 241- [324,1] - 199 respectively) or a recombinant clone of a SNMV virusoid double cDNA Sau3Al fragment in M13mp8 was transcribed with the Klenow

fragment of E. coli polymerase I in 20 µl reactions (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.5 mM each of dGTP and dTTP, and 50 µCi each of alpha-^{32}P-dATP and alpha-^{32}P-dCTP) after annealing with an M13-specific 17 nucleotide residue primer. After transcription at 37°C for 15 min, 1 µl of 10 mM dATP, dCTP was added for a further 15 min. Incubation was terminated by heating at 70°C for 1 min and the reaction mixture digested with 20 U EcoRI restriction endonuclease at 37°C for 60 min after addition of 1 µl 4 M NaCl. After heating the reaction mixtures at 100°C for 4 min with an equal volume of 95% (v/v) formamide, 10 mM EDTA pH 8.0, 0.02% bromophenol blue and xylene cyanol FF) the restriction endonuclease fragments were fractionated by 5% polyacrylamide/7 M urea/TBE gel electrophoresis. The appropriate fragments were excised, the DNA eluted by soaking, ethanol precipitated and stored in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 and 5 mM 2-mercaptoethanol.

An LTSV-NZ RNA 1 specific probe was generated as above using a double-stranded cDNA clone of LTSV-NZ RNA 1 (made as in Chapter 4 using a random primer to initiate a first strand synthesis, snap-back second strand synthesis and digesting with S_1 nuclease before ligating into SmaI cut M13mp93 replicative form). A single-stranded radiolabelled cDNA transcript complementary to LTSV-NZ RNA 1 was achieved by digesting

with HindIII restriction endonuclease before fractionation by polyacrylamide gel electrophoresis.

6-3 Dot-blot hybridisation procedure

The methods used were adapted from those of Thomas (1980). A pre-stamped sheet of GeneScreen was soaked in water for 5 min and then in 20 x SSC (SSC; 0.15 M NaCl, 0.015 M sodium citrate) for 30 min before drying. 2 µl samples were spotted, dried and baked at 80°C for 2 h to immobilise nucleic acids.

Prehybridisation for 4-20 h at 42°C was carried out in a buffer of 50% (v/v) deionised formamide, 5 x SSC, 50 mM sodium phosphate pH 6.5, 250 µg/ml sonicated, denatured salmon sperm DNA, 1x Denhardts solution (0.02% [w/v] each of bovine serum albumin, Ficoll 400, Sigma polyvinylpyrrolidone 40,000), 5 mM EDTA, 0.2% (w/v) SDS. Hybridisation buffer contained 9 parts of hybridisation concentrate (44% [v/v] deionised formamide, 4.4 x SSC, 44 mM sodium phosphate pH 6.5, 220 µg/ml sonicated denatured salmon sperm DNA, 0.8 x Denhardts solution, 5 mM EDTA, 0.2% (w/v) SDS, 11% (w/v) dextran sulphate) plus 1 part of radiolabelled DNA probe (final concentraion 0.5 x 10^6 cpm/ml). Prehybridisation and hybridisation buffers were used at 0.075 ml/cm^2 of membrane filter. The DNA probes were denatured in 50% (v/v) deionised formamide at 100°C for 3 min, rapidly cooled in ice and added to the hybridisation concentrate.

Hybridisation was carried out in sealed plastic bags in a shaking water bath at 55°C for 20-24 h. The membrane filters were then washed three times for 10 min at room temperature in 2 x SSC, 0.1% (w/v) SDS and two times for 20 min at 55°C in 0.1 x SSC, 0.1% (w/v) SDS before autoradiographing at -70°C for 12-48 h.

6-4 Restriction endonuclease cleavage of viral double-stranded cDNA

Random-primed first strand cDNA was transcribed from LTSV-NZ RNA 1, VTMoV RNA 1, SNMV RNA 1 and SCMoV RNA 1 essentially as described by Taylor *et al.* (1976). Purified RNA (0.5-1 µg) was resuspended in 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each of dATP, dGTP, and dTTP, 50 µCi alpha-^{32}P-dCTP and 22 units of avian myeloblastosis reverse transcriptase. Transcription was terminated after 60 min by boiling for 2 min. Second strand synthesis was as in Chapter 4, Methods 5-2, C. Synthesised double-stranded cDNA was digested with different restriction endonucleases and fractionated by 6% polyacrylamide/2 M urea/TBE gel elctrophoresis.

RESULTS AND DISCUSSION

6-1 Sequence homology between LTSV-NZ RNA 1 and 2

LTSV-NZ RNA 1 and partially purified nucleic acid extracts of healthy *C. quinoa* were probed by the dot

blot hybridisation procedure with radiolabelled probes complementary to the RNA sequence of the virusoid of LTSV-Aus (which is 98% sequence homologous with the virusoid of LTSV-NZ) to test for sequence homology between the the virusoid and the RNA 1 component. For this purpose two probes synthesised from recombinant clones were used that overlapped in sequence such that they encompassed the entire RNA sequence of the virusoid of LTSV-Aus. The TaqI restriction endonuclease fragment of LTSV-Aus virusoid double-stranded cDNA was inserted into M13mp73 corresponding to nucleotide residues 56-324 of the virusoid of LTSV-Aus, while nucleotide residues 1-55 were part of a MspI insert. Radiolabelled probes from both of these clones readily hybridised to purified virusoid RNA, total viral RNA from virions with the virusoid present and nucleic acids from infected plants (Figure 6-1). The probes failed to hybridise detectably with purified viral RNA from virions free of the virusoid and nucleic acid from healthy plants. Under the hybridisation and washing conditions used, the LTSV-NZ RNA 1 specific probe hybridised to viral RNA with the virusoid either present or absent (Figure 6-1). The results show that LTSV-NZ RNA 1 does not contain homologous sequences to the virusoid such as expected of a subgenomic RNA. However, the possibility of low but significant sequence homology of the virusoid with RNA 1 of LTSV-NZ cannot be excluded.

Figure 6-1 Detection of sequence homology between RNA 1 and the virusoid of LTSV-NZ

The presence of homologous sequences between RNA 1 and the virusoid of LTSV-NZ was tested by blot hybridisation, using ^{32}P-labelled cDNA probes as described in Methods.

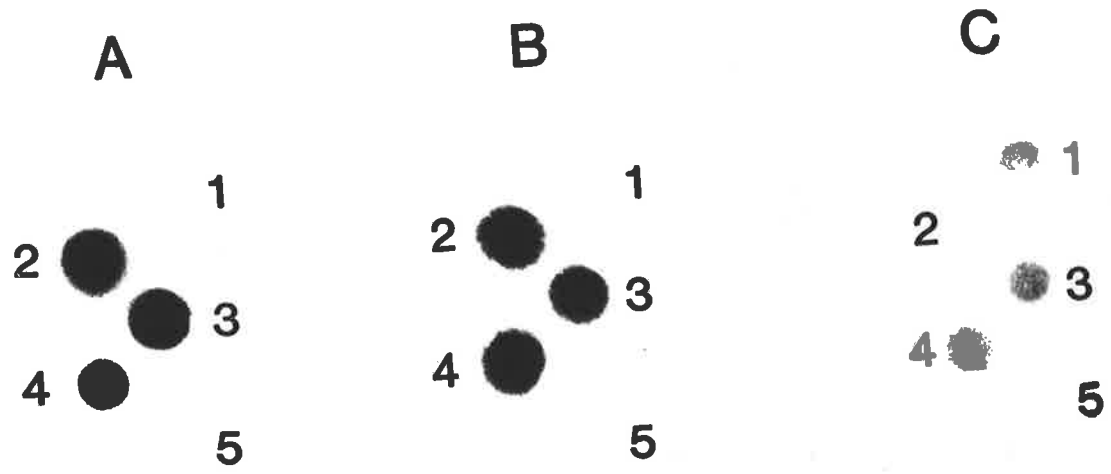
A, ^{32}P-labelled probe, from a TaqI insert in M13mp73 of LTSV-Aus virusoid cDNA with a DNA sequence complementary to nucleotide residues 56-324 of the virusoid.

B, ^{32}P-labelled probe, from a MspI insert in M13mp73 of LTSV-Aus cDNA, with a DNA sequence overlapping that of A and including complementary DNA sequence to nucleotide residues 1-55 of the virusoid of LTSV-Aus.

C, ^{32}P-labelled probe, from a S_1 generated LTSV-NZ RNA 1 double-stranded cDNA insert in M13mp93, with a DNA sequence complementary to about 280 nucleotide residues of LTSV-RNA 1.

1 µl of samples of the following unlabelled nucleic acids were spotted onto three filters and probed individually with A, B, and C.

1 0.5 µg LTSV-NZ viral RNA free of the virusoid and generously provided by Dr. R.L.S. Forster.
2 0.2 µg of virusoid from LTSV-NZ, isolated by gel electrophoresis.
3 0.5 µg of LTSV-NZ viral RNA with the virusoid present.
4 nucleic acid extract of N. clevelandii infected with LTSV-NZ with the virusoid present, prepared essentially as in Chapter 2, Methods 2-1.
5 nucleic acid extract of a healthy N. clevelandii.


A
1
2
3
4
5
B
1
2
3
4
5
C
1
2
3
4
5

Using a sensitive bioassay based on the ability of the virusoid of LTSV-NZ to modulate symptom expression, LTSV-NZ RNA 1 was shown to replicate in the absence of the virusoid but the virusoid failed to multiply detectably in the same hosts (Jones et al., 1983). It would thus appear that the virusoid of LTSV-NZ satisfies all of the charactersitic of a plant viral satellite RNA.

6-2 Independent replication of VTMoV RNA 1

During two passages of LTSV-NZ through N. clevelandii the yield of virus increased but the levels of the virusoid of LTSV-NZ decreased. No virusoid was detectable by staining with toluidine blue-O on the third passage. When the virus was examined by Dr. R.I.B. Francki, it was found to react serologically to antibodies raised to either VTMoV or SNMV but not to LTSV or any other spherical virus kept in the glasshouse (personal communication). In order to distinguish between VTMoV and SNMV, radiolabelled cDNA was synthesised to the unknown viral RNA using a random DNA primer and avian myeloblastosis virus reverse transcriptase. After conversion to double-stranded DNA, restriction endonuclease cleavage was performed with HaeIII, Sau3AI (results not shown), MspI and TaqI. Fractionation of the cDNA fragments by polyacrylamide gel electrophoresis revealed an identical pattern of the

unknown viral RNA to that obtained with VTMoV (Figure 6-2).

Further passaging of this isolate of VTMoV at high titre (7 mg/ml) resulted in the appearance of an RNA species that co-migrated during polyacrylamide gel electrophoresis with the virusoid of VTMoV (result not shown). It was therefore assumed that trace amounts of the virusoid of VTMov were still present or acquired during passaging.

One puzzling finding with this isolate was the lack of detectable virusoid when passaged at 50-100 µg/ml except when co-inoculated with 50 µg/ml of the virusoids of LTSV-NZ or SCMoV. High levels of the virusoid of VTMoV (about 70-80% of total viral RNA) were then detected although no LTSV-NZ or SCMoV virusoid RNA could be detected by staining with toluidine blue-O (Figure 6-3).

