The Viroids

THE VIRUSES

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Edited by T. O. Diener

The Viroids

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Preface

More than seven years have passed since the first monograph on viroids was published. At that time, the existence of viroids as a novel type of pathogen far smaller than viruses had been amply demonstrated and some of their unusual molecular properties had been elucidated, but the entry of molecular biology into viroid research was still in its infancy.

Since that time, our knowledge of the molecular properties of viroids has increased exponentially and viroids have become even more fascinating than was the case seven years ago. Today, aside from transfer RNA, viroids are probably the best known type of RNA—at least from a structural standpoint. Much less is known of the mechanisms of viroid function, such as the exact pathway and enzymology of viroid replication and the biochemistry of viroid pathogenesis. Recently, however, emphasis in viroid research has shifted from structural to functional themes and important beginnings have been made in the elucidation of viroid structure–function relationships.

With the discovery of viroidlike RNAs within the capsids of certain plant viruses and the finding of surprising structural similarities between viroids and plant satellite RNAs, the conceptual gap between viroids and conventional viruses has significantly narrowed. Even beyond virology, connecting links with cellular RNAs have come to light and the long isolation of viroids (and "viroidologists") has come to an end.

It is hoped not only that the present volume brings the reader up to date on our present knowledge of viroids and viroid diseases, but that it also reflects some of the excitement and enthusiasm that motivates viroid investigators and permeates the field.

It has been pointed out before that, in contrast to many other areas of virology or plant pathology, our knowledge of the molecular aspects of viroids is far greater than that of more traditional aspects, such as the mechanisms of viroid spread in the field, possible vector transmission of viroids, and all aspects of disease epidemiology.

It is for this reason that this volume is divided into two parts: a general section that is mostly dedicated to the physical–chemical and biological properties that viroids have in common and a special section that treats each viroid in turn with emphasis on those properties that are peculiar to each and concentrating on the plant pathological aspects of the diseases that each viroid causes.

I wish to thank all the authors who contributed to the volume for their excellent and up-to-date treatments, and I hope that the book will prove useful not only to viroid investigators but also to scientists in other fields who wish to learn of the newest results regarding viroids and viroid diseases.

T. O. Diener

Beltsville, Maryland

Contents

Chapter 2

Physical–Chemical Properties: Molecular Structure (Primary and Secondary)

Paul Keese and Robert H. Symons

Chapter 3

Physical–Chemical Properties: Structure Formation

Detlev Riesner

Chapter 4

Viroid Function: Subcellular Location and *in Situ* Association with Cellular Components

Detlev Riesner

Chapter 5

Viroid Function: Viroid Replication

Heinz L. Sänger

Chapter 6

Viroid Function: Molecular Biology of Viroid–Host Interactions

Robert A. Owens and Rosemarie W. Hammond

Chapter 7

Possible Viroid Origin: Viroids, Virusoids, and Group I Introns

Gail Dinter-Gottlieb

Chapter 8

Possible Viroid Origin: Encapsidated Viroidlike RNA

R. I. B. Francki

II. Special Section

Chapter 9

Potato Spindle Tuber

T. O. Diener

Chapter 10

Citrus Exocortis

Patricia Broadbent and S. M. Garnsey

Chapter 11

Chrysanthemum Stunt

Roger H. Lawson

Chapter 12

Cucumber Pale Fruit

T. O. Diener

Chapter 13

Coconut Cadang-Cadang

John W. Randles

Chapter 14

Hop Stunt

Eishiro Shikata

Chapter 15

Chrysanthemum Chlorotic Mottle

R. Kenneth Horst

Chapter 18

Tomato Planta Macho

Jorge Galindo A.

Chapter 19

Tomato Apical Stunt

B. Walter

Chapter 20

Tomato Bunchy Top

T. O. Diener

Chapter 21

Burdock Stunt

Tien Po and Chen Wei

Viroid Abbreviations

ASBV	Avocado sunblotch viroid
BSV	Burdock stunt viroid
CCCV	Coconut cadang-cadang viroid
CCMV	Chrysanthemum chlorotic mottle viroid
CEV	Citrus exocortis viroid
CLV	Columnea latent viroid
CPFV	Cucumber pale fruit viroid
CSV	Chrysanthemum stunt viroid
HSV	Hop stunt viroid
PSTV	Potato spindle tuber viroid
TASV	Tomato apical stunt viroid
TBTV	Tomato bunchy top viroid
TPMV	Tomato planta macho viroid

Introduction

T. O. DIENER

Viruses have played a pivotal role in the dramatic elucidation of life's chemical foundation during the past three decades. Their borderline position between living and inanimate matter and their relative simplicity have made them ideal objects for the study of basic life processes.