In order to establish that VTMoV RNA 1 was replicating without the assistance of even low levels of virusoid, the above isolate was inoculated onto N. clevelandii at 10 µg/ml. Seven days after infection, sections (in square mm) of leaf material, to which the original inoculum had been applied, were harvested. After grinding in a minimal volume of 50 mM sodium phosphate, 1 mM EDTA pH 7.0, the slurry was inoculated onto further plants. After preliminary screening of infected plants on the basis of symptom expression, four

Figure 6-2 Restriction endonuclease digestion of double-stranded viral RNA

Double-stranded cDNA was synthesised to about 0.5-1 ug RNA 1 from LTSV-NZ (tracks 1 and 6), SNMV (tracks 3 and 7), VTMoV (tracks 4 and 8) and a viral contaminant (x, tracks 5 and 9), digested with either TaqI or MspI restriction endonucleases and fractionated by 6% polyacrylamide/2 M urea/TBE gel electrophoresis. Track 1 is M13mp93 replicative form digested with MspI. The sizes of the ^{32}P-labelled fragments are 1596, 829, 818, 652, 545, 543, 472, 454, 357, 183, 176, 156, 129, 123, 79, 60, 30 base pairs.

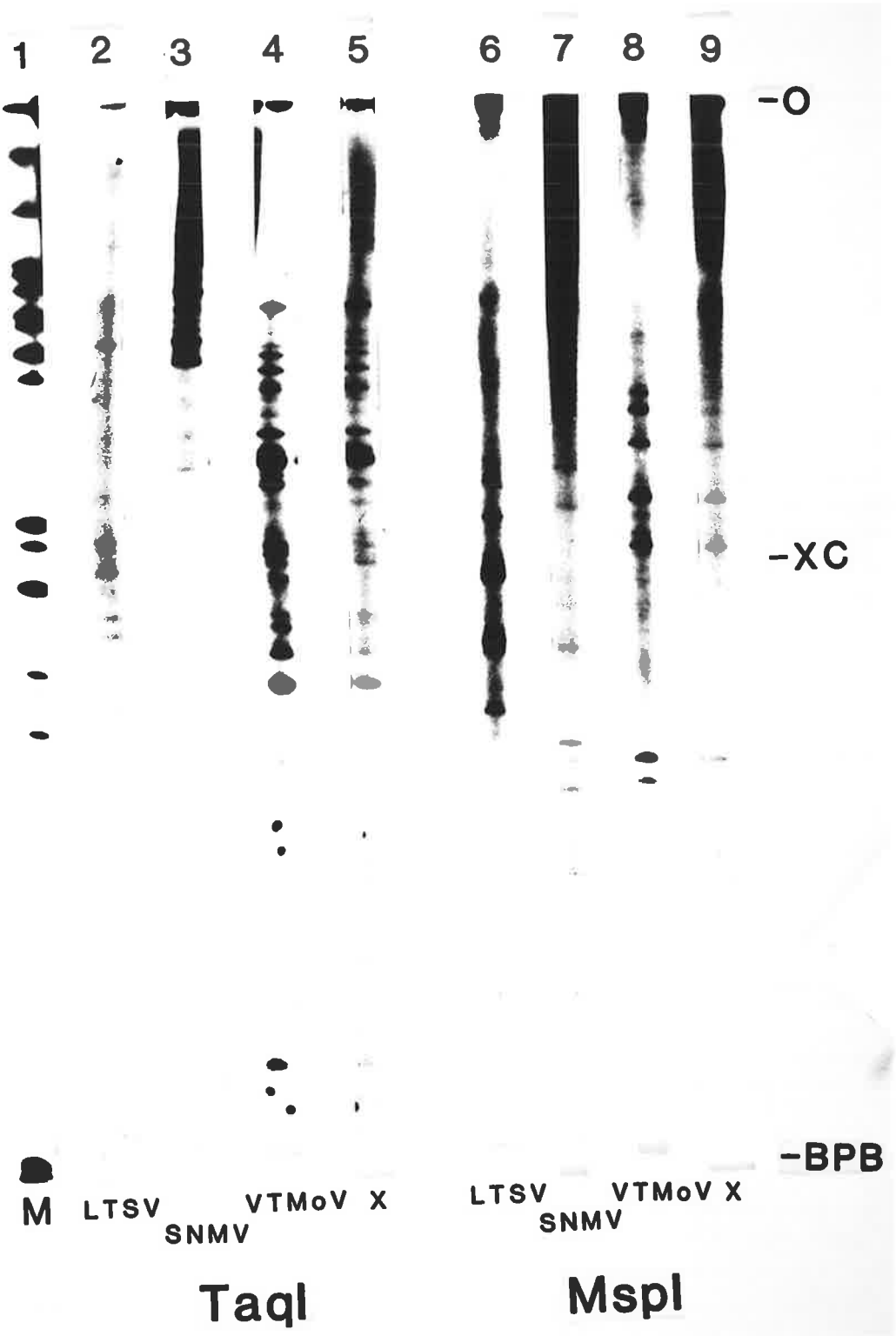
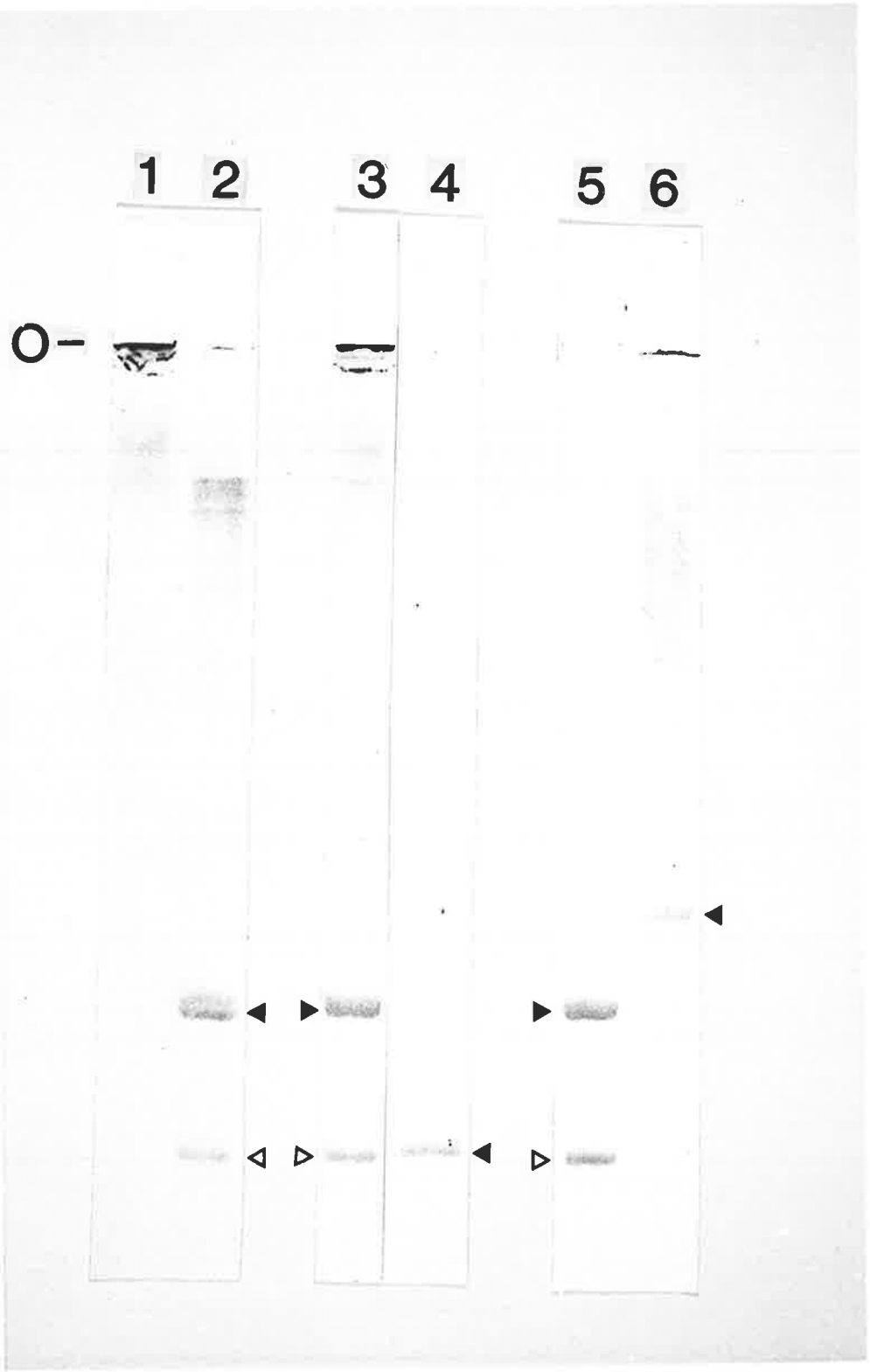

1
2
3
4
5
6
7
8
9
-O
-XC
-BPB
M
LTSV
SNMV
VTMoV
X
LTSV
SNMV
VTMoV
X
TaqI
MspI

Figure 6-3 Co-inoculation of VTMoV and the virusoids of LTSV-NZ and a 388 nucleotide residue virusoid variant of SCMoV

A VTMoV viral RNA preparation largely free of the virusoid was co-inoculated onto N. clevelandii at 50 µg/ml with 50 µg/ml of either the virusoid of LTSV-NZ or a virusoid of SCMoV. Virus was extracted after 10 days and the viral RNA purified and subjected to 4.5% polyacrylamide/7 M urea/TBE gel electrophoresis. Staining was with toluidine blue-O.

1. VTMoV viral RNA (partially virusoid free) inoculated alone.
2. VTMoV viral RNA (standard strain with virusoid present).
3. VTMoV viral RNA (partially virusoid free) and co-inoculated with the virusoid of LTSV-NZ.
4. LTSV-NZ.
5. VTMoV viral RNA (partially virusoid free) and co-inoculated with the 388 nucleotide residue virusoid of SCMoV.
6. SCMoV.

The closed and open triangles mark the circular and linear forms, respectively, of each virusoid where detected.


1 2 3 4 5 6
O-

isolates (A-D) were investigated further. One of these isolates VTMoV-A, when passaged three times at 7 mg/ml appeared to remain free of the virusoid. The virusoid of VTMoV could not be detected by a biological assay or dot blot hybridisation with a probe to the virusoid of SNMV (Figure 6-4). The probe readily cross-hybridised to the virusoid of VTMoV due to 93% overall sequence homology shared by both RNAs.

It was previously noted that the absence of the virusoid from infections with VTMoV led to reduction in severity of symptoms. VTMoV usually induced necrotic lesions on inoculated leaves of N. clevelandii together with severe leaf epinasty of systematically infected leaves and overall stunting. In the apparent absence of the virusoid, such as with VTMoV-A, induced only slight stunting and occasional faint mottling and mild leaf epinasty. No necrotic lesions with VTMoV-A were observed (Figure 6-5). These changes occurred despite similar virus yield between VTMoV-A and VTMoV with virusoid present (about 0.5-1 mg/g infected tissue).

VTMoV-A was derived from the original stock of virus that contaminated LTSV-NZ. Although the viral RNA had an identical double-stranded cDNA restriction endonuclease pattern as VTMoV, it could not be ascertained that VTMoV-A was free of virusoid due to a small or point mutation. This possibility seems unlikely since VTMoV-A when co-inoculated with 50 µg/ml of the

Figure 6-4 Detection for the presence of virusoid in VTMoV-A by dot blot hybridisation

A ^{32}P-labelled DNA transcript complementary to a virusoid of SNMV cDNA insert in M13mp8 (that cross reacts with the virusoid of VTMoV) was used to probe for the presence of virusoid in an apparently virusoid-free preparation of VTMoV (VTMoV-A) by dot blot hybridisation, see Methods.

1 µl samples of the following nucleic acids were probed.

1. nucleic acid extract from a healthy N. clevelandii plant.
2. 1 µg of VTMoV-A viral RNA.
3-5. 10 ng, 1 ng, 0.1 ng of standard VTMoV viral RNA with virusoid present.

H VTMoV-A

1 2

3 4 5

VTMoV

10 1 0.1 ng

purified virusoid of VTMoV, readily produces symptoms typical of VTMoV (Figure 6-5), and an RNA with the same electrophoretic mobility as the virusoid of VTMoV (results not shown).

The finding of apparent independent replication of VTMoV RNA 1 seems at variance with Gould et al., (1981) who reported a dependence of VTMoV RNA 1 on the virusoid for detectable multiplication. More recently however Dr. R.I.B. Francki (personal communication) has obtained, during insect transmission trials of VTMoV, an isolate that is free of the virusoid. Insect transmission previously reported for VTMoV by Randles et al. (1981) may also have been responsible for the original contamination reported here.

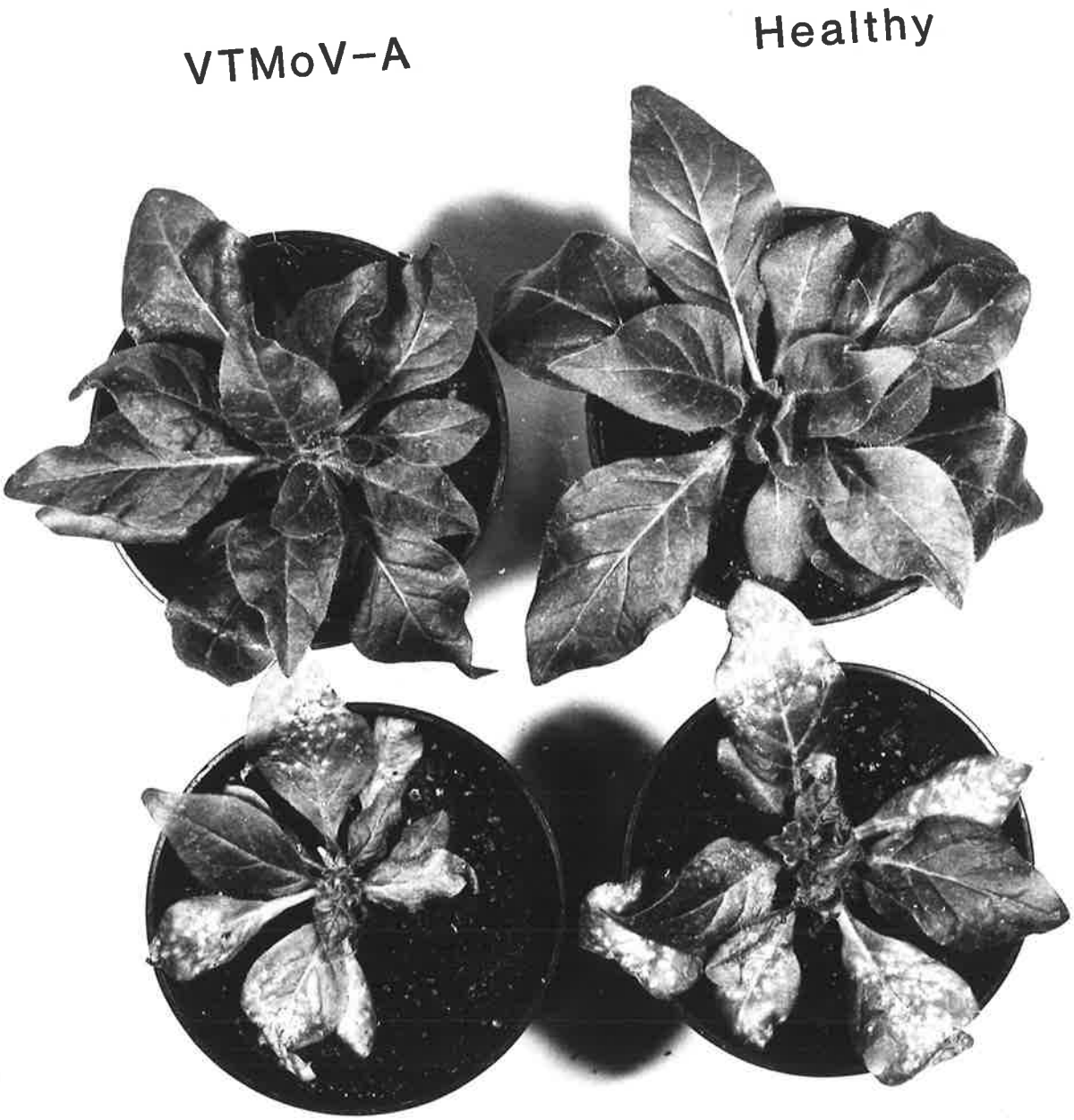
It now seems that the RNA 1 components of VTMoV, SNMV (Jones and Mayo, 1984) and LTSV (Jones et al., 1983) replicate independently of their respective virusoids. The virusoids of these viruses also lack significant sequence homology with their respective helper viruses (Gould, 1981; Gould and Hatta, 1981) and are unable to independently multiply to detectable levels (Jones et al., 1983; Jones and Mayo, 1984) thus providing stronger evidence of their satellite RNA nature.

6-3 Replication of the virusoid SCMoV with LTSV-Aus

During passaging of LTSV-Aus in C. quinoa, one viral RNA preparation revealed the presence of two RNAs

Figure 6-5 Symptoms expression of N. clevelandii infected with VTMoV

Symptoms of VTMoV, VTMoV-A, VTMoV-A plus virusoid on experimentally infected N. clevelandii plants showing mild symptoms for VTMoV-A and necrotic lesions and leaf crinkling for VTMoV and VTMoV-A co-inoculated with the virusoid of VTMoV.

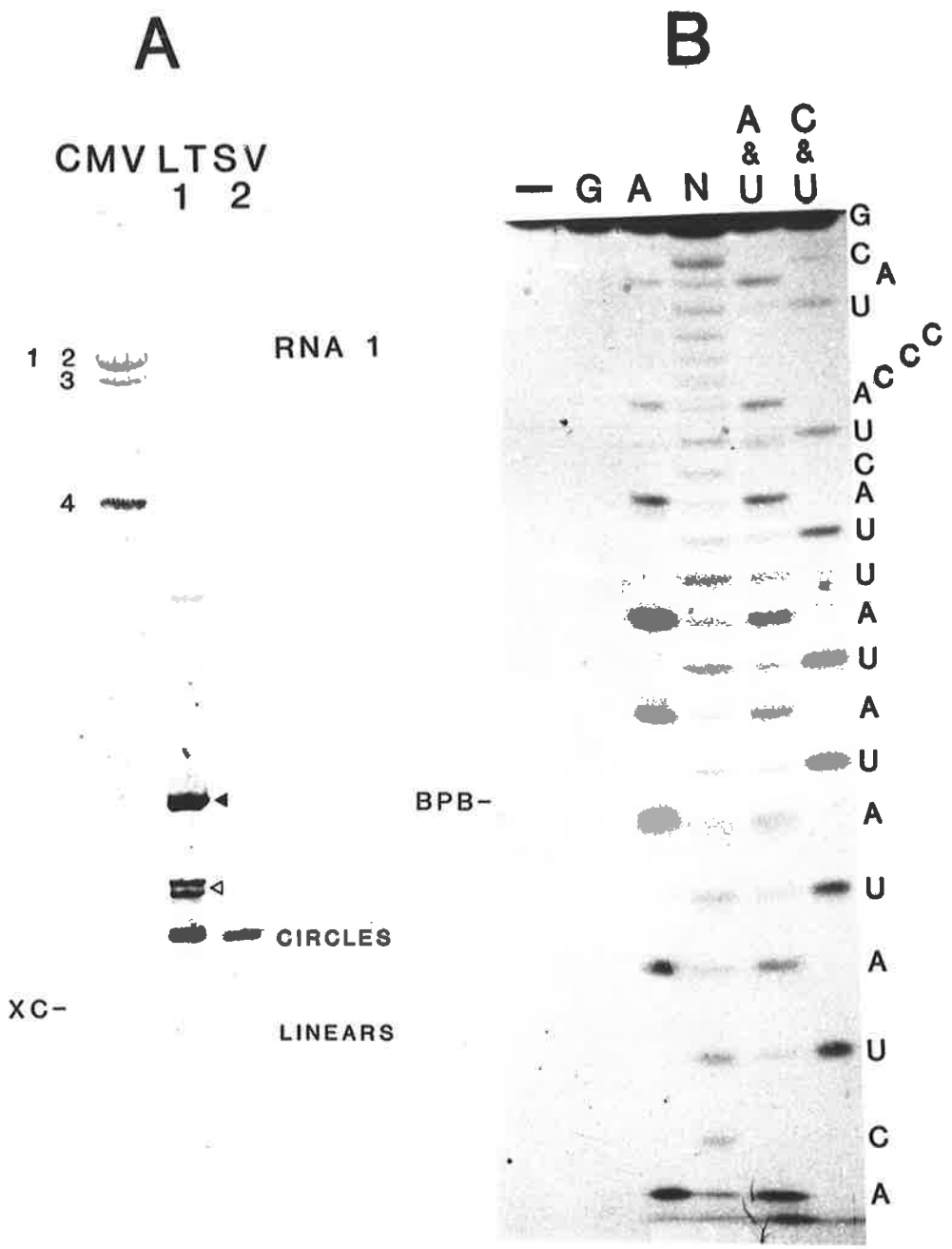

VTMoV-A
Healthy
VTMoV-A
+
virusoid
VTMoV

with slower electrophoretic mobility than the circular form of the virusoid of LTSV-Aus (Figure 6-6A). Sequence analysis by RNA partial enzymic hydrolysis as in Chapter 2, Methods 2-3, revealed that the RNA corresponded to the 388 nucleotide residue virusoid of SCMoV (Figure 6-6B). It was subsequently determined that the two extra RNA components of LTSV-Aus presumably are circular and linear forms of the virusoid (388) of SCMoV due to the identical migration of both bands during non-denaturing polyacrylamide gel electrophoresis (results not shown).

Restriction endonuclease digestion was performed as above on double-stranded cDNA to LTSV-Aus RNA 1, SCMoV RNA 1 and the RNA 1 of the LTSV isolate with both LTSV-Aus and SCMoV virusoid components. No SCMoV RNA 1 cDNA specific fragments were detected, only those of LTSV-Aus RNA 1 cDNA (results not shown). This absence of SCMoV RNA 1 is not surprising since SCMoV is unable to infect C. quinoa (Francki et al., 1983b; Francki et al., 1985). Thus LTSV appears to be able to support the replication of the virusoid (388) of SCMoV as well as the virusoid of SNMV (Jones and Mayo, 1983). In addition. viruses of the Sobemovirus group such as southern bean mosaic virus and sowbane mosaic virus are able to support the virusoids of LTSV (Paliwal, 1984; Francki et al., 1983a). These results suggest that all virusoids contain homologous signals for recognition by a range of serologically unrelated viruses. The GAUUUU sequence

FIGURE 6-6 Contamination of LTSV-Aus with the 388 nucleotide residue virusoid variant of VTMoV

When viral RNA of LTSV-Aus was subjected to 4% polyacrylamide gel electrophoresis and stained with toluidine blue-O, additional bands were detected (A, LTSV track 1, closed and open triangles). These RNAs corresponded to the circular and linear forms of the 388 nucleotide residue virusoid variant of SCMoV (Haseloff, 1983) when sequenced by RNA enzymic hydrolysis of fragments from a partial RNase T_1 digest as in Chapter 2 (B).