With the discovery of the viroids, a group of replicating entities still simpler than viruses has become available to the molecular biologist; and it has become evident that their study will equally contribute to our knowledge of basic biological phenomena.

Viroids are low-molecular-weight RNAs ($1.1–1.3 \times 10^5$) of a unique, previously unknown structure that have been isolated from certain higher plant species afflicted with specific maladies. They are not found in healthy individuals of the same species, but when introduced into such individuals, they are replicated autonomously despite their small size and cause the appearance of the characteristic disease syndrome. Thus, viroids are the causative agents of the diseases in question. Unlike viral nucleic acids, viroids are not encapsidated, i.e., no virionlike particles can be isolated from infected tissue.

Although viroids have been discovered owing to the readily recognizable disease symptoms produced in certain hosts, in certain other species viroids sometimes are replicated without causing obvious damage to the host. Recent results, in fact, suggest that viroids may be more common in nature than previously believed, and that they are not restricted to diseased plants.

Originally, the term *viroid* was introduced on the basis of newly established properties of the infectious agent responsible for the potato

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spindle tuber disease (Diener, 1971). These properties were found to differ basically from those of conventional viruses in five important respects:

1. The pathogen exists *in vivo* as an unencapsidated RNA.
2. Virionlike particles are not detectable in infected tissue.
3. The infectious RNA is of low molecular weight.
4. Despite its small size, the infectious RNA is replicated autonomously in susceptible cells, ie., no helper virus is required.
5. The infectious RNA consists of one molecular species only.

As is demonstrated in this volume, subsequent work from several laboratories has amply confirmed these original postulates and has led to a vast increase in our knowledge of the structural and functional properties of these unusual pathogens.

Although the five criteria listed above constituted ample justification to classify these pathogens as a taxon separate from viruses, more recent work has shown that this disparity is even greater than could initially have been imagined. In this introductory chapter, the fundamental distinctions between organisms and viruses on the one hand and viroids on the other are examined and the biochemical and possible evolutionary significance of viroids are discussed.

I. ORGANISMS, VIRUSES, AND VIROIDS

Viruses, like organisms, are genetic systems that contain, in the form of the universal genetic code, information for the synthesis of specific proteins. Unlike organisms, however, viruses do not code for a translational machinery, but instead utilize that of their host cells. In this sense, viruses may be regarded as obligate parasites of the cell's translational system. Because of this genetic deficiency, viruses require far less genetic information than do organisms. Indeed, the genomes of the smallest viruses known consist of barely more than 3000 nucleotides (corresponding to a molecular weight of about 1×10^6) and this size has long been assumed to represent the minimal amount required for a translationally defective, but autonomously replicating, genetic system.

With the discovery of viroids, however, a form of parasitism has come to light that relies to an even greater extent than viruses on the biochemical capacities of host cells. Whereas viruses contain some or most of the genetic information required for their replication in susceptible cells, viroids rely entirely on host systems. In this sense, viroids may be regarded as obligate parasites of the cell's transcriptional machinery. Being relieved of the necessity to code for specific enzymes (or enzyme subunits) required for their replication, viroids get by with still less genetic capacity than do viruses.

But viroids differ from viruses in another fundamental characteristic. Their lack of a capsid frees them of the need to code for the corresponding

structural proteins, thus further reducing the amount of genetic information required. This, together with their total transcriptional dependence on the host cell, relieves viroids from coding for any proteins at all.

Theoretically, at least, all that viroids need are appropriate signal sequences to trigger their replication in susceptible cells. Hence, viroids represent the most extreme form of parasitism imaginable.

II. THE BIOCHEMICAL SIGNIFICANCE OF VIROIDS

Aside from their obvious agricultural and plant pathological importance (see Part II, this volume), viroids are of considerable interest to the microbiologist and molecularly oriented biologist.