A
CMV LTSV
1 2
1 2 3
4
RNA 1
XC-
CIRCLES
LINEARS
B
— G A N A & U C & U
BPB-
G
C A
U
A C C C
A
U
C
A
U
U
A
U
A
U
A
U
A
U
C
A

common to all virusoids (see Chapter 5) may constitute part of that signal. This sequence cannot, however, represent the entire recognition signal since some heterologous mixtures are incompatible. For example, SNMV RNA 1 is unable to support the virusoid of LTSV-NZ (Jones and Mayo, 1984).

CHAPTER 7

REPLICATIVE STRATEGIES OF VIRUSOIDS

INTRODUCTION

Viroids and virusoids show some structural similarities. Although specific regions of noteworthy sequence homology are less apparent, these two groups of molecular parasites may demonstrate some functional homology. For example, a common mechanism of replication between viroids and virusoids has been suggested (Chu et al., 1983; Branch et al., 1985; Hutchins et al., 1985; Symons et al., 1985).

Replication of viroids proceeds via complementary RNA intermediates. Both greater-than-unit length viroid (arbitrarily designated plus RNA) and complementary (minus) RNA sequences have been described (Grill and Semancik, 1978; Grill et al., 1980; Branch et al., 1981, 1985; Bruening et al., 1982; Owens and Diener, 1982; Mülhbach et al., 1983; Spiesmacher et al., 1983; Branch and Robertson, 1984; Ishikawa et al., 1984; Hutchins et al., 1985; Symons et al., 1985). Similar findings have been reported for virusoids where multimeric plus RNAs of the virusoids of SNMV and VTMoV, and high molecular weight minus RNA of the virusoid of VTMoV were observed (Chu et al., 1983; Haseloff, 1983).

Detection of viroid and virusoid related sequences has usually employed hybridisation analysis of nucleic acids from plant extracts that have been transferred to membrane filters following size fractionation by gel electrophoresis (referred to hereafter as blot

hybridisation). This method was used for examining possible replicative strategies of the virusoids of LTSV, SNMV and VTMoV to test relationships with viroids and extend rolling circle models of viroid/virusoid replication.

MATERIALS

Enzymes

SP6 RNA polymerase was obtained from BRESA Pty. Ltd. Lysozyme was obtained from the Sigma Chemical Co.

Radiosotope

Alpha-^{32}P-GTP (1500 Ci/mmol) was obtained from BRESA Pty. Ltd.

Bacterial strains and cloning vectors

E. coli. MC1061 was generously provided by Dr. R. Harvey. Plasmid vector pSP6-4 was obtained from BRESA Pty. Ltd.

Growth media for E. coli MC1061

Luria (L) broth : 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl per litre. Where appropriate, the media was supplemented with ampicillin (50 µg/ml).

L-amp-agar consists of L-Broth with 1.5% (w/v) bacto-agar and 50 µg/ml ampicillin.

Chemicals

Ampicillin was obtained from the Sigma Chemical Co. Glyoxal was deionised by Dr. R.H. Symons according to McMaster and Carmichael (1977).

METHODS

7-1 Preparation of virusoid double-stranded cDNA clones in the plasmid pSP6-4

A. Preparation of virusoid double-stranded cDNA

Recombinant bacteriophage (10 ug) M13mp73 DNA with a TaqI insert of LTSV-Aus virusoid cDNA (nucleotide residues 56-324) or M13mp8 DNA with a Sau3AI insert of SNMV virusoid cDNA (nucleotide residues 131-216, a gift from Dr. J. Haseloff) were transcribed after annealing with a M13-specific 17 nucleotide residue primer (10 ul reactions) in 50 mm Tris-HCl pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.25 mM of each dNTP, 20 uCi alpha-^{32}P-dATP and 1 unit of the Klenow fragment from E. coli DNA polymerase I at 37^{o}C for 30 min. Transcription was terminated by heating at 70^{o}C for 2 min. The LTSV-Aus virusoid cDNA insert was excised with BamHI restriction endonuclease and SNMV virusoid cDNA insert with Sau3AI restriction endonuclease. After fractionation by 6% polyacrylamide/2 M urea/TBE gel electrophoresis the appropriate fragments were excised, eluted, ethanol precipitated and resuspended in sterile water.

B. Transformation of E. coli MC1061

E. coli MC1061 was grown in L-broth at 37°C overnight and then diluted 1/100 into fresh L-broth and grown to an OD_{600} of 0.3. The cells were chilled on ice for 10 min, pelleted by centrifugation at 3,000 rpm for 5 min and resuspended in 0.5 volume of cold 0.1 M $CaCl_2$. The cells were left at 0°C for 20 min, collected by centrifugation and resuspended in 0.05 volume of 0.1 M $CaCl_2$. They were then left at 0°C for a minimum of 1 h.

Competent cells (100 µl) were mixed with 2.5-5 µl of virusoid double-stranded cDNA ligated into the BamHI site of pSP6-4 and left at 0°C for 10 min. The mixture was heat-shocked at 37°C for 5 min and left at 0°C for a further 10 min. L-broth (1 ml) was added to the transformed cells which were then incubated at 37°C for 1 h before spreading on L-agar plates containing 50 µg/ml ampicillin. The plates were incubated overnight at 37°C.

C. Preparation of recombinant plasmid DNA

Single colonies were grown in 1.8 - 40 ml of L-broth containing 50 µg/ml ampicillin at 37°C overnight. The 1.8 ml cultures were transferred to an eppendorf tube, microcentrifuged for 1 min and the cells resuspended in 100 µl solution consisting of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, 5 mg/ml lysozyme (freshly added). After incubation for 5 min at room temperature, 200 µl of freshly made 200 mM NaOH, 1% (w/v) SDS was added and the mixtures left at 0°C for 5

min. The viscous solutions were neutralised by the addition of 150 ul of precooled 3 M potassium acetate, pH 4.8, and left at 0°C a further 5 min. Cell debris was removed by centrifugation for 5 min and nucleic acid in the supernatant was ethanol precipitated, washed with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 0.1 mM EDTA, pH 8.0.

Bacteria from 40 ml cultures were pelleted and resuspended in 0.4 ml of 15% sucrose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, 5 mg/ml lysozyme (freshly added) and incubated at 0°C for 30 min. An alkali/SDS solution (0.8 ml) containing 0.2 M NaOH, 1% (w/v) SDS was added and the mixture was then neutralised with 0.5 ml of 3 M sodium acetate, pH 4.6, for 40 min at 0°C. Bacterial debris was removed by microcentrifugation (10,000 rpm for 10 min at 4°C) and the supernatant treated with 1 ul of 1 mg/ml RNase A (DNase free) for 20 min at 37°C. The mixture was extracted with phenol:chloroform (1:1) and ethanol precipitated. The pellet was resuspended in 0.16 ml of water and the DNA precipitated for 1 h at 0°C after the addition of 0.04 ml of 4 M NaCl and 0.2 ml of 13% PEG. Plasmid DNA was pelleted by microcentrifugation (10,000 rpm for 10 min at 4°C), washed with 70% ethanol, dried *in vacuo* and resuspended in 0.1 mM EDTA, pH 8.0. The solution was then extracted with phenol:chloroform (1:1), followed by ethanol precipitation and spermine

precipitation. The pellet was resuspended in 100 ul of 0.1 mM EDTA, pH 8.0.

Recombinant clones and their orientation were determined by restriction endonuclease digestion.

7-2 Preparation of ^{32}P-labelled probes

A. Preparation of ^{32}P-labelled cDNA probes

Single-stranded ^{32}P-labelled cDNA transcripts of the virusoids of LTSV-Aus and SNMV were prepared from recombinant clones as described in Chapter 6, Methods 6-2 except that a HaeIII insert of LTSV-Aus (nucleotide residues 206-324/1-15) double-stranded virusoid cDNA in bacteriophage M13mp8 was used for generating ^{32}P-labelled DNA transcripts. By linearising the recombinant bacteriophage DNA after transcription at a unique EcoRI restriction endonuclease site, a low molecular weight ^{32}P-labelled transcript corresponding in sequence to the virusoid (or its complement) could be readily separated from the high molecular weight unlabelled complementary strand by denaturing electrophoresis (Bruening et al., 1982).

B. Preparation of ^{32}P-labelled RNA transcripts

Recombinant clones of the virusoids of LTSV-Aus and SNMV in pSP6-4 were linearised downstream from the insert by restriction endonuclease digestion with EcoRI. Linear DNA template (about 1-2 ug) was transcribed (Melton et al., 1984) in a reaction mixture (25 ul) containing 40 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 10 mM DTT,

100 µg/ml BSA, 0.5 mM ATP, CTP, UTP, 0.01 mM unlabelled GTP, 0.0025 mM alpha-^{32}P-GTP and 5 units of SP6 RNA polymerase. The reaction was incubated at 40^{o}C for 1-4 h, terminated by the addition of 25 µl 95% (v/v) deionised formamide, 10 mM EDTA pH 8.0, 0.02% bromophenol blue and 0.02% xylene cyanol FF, heated at 100^{o}C for 1 min and electrophoresed in a 6% polyacrylamide gel containing 7 M urea and using TBE buffer. Following autoradiography, the RNA transcripts were excised, eluted, ethanol precipitated and resuspended in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, 0.1% (w/v) SDS, 5 mM 2-mercaptoethanol.

7-3 Blot hybridisation analysis

Partialy purified nucleic acid extracts (Hutchins et al., 1985) were prepared as described in Chapter 2, Methods 2-1, except that 3 volumes of AMES buffer and 1.5 volumes of redistilled phenol were used for homogenisation. These extracts together with viral RNA and virusoid RNA markers were denatured by glyoxalation (McMaster and Carmichael, 1977). Reaction mixtures (24 µl) consisted of 0.2-20 µg of nucleic acids, 1 M deionised glyoxal and 10 mM sodium phosphate, pH 6.5. Reaction mixtures were incubated at 50^{o}C for 60 min, after which 10 µl of 40% (w/v) sucrose, 0.02% bromophenol blue, 0.02% xylene cyanol FF was added. Samples were fractionated by electrophoresis in 1.9% agarose gels (14 x 14 x 0.3 cm) containing 10 mM sodium

phosphate pH 6.5, at 30 mA. Nucleic acids were transferred by capillary action to nylon filters (GeneScreen) which were then baked *in vacuo* at 80°C for 2 h (Thomas, 1980). Blot transfer of non-denatured nucleic acids were strand separated and fixed on the filter prior to baking as adapted from Grunstein and Hogness (1975) by layering the filter on a sheet of Whatman 3MM paper soaked in 50 mM NaOH. After 1 min the filter was neutralized by infusion with 1.0 M Tris HCl pH 7.4 (2 x 2 min) and equilibrated in 10 x SSC (2 x 5 min). Conditions for prehybridisation, hybridisation and subsequent washing of filters were essentially as described in Chapter 6, Methods 6-3, except that prehybridisation was at 55°C while hybridisation and washing of filters were carried out at 65°C for RNA:RNA hybridisation. Hybridisation to plus sequences of VTMoV and SNMV was done in conjunction with Dr. J.L. McInnes.

RESULTS

7-1 Plus and minus RNA sequences of the virusoids of LTSV-Aus and LTSV-NZ.

Total viral RNA and partially purified nucleic acid extracts of plants infected with LTSV-Aus and LTSV-NZ were glyoxalated and subjected to blot hybridisation analysis using plus and minus ^{32}P-labelled DNA or RNA probes prepared from partial length DNA clones. LTSV-Aus virusoid clones were used to synthesise probes specific

for both the virusoid of LTSV-Aus and LTSV-NZ, since these two virusoids share 98% sequence homology.

The virusoids of both LTSV-Aus and LTSV-NZ gave the same pattern of an oligomeric series of plus RNA sequences up to octamers when compared with markers (Figure 7-1A, C) or by regression analysis as described by Bruening et al. (1982) [Figure 7-2]. The minus RNA sequences of the virusoids gave an identical pattern with the same relative abundance to the plus sequences (Figure 7-1B). This appeared to be due to some form of cross-hybridisation effect which appeared to be overcome by heating the baked filter in water at 90^{o}C for 10 min. After this procedure the major minus RNA component was still a form that co-migrated with the monomeric plus RNA but a regular oligomeric minus series was no longer discernible (Figure 7-1D).

Both the plus and minus LTSV-Aus and LTSV-NZ specific sequences disappeared if viral RNA and partially purified extracts were incubated with RNase A (results not shown). The detection of high molecular weight plus RNA oligomers in virions probably accounts, as suggested by Kiefer et al. (1982), for the difficulty in eliminating satellite RNA sequences, such as the virusoids of LTSV, from the helper virus by gel electrophoresis.

7-2 Plus and minus RNA sequences of the virusoids of VTMoV and SNMV

Figure 7-1 Plus and minus species of the virusoids of LTSV-Aus and LTSV-NZ as detected using both RNA and DNA probes

Glyoxalated nucleic acid extracts were fractionated by agarose gel electrophoresis, bi-directionally transferred to GeneScreen and probed with partial length ^{32}P-DNA probes in A and B and with ^{32}P-RNA probes in C and D. Filters in C and D, but not in A and B were washed prior to prehybridisation (see METHODS). Tracks 1 and 7, healthy leaf extract; tracks 2 and 8, LTSV virusoid marker; tracks 3, 9, 14 and 16, extract of LTSV-NZ infected leaves; tracks 4, 10, 13 and 15, LTSV-NZ viral RNA; tracks 5 and 11, LTSV-Aus viral RNA; tracks 6 and 12, size markers of *Bacillus subtilis* phage SPP1 DNA digested with EcoRI and end-filled with alpha-^{32}P-dATP (DNA and sizes of restriction fragments in nucleotide residues kindly provided by Peter Reeves).

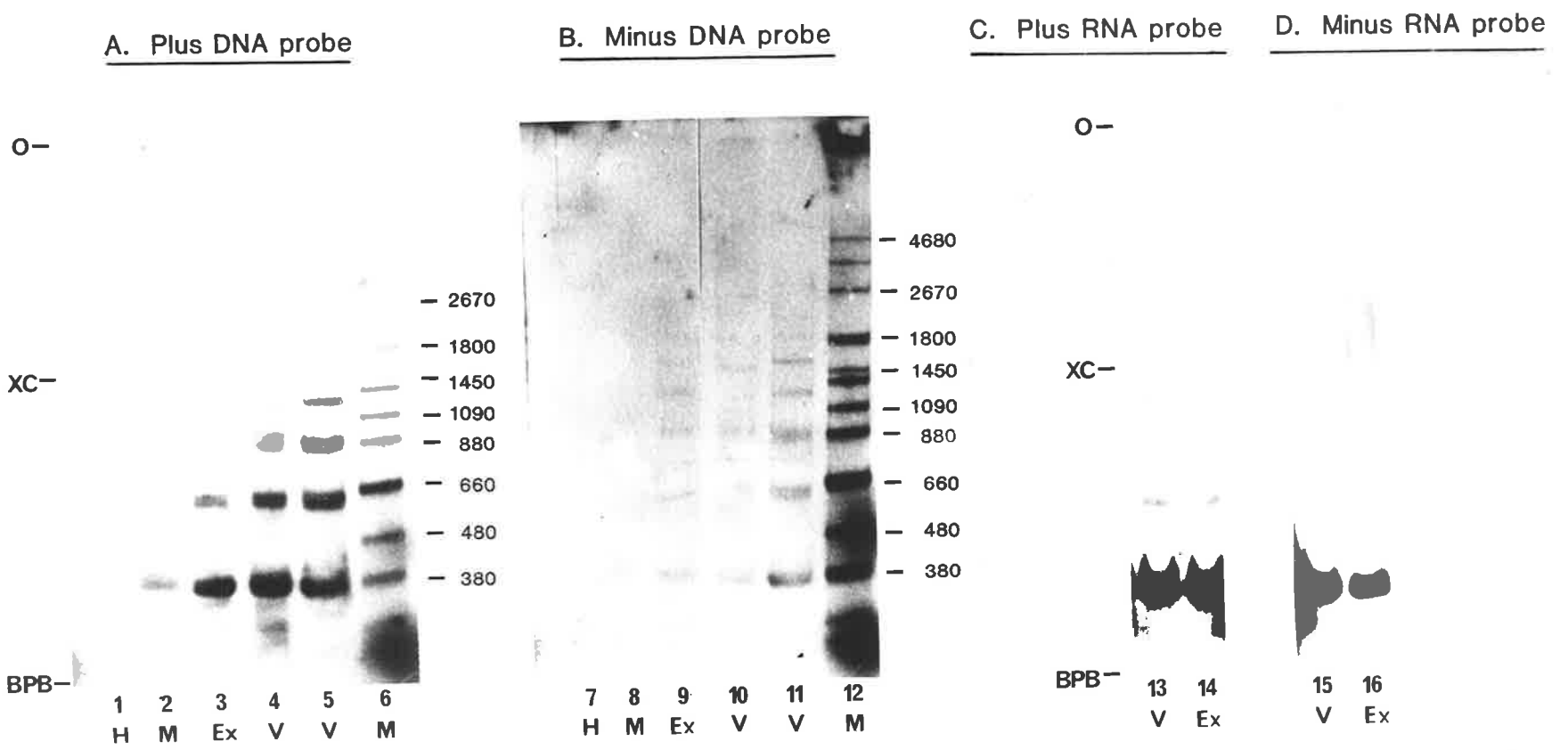
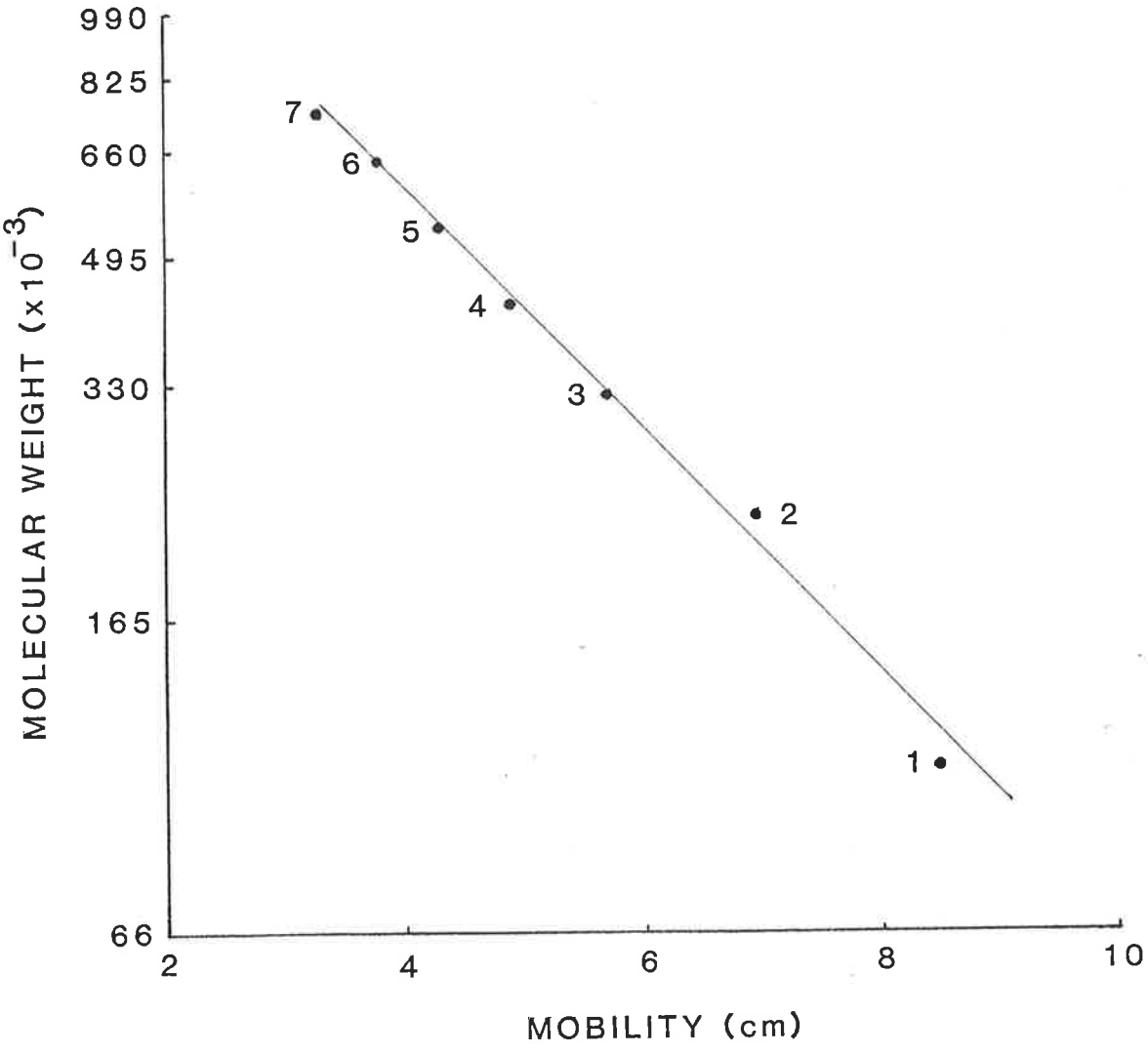

A. Plus DNA probe
B. Minus DNA probe
C. Plus RNA probe
D. Minus RNA probe
O–
XC–
BPB–
2670
1800
1450
1090
880
660
480
380
4680
1 H
2 M
3 Ex
4 V
5 V
6 M
7 H
8 M
9 Ex
10 V
11 V
12 M
13 V
14 Ex
15 V
16 Ex

Figure 7-2 Plot of the logarithm of the presumed molecular weight values of the oligomeric series of the virusoid of LTSV-Aus bands against mobility

Oligomeric virusoid of LTSV-Aus series, 1-7, (taken from Figure 7-1A track 5) based on molecular weight of 108,000 for LTSV-Aus RNA 2.


990
825
660
495
330
165
66
MOLECULAR WEIGHT (x10^{-3})
7
6
5
4
3
2
1
2
4
6
8
10
MOBILITY (cm)

A virusoid of SNMV clone was used to synthesise probes specific for both the virusoids of VTMoV and SNMV since these two virusoids share 93% sequence homology (Haseloff and Symons, 1982).

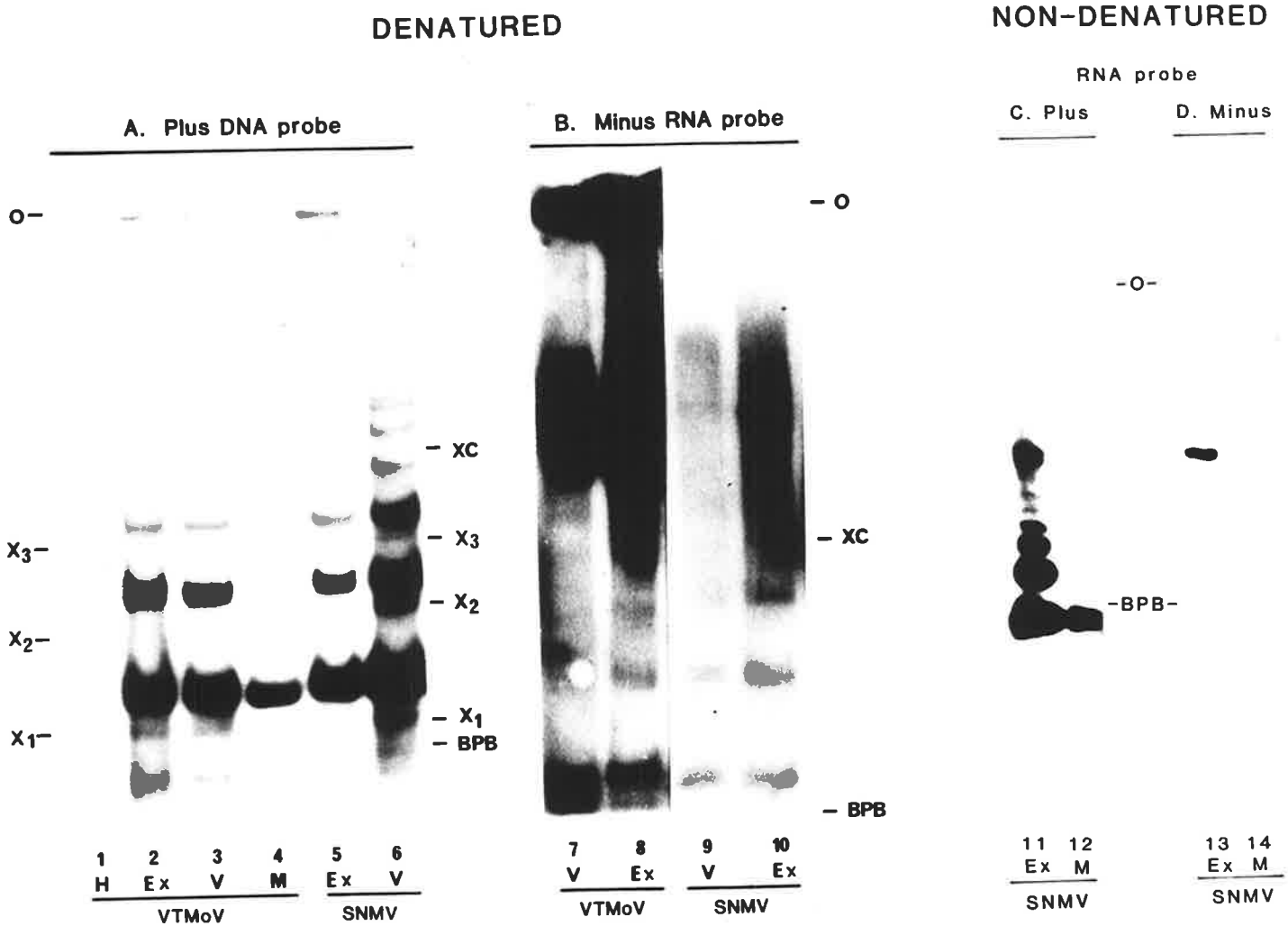
Similar to the findings of the virusoids of LTSV, and in accordance with Chu et al. (1983) and Haseloff (1983) an oligomeric series of plus VTMoV and SNMV virusoid RNA sequences up to decamers were detected in nucleic acid extracts from infected plants as well as in total viral RNA. In addition to this major oligomeric series based on the monomeric unit of molecular weight of 121,000 (virusoid of VTMoV) or 125,000 (virusoid of SNMV) a minor oligomeric series was observed (Figure 7-3A). This series of 'X' bands is analogous to that reported for ASBV (Bruening et al., 1982) but were not observed for the plus sequences of the virusoids of LTSV (Figure 7-1A). It would seem unlikely that this series of 'X' bands represents a conformational difference rather than a size difference with respect to the major oligomeric series since the nucleic acids were fully denatured by glyoxalation (McMaster and Carmichael, 1977) and the marker virusoid RNA (Figures 7-1A, track 2; 7-3A, track 4), circular and linear forms of the same RNA migrate as a single band in the agarose gel system.

The dominant minus RNA sequences of the virusoid of SNMV are high molecular weight forms similar to that observed for the virusoid of VTMoV (Figure 7-3B, D) and

Figure 7-3 Plus and minus species of the virusoids of VTMoV and SNMV detected by blot hybridisation in nucleic acid extacts of infected N. clevelandii plants and in total viral RNA

Glyoxalated nucleic acids (A and B) were fractionated by agarose gel electrophoresis, transferred to GeneScreen and probed with a partial length ^{32}P-DNA plus probe in (A) and a ^{32}P-RNA minus probe in (B). Non-denatured nucleic acids (C and D) were denatured after bi-directional transfer to the filter to enhance binding to the filter and then probed with a partial length ^{32}P-RNA minus probe. Track 1, healthy plant extract; tracks 2 and 8, VTMoV-infected leaf extract; tracks 3 and 7, total VTMoV viral RNA; track 4, virusoid marker of VTMoV; tracks 5, 10, 11 and 13, SNMV-infected leaf extract; tracks 6 and 9, total SNMV viral RNA; tracks 12 and 14, virusoid marker of SNMV.

The bands at the origin of B, tracks 7 and 8 is not usually seen and may have been due to overloading of the sample or non-specific aggregation after glyoxalation as indicated by the occassional difficulty in redissolving ^{32}P-labelled glyoxalated RNA.


DENATURED
NON-DENATURED
A. Plus DNA probe
B. Minus RNA probe
RNA probe
C. Plus
D. Minus
O–
X3–
X2–
X1–
– XC
– X3
– X2
– X1
– BPB
– O
– XC
– BPB
-O-
-BPB-
1 H
2 Ex
3 V
4 M
5 Ex
6 V
7 V
8 Ex
9 V
10 Ex
11 Ex
12 M
13 Ex
14 M
VTMoV
SNMV
VTMoV
SNMV
SNMV
SNMV

reported by Chu et al. (1983). This finding is in marked contrast to the dominant monomeric minus RNA form of the virusoids of LTSV (Figure 7-1D) but like the virusoids of LTSV, the minus sequence disappear upon incubation with RNase A (results not shown). The presence of high molecular weight SNMV virusoid minus RNA is supported by blot hybridisation analysis in non-denaturing gels whereby a dominant high molecular weight plus RNA band (Figure 7-3D,track 11) co-migrated with a single high molecular weight minus RNA band (Figure 7-3D, track 13). An equivalent high molecular weight plus RNA band could not be detected by denaturing blot hybridisation analysis (Figure 7-3A, tracks 5 and 6). This indicates that glyoxalation is able to denature double-stranded DNA. No prominent high molecular weight plus RNA sequences were detected by non-denaturing blot hybridisation of the virusoids of LTSV (results not shown).

7-3 Binding efficiency of nucleic acids transferred to a nylon-based filter (GeneScreen)

A ^{32}P-labelled RNA transcript complementary to plus sequences of the virusoid of LTSV-NZ was synthesised by SP6 transcription of the TaqI cloned insert. It was glyoxalated, electrophoresed in a 1.9% agarose, 10 mM sodium phosphate, pH 6.5 gel and transferred to GeneScreen. Unlabelled, glyoxalated total viral RNA and purified virusoid RNA of LTSV-NZ were co-electrophoresed

with the ^{32}P-labelled RNA transcript. After baking at 80^{o}C for 2 h, the filter underwent prehybridisation, hybridisation and washings as described in Methods 7-3, except that no probe was added at the hybridisation step. After autoradiography, however, not only did the ^{32}P-labelled RNA transcript give a signal but also the nucleic acids in the unlabelled tracks (Figure 7-4). This result was attributed to the release of loosely-bound ^{32}P-labelled RNA transcript from the filter during prehybridisation and/or hybridisation which then hybridised to unlabelled nucleic acids bound to the filter.

In order to quantify this loss of nucleic acids from the nylon-based filter during the blot hybridisation procedure, a ^{32}P-labelled RNA transcript as used above was monitored for losses during various steps of the blot hybridisation procedure (Table 7-1). Of the ^{32}P-labelled RNA transcript that was transferred to the filter, only 38%-45% was retained after the final washing step; most nucleic acid was lost during prehybridisation. As indicated in Figure 7-4, the released nucleic acid is available for hybridisation to complementary sequences still bound elsewhere on the filter and as such may give rise to misleading hybridisation signals (see Discussion). Most of the loosely bound nucleic acid appeared to be removed by

Figure 7-4 Nucleic acid transfer during blot hybridisation

A ^{32}P-labelled partial length RNA transcript for plus sequences of the virusoid of LTSV-NZ was glyoxalated and gel electrophoresed in 1.9% agarose/10 mM sodium phosphate pH 6.5 (track 1) together with unlabelled healthy extract (track 2), infected leaf nucleic acid extract (track 3) and virusoid marker RNA (track 4). After transfer to GeneScreen, baking and prehybridisation, the hybridisation step was performed without adding probe. After the washing steps the filter was autoradiographed at -70^{o}C for 12 h (track 1) and 120 h (tracks 2-4).


1 2 3 4
O-
32P H Ex M

Table 7-1 Binding efficiencies of a glyoxalated RNA transcript transferred to a nylon-based membrane filter

	Gel 1		Gel 2	
	Top Filter	Bottom Filter	Top Filter	Bottom Filter
Initial binding	11 %	15 %	13 %	15 %
After prehybridisation	6 %	7 %	5 %	8 %
After hybridisation and washings	5 %	6 %	5 %	6 %

The binding efficiencies of a glyoxalated ^{32}P labelled RNA plus RNA from a pSP6-4 clone with a virusoid of LTSV-NZ double-stranded cDNA TaqI insert were estimated at different stages during blot hybridisation (see Methods) with a mini-monitor, g-m meter, Mini-Instrument Pty, Ltd. The percent efficiencies were calculated from the two gels as the number of counts on the filter after treatment (first detected by autoradiography) divided by the total number of counts loaded on the gel (estimated by spotting onto a filter an equivalent amount of ^{32}P-labelled RNA transcript to that used for loading the gel) x 100.

heating the filter prior to prehybridisation at 90^{o}C for 10 min in water.

DISCUSSION

Two problems handicap interpretation of the hybridisation data presented here. One is the technical problem of differentiating between plus and minus RNA sequences and the second is a more theoretical one of giving temporal significance to the static forms detected by blot hybridisation and so reflecting the dynamic nature of the replication process.

The difficulty in distinguishing between plus and minus RNA sequences may have been due to the inability to fully denature double-stranded RNA sequences. However, attrition of apparently greater than 50% of virusoid-related sequences from the filters during prehybridisation and hybridisation (Table 7-1) may provide a more likely explanation. There are several ways that spurious results may arise due to the interference of unlabelled nucleic acid lost from the filter with the radioactively labelled probes. For example, the minus RNA sequences of the virusoids of VTMoV and SNMV could not be detected when using a ^{32}P-labelled DNA probe and no washing of the filter prior to prehybridisation. It is suggested that this was due to high concentrations of plus sequences shed from the filter during prehybridisation that then formed stable

RNA:RNA hybrids with filter bound minus RNA. These hybrids may have prevented formation of less stable DNA:RNA hybrids with ^{32}P-labelled DNA minus probe but not with a ^{32}P-labelled RNA probe (Figure 7-3B; Chu et al., 1983).

A second possible effect of the release of high concentrations of plus sequences from the filter is competition with the plus probe in solution. Paradoxically this could result in the signal detected on the filter diminishing with the increasing amount of nucleic acids loaded onto the gel and transferred to the filter.

A third possible effect of the presence of unbound nucleic acids is 'sandwich' hybridisation. For example, unbound minus RNA sequences may bind to the abundant, bound plus RNA sequences. The specific hybridisation of the minus probe to these minus RNA sequences which are hybridised to bound plus RNA sequences would give rise to the appearance of cross-hybridisation. Cross-hybridisation hampered characterisation of virusoid minus RNA sequences and has been reported to occur with both the HSV plus and minus specific probes (Ishikawa et al., 1984) and the minus probe of STobRV (Kiefer et al., 1982). Cross-hybridisation may have been mistakenly attributed to the probes not being highly specific for one particular orientation, citing the high degree of self complementarity of these pathogenic RNAs. The true

cause of cross-hybridisation could be tested by probing *in vitro* synthesised plus and minus RNA transcripts that are transferred to separate filters and to the same filter. A minus probe that is capable of cross-hybridising to plus sequences will generate a signal when hybridised to both the filter with only minus RNA transcript present as well as to the filter with both plus and minus RNA transcripts. The 'sandwich' effect will only give false hybridisation to plus RNA sequences on the latter filter. Possible examples of the 'sandwich' effect is the identical pattern detected for minus and plus RNA sequences of the virusoids of LTSV (FIgure 7-1A, B). Furthermore, presumably spurious monomeric, dimeric and trimeric minus RNA sequences of the virusoids of VTMoV and SNMV were more readily detected if the filters were not washed prior to prehybridisation (results not shown).

Finally, interference by unlabelled nucleic acids may explain the gap effect described by Branch and Robertson (1984); Branch *et al.* (1985) in which a gap in hybridisation to minus RNA sequences of PSTV was observed in the region corresponding to unit length plus RNA sequences. Similar findings were obtained with hybridisation analysis of CCCV-related minus RNA sequences (Hutchins *et al.*, 1985). In the case of PSTV this effect was shown to be due to the presence of an excess of plus over minus sequences. It may have

resulted from hybridisation of released or partially released unlabelled unit length plus sequences to nearby unit length minus RNA sequences. Hybridisation between same sized molecules may preclude the 'sandwich' effect described above.

One partially successful solution to misleading hybridisation included heating of the filters in water after baking, but prior to prehybridisation, to release loosely bound nucleic acids. Covalent coupling to chemicall prepared papers (Wahl et al., 1979) does not appear to reduce this effect (Rathjen, 1984).

An alternative approach is the isolation of double stranded RNA from infected plants by CF-11 cellulose chromatography (Owens and Diener, 1982; Branch and Robertson, 1984; Branch et al., 1985). The lack of disparity between the levels of plus and minus RNA sequences avoids most of the above problems and has allowed detection of unit length minus RNA sequences of PSTV.

Despite these difficulties the results do suggest the existence of greater-than-unit-length plus and minus virusoid-related sequences with similar characteristics to those found from viroid infections (discussed below). As such they are consistent with a rolling circle mechanism of replication. Indeed two cornerstones of rolling circle replication are supported by experimental evidence. These include the ability of greater-than -

unit-length sequences to process to unit length, as demonstrated by specific autocatalytic cleavage of plus and minus RNA transcripts of the virusoid of LTSV-Aus (A.C. Forster, personal communication; D.B. Mitchell, 1985). Secondly, circularisation of linear RNA forms that may be carried out by known plant RNA ligases that utilise 2':3'-cyclic phosphodiester groups (Branch et al., 1982; Kornaska et al., 1982). This form of ligation step has been implicated by the presence of a 2'-phosphomonoester, 3'-5' phosphodiester bond (Kiberstis et al., 1985) across the postulated processing site of the virusoids of VTMoV and SNMV (Haseloff, 1983). Alternatively the ligation may be autocatalytic as shown by the reversibility of the cleavage reaction of STobRV (Prody et al., 1986).

Interestingly, the mechanism of replication appears to differ in some regards between virusoids. The virusoids of VTMoV and SNMV reveal non-integral multimeric plus forms in infected tissue ('X' bands) analogous to those detected for ASBV. No such bands were observed from tissues infected with the virusoids of LTSV. This may have been due to the low levels of 'X' bands in the latter case. A more significant difference lies in the nature of the major minus RNAs detected from infected tissue. Virusoid minus RNA of VTMoV and SNMV is dominated by the presence of high molecular weight components ($2\text{-}4 \times 10^6$). In contrast, unit length LTSV

virusoid minus RNA is the predominant form together with low concentrations of low molecular weight oligomers. Probes for plus virusoid-related RNA sequences revealed similar oligomeric patterns in all cases with a preponderance of monomeric RNA.

The characteristics of the virusoid minus RNAs of LTSV are more akin to those reported for ASBV and STobRV. In common with these latter RNAs, minus RNA transcripts of the virusoid of LTSV-Aus will cleave *in vitro* (A.C. Forster, personal communication). It is suggested, therefore, that the virusoid minus RNAs of VTMoV and SNMV lack recognition signals for processing.

The virusoids of LTSV and SNMV are biologically similar; they are both supported by viruses of the Sobemovirus group and are even able to multiply and be encapsidated by the same helper virus, LTSV, where they cause the same changes in symptom expression (Jones and Mayo, 1984). Therefore any variation in the mechanism of replication between virusoids may reflect differences in virusoid RNA-specific sequences and not a fundamental difference regarding interaction of virusoids with viral or host components that might be responsible for their replication.

One puzzling finding was the detection of minus RNA sequences amongst total viral RNA (Figure 7-1D,track 15; 7-3B track 7). It seems unlikely to be due to cross-hybridisation with viral plus RNA sequences since the

mobility of minus RNA sequences differs markedly from the plus RNA sequences (in the case of the virusoids of VTMoV and SNMV). One possibility is that the viral preparations were impure and included non-encapsidated RNAs.

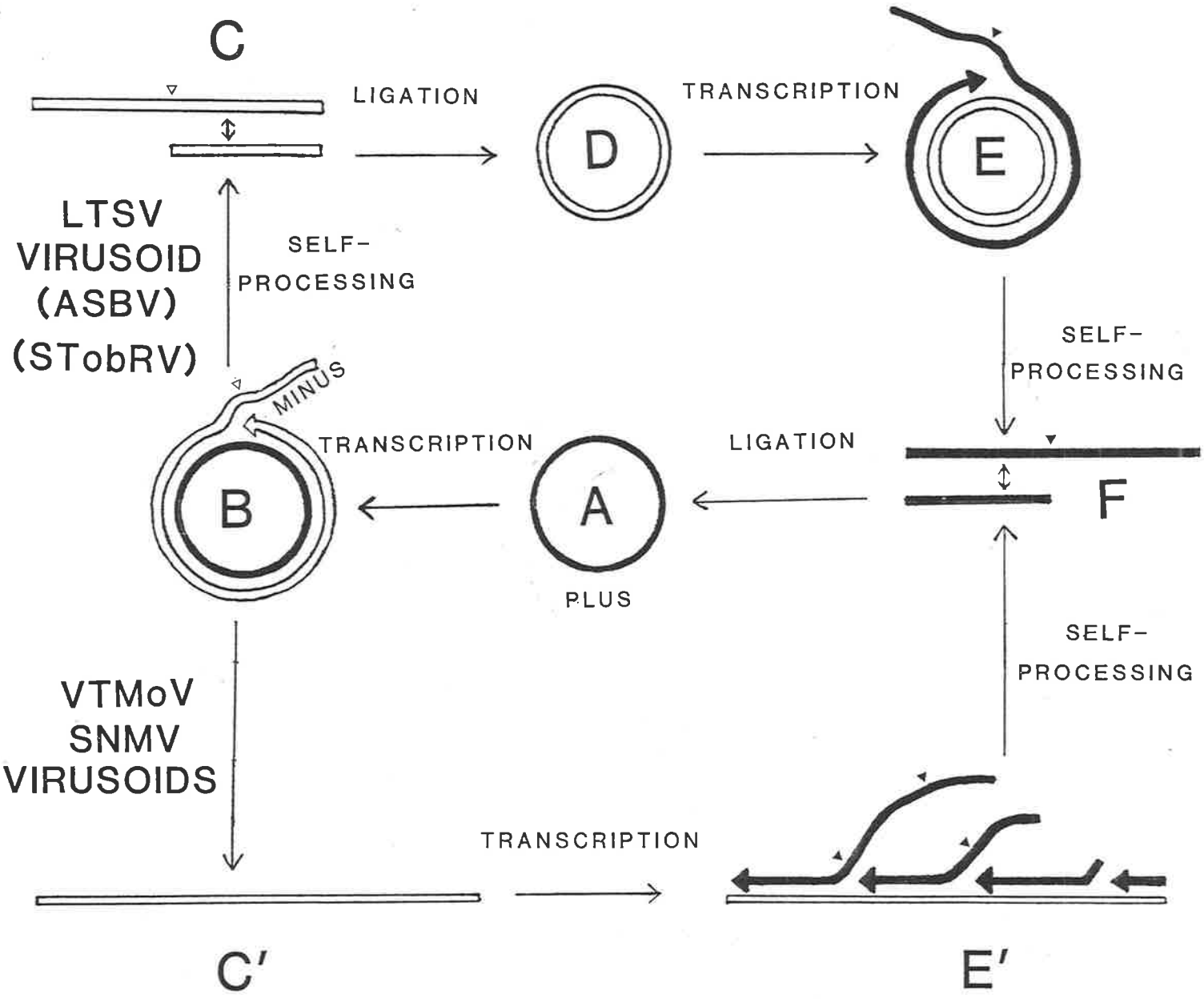
The model of virusoid replication presented in Figure 7-5 attempts to account for the existence of the virusoid-related sequences observed by blot hybridisation. As such it has several features in common with models presented for the replicative cycle of viroids (Bruening et al., 1982; Owens and Diener, 1982; Branch and Robertson, 1984; Ishikawa et al., 1984; Hutchins et al., 1985). This model, however, lacks temporal and mechanistic information regarding the generation of each RNA sequence detected by blot hybridisation. As such the model raises several questions:

1) Do the multimeric forms of pathogenic RNAs observed by blot hybridisation analysis arise out of inefficient processing or by reversibility of the ligation reaction as suggested by C. J. Hutchins (in Hutchins et al., 1985) and shown by Prody et al. (1986) and Buzayan et al. (1986a).

2) Are two or more of the plus and minus RNA sequences detected by blot hybridisation able to act as templates for replication?

Figure 7-5 Model of rolling circle replication for virusoids

After inoculation with the infectious plus RNA sequences of the virusoid (A) rolling circle transcription of the template allows formation of greater-than-unit-length minus RNA (B). In the case of the virusoids of LTSV, these minus forms process to the monomeric form (C) which is circularised (D) and allows rolling circle replication of plus RNA sequences (E). In the case of the virusoids of VTMoV and SNMV the minus form is not processed and is of high molecular weight (C') which allows direct transcription of greater-than-unit-length plus RNA sequences (E'). The greater-than-unit-length plus sequences are then processed to the monomeric form (F) and then ligated to return to the infectious monomeric circular form (A). The proposed replication cycle for the virusoids of LTSV is compatible with the plus and minus forms detected for ASBV (Bruening et al., 1982) and STobRV (Keifer et al., 1982).


C
LIGATION
D
TRANSCRIPTION
E
LTSV
VIRUSOID
(ASBV)
(STobRV)
SELF-
PROCESSING
SELF-
PROCESSING
MINUS
TRANSCRIPTION
LIGATION
B
A
F
PLUS
VTMoV
SNMV
VIRUSOIDS
SELF-
PROCESSING
TRANSCRIPTION
C′
E′

3) What is the structural nature of the minus template? The minus RNAs alone of STobRSV, HSV and PSTV do not appear to be readily infectious. Ishikawa et al. (1984) suggest that a double stranded template is required for transcription of the minus strand since multimeric minus RNA transcripts of HSV were infectious only in the presence of a non-infectious form of plus RNA. However, double-stranded RNA of STobRSV is not infectious unless denatured and the HSV infectivity results may have arisen by non-specific primer extension.

4) Can the site of initiation of transcription explain features such as the anomalous 'X' bands? It cannot be determined whether this series of 'X' bands represent a true oligomeric series based on a lower molecular weight form (estimated to be 105,000 in the case of the virusoid of SNMV) or whether X_2 and X_3 represent part of an oligomeric series based on the dominant molecular weight (estimated to be 125,000 for the virusoid of SNMV) but with a single low molecular weight unit of 105,000 covalently attached. This latter possibility may be expected to occur if initiation of multimeric transcripts occurs at a different site to that of processing.

One of the most promising recent insights into the possible replicative strategies of virusoids, viroids and satellite RNAs is the reports of autocatalytic cleavage of these RNAs (Buzayan et al., 1986; Hutchins

et al., 1986; Prody et al., 1986; A.C. Forster, personal communication). As described above, processing of the virusoid minus RNAs of LTSV but not those of VTMoV and SNMV may explain the differences in minus forms detected.

These in vitro cleavage reactions may also help to explain the exact nature of the unusual double-stranded RNAs isolated by CF-11 cellulose chromatography of PSTV infected tissue (Owens and Diener, 1982; Branch et al., 1981, 1985) and STobRV infected tissue (Sogo and Schneider, 1982). For example, fully denatured double-stranded RNAs of STobRV are mainly unit length plus and minus RNA with low concentrations of circular and linear oligomeric forms. Non-denatured double-stranded RNA revealed higher concentrations of circular and linear oligomeric forms as well as the appearance of racket-shaped structures with a circular head (mainly monomeric in size) and a linear tail of variable size. A model for the origins of these double-stranded RNA structures centred on speculation of the minus RNAs having additional sequences not complementary to the plus RNA (Bruening, 1981). A more ready explanation is dependent upon cleavage site of the minus RNA that has now been located 48 nucleotide residues away from the cleavage site in the plus RNA. Hybridisation of the unit length minus RNA to unit length plus RNA would generate either single-stranded 5' overhangs of 48 nucleotide residues

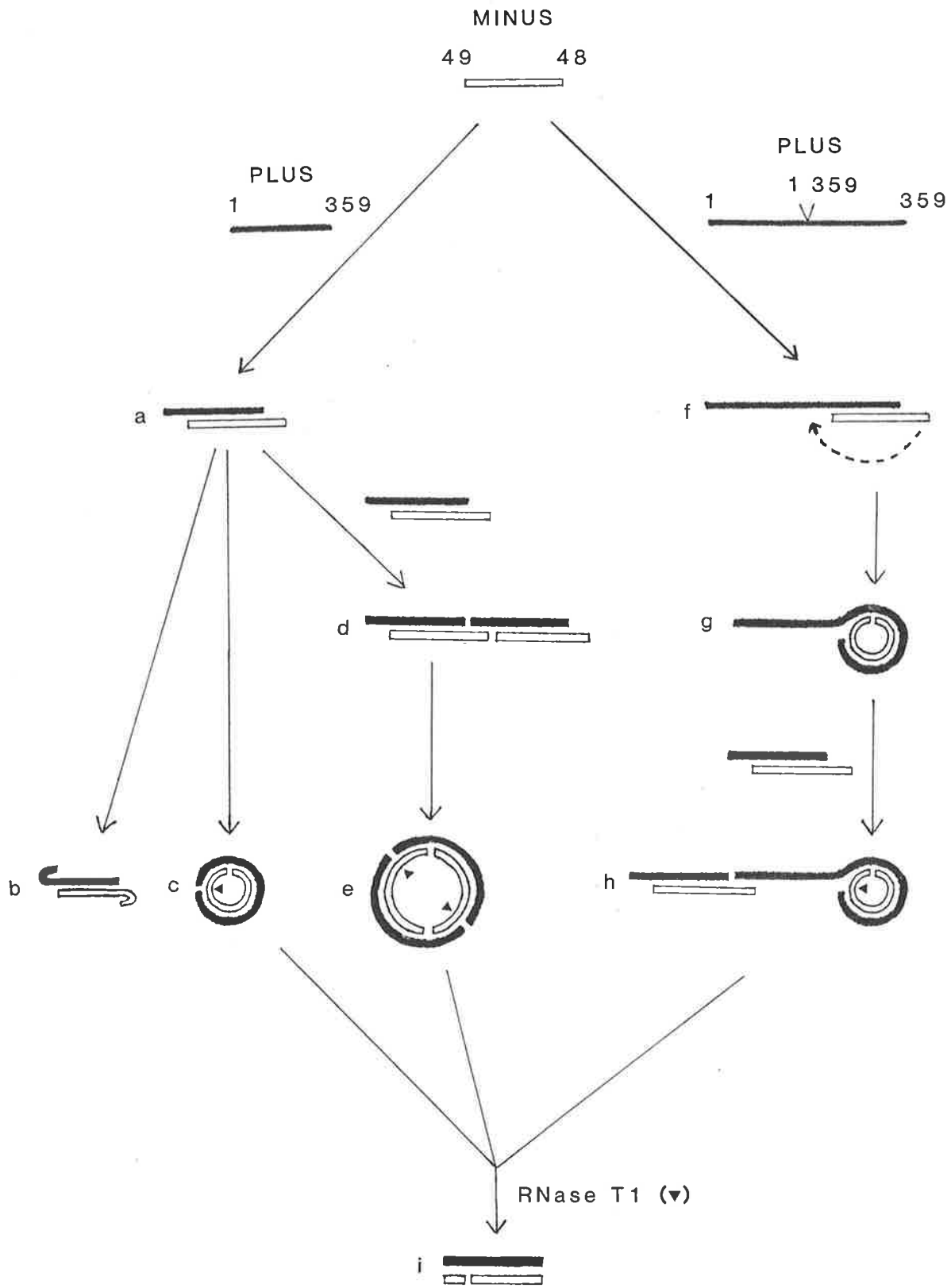
or 3' overhangs of 311 nucleotide residues. These together with oligomeric forms (Kiefer et al., 1982; Sogo and Schneider, 1982) can be used to show the generation of all non-denatured double-stranded RNA forms described by Sogo and Schneider (1982) [Figure 7-6].

Finally, how closely does the replication cycle of virusoids mirror that of viroids and the possibly related STobRV. All of these RNAs appear to be replicated by some form of rolling circle mechanism. They all reveal, in nucleic acid extracts of infected tissue, a series of oligomeric plus-related sequences with diminishing concentration of the higher molecular RNAs and complementary RNAs some of which are greater-than-unit-length. Furthermore, there is no evidence as yet that helper viral RNA encoded polymerases are responsible for the replication of virusoids or satellite RNA. Indeed, there are several lines of evidence that suggest virusoids and STobRV share replicative strategies distinct from that of their respective helper viruses. For example. virusoids can be supported by serologically distinct viruses (Jones and Mayo, 1983) some of which have never been previously found associated with a satellite RNA (Francki et al., 1983a ; Paliwal, 1984b). Unlike other satellite RNAs such as those of cucumber mosaic virus or peanut stunt virus whose termini mimic that of the helper virus

Figure 7-6 Model for the origin of double-stranded RNAs of STobRV (Sogo and Schneider, 1982)

Non-denatured double-stranded RNAs detected by electron microscopy (Sogo and Schneider) may arise as following: overlapping monomeric plus and minus strands could hybridise to form the major observed unit length linear form with single-stranded overhangs (a). These single-stranded overhangs may either form internal secondary structure (b) or hybridise with itself to form a circle (c) or a larger linear form (d) which may then also circularise non-covalently to form multimeric circular forms (e). Hybridisation of a dimeric (for example, plus) form with a unit length minus (f) allows the possibility to form racket like structures (g) with a monomeric 'head' and a tail which is variable in length (h) depending on the degree of base-pairing with forms such as (a).

Digestion with RNase T_1 generates largely monomeric forms which are infectious after denaturation. This is presumably due to resistance to digestion of the single-stranded overhangs by internal secondary structure (b) and nicking of primarily the minus template of the multimeric forms (closed triangles) to form structures such as (i). This could be due to a more accessible guanidylate residue in the minus strand near the junction of the plus cleavage site if some strand separation at an A:U base pair at the termini occurs.


MINUS
49
48
PLUS
1
359
PLUS
1
1 359
359
a
f
d
g
b
c
e
h
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(Gordon and Symons, 1983; Collmer et al., 1985), the termini of virusoids and STobRV lack the low molecular weight protein attached covalently to the RNA of their respective helper virus. Lastly, the helper virus of VTMoV appears to replicate through a unit length linear double-stranded form (Chu et al., 1983) and not by a rolling circle mechanism which requires circular forms. Whereas circular RNAs have been detected for STobRV, they are not present in tissue infected with the satellite RNA of peanut stunt virus (Linthorst and Kaper, 1984).

It is the circular nature of virusoids and STobRV, however, that may be responsible for functional analogy rather than functional homology with viroids. W. Rohde (personal communication) has observed that cDNA synthesis of the circular monomeric forms of the virusoids of VTMoV and SNMV with avian myeloblastosis virus reverse trancriptase and a specific oligodeoxyribonucleotide primer can yield oligomeric transcripts. Thus, it is the circular nature of these RNAs, rather than the type of polymerase, that could be solely responsible for determining a rolling circle mechanism of replication.

Fucntional similarities between the virusoids, STobRV and ASBV is supported by sequence homology but it remains to be established that they, together with ASBV, share a common ancestor that is phylogenetically

separate from the PSTV-like viroids. The answer may lie in the discovery of the specific viral and host factors that these parasitic RNAs interact with.

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