Among the biochemically intriguing questions that the existence of viroids raises, the following might be mentioned:

1. What are the molecular signals that viroids possess, and cellular RNAs evidently lack, that induce an as yet inadequately specified host enzyme(s) to accept viroids as templates for transcription into RNA strands of opposite polarity?
2. What are the molecular mechanisms of viroid replication? Are these mechanisms also operative in uninfected cells? If so, what are their functions?
3. How do viroids induce disease in infected organisms? In the absence of viroid-specified proteins one is forced to assume that viroids (or their complements) directly interact with certain host constituents (Diener, 1981). The nuclear location of viroids suggests an interaction with the host genome—with potentially important lessons for the understanding of gene regulation in eukaryotic cells.
4. Why are viroids restricted to higher plants? Or do viroids, after all, have counterparts in animals?
5. How did viroids originate?

Chapters in this volume address most of these questions. Although relatively few definitive answers are available, comparison with earlier reviews (Diener, 1979a,b, 1981, 1982; Sänger, 1979, 1982; Gross and Riesner, 1980) illustrates the dramatic advances made in the last several years.

No chapter is devoted to putative animal viroids, simply because evidence for their existence has not been forthcoming. Although one group of animal diseases, the subacute spongiform encephalopathies, are incited by agents with unusual properties (Gajdusek, 1977) superficially similar to those of viroids, recent results (Diener and Prusiner, 1982; Prusiner, 1984) have demonstrated that, contrary to earlier speculations (Diener, 1972), these agents differ fundamentally from viroids.

The question of viroid origin is discussed in connection with reported

sequence similarities of viroids with group I introns (see Chapter 7, this volume). Here, a more general (and more speculative) summary of possible viroid evolution is given.

III. THE POSSIBLE EVOLUTIONARY SIGNIFICANCE OF VIROIDS

At the time when the viroid concept was advanced (Diener, 1971), viroids could be regarded as relatives of conventional viruses, being either very primitive or degenerate representatives of the latter. Knowledge accumulated since that time has rendered this concept increasingly less likely. Their apparent lack of mRNA activity and novel molecular structure imply a far greater phylogenetic distance from viruses than could be imagined previously.

Comparative sequence analysis of five related viroids (PSTV, CEV, CSV, TASV, and TPMV) has revealed striking similarities with the ends of transposable genetic elements (Kiefer *et al.*, 1983). The presence of inverted repeats often ending with U-G and C-A and flanking imperfect direct repeats suggests that viroids may have originated from transposable elements or retroviral proviruses by deletion of interior sequences. Alternatively, these similarities between viroids and transposable genetic elements could be a consequence of convergent evolution. In the former view, viroids would represent (or be derived from) RNA elements with the capacity to integrate into cellular RNA. No evidence for RNA integration mechanisms exists, but Zimmern (1982) has speculated that viroids and RNA viruses may have evolved comparatively recently from two interacting classes of RNA molecules—mobile, circular, viroidlike "signal RNAs" and linear "antenna RNAs." These two classes of RNA are assumed to be normally involved in genetic exchange between cells via RNA recombination and amplification.

On the other hand, it is also possible that viroids originated early in precellular (or early cellular) evolution when the primary genetic material was RNA. Two lines of evidence, recent studies of protein-free RNA processing reactions (Cech, 1986; Chapter 7, this volume) and eukaryotic gene structure, as well as comparative sequence analysis of prokaryotic and eukaryotic ribosomal RNAs, have led Darnell and Doolittle (1986) to propose a hypothesis in which the precursor of the eukaryotic nuclear genome is traced back to the earliest stages in evolution. In this scheme, protein-free RNA synthesis as well as site-specific RNA cleavage and splicing could all have been available for use in primitive RNA-dominated genetic systems, and fairly sophisticated RNA molecules are envisioned as early participants in evolution. In this view, viroids may be *living fossils*, RNA molecules that have survived (and evolved) since their origin during the very early prebiotic stages of evolution.

These exciting new horizons are drastically changing the position of

viroids within the scientific enterprise. Whereas earlier, viroids were generally regarded as agriculturally important, but scientifically rather esoteric entities, their scientific significance and potential evolutionary importance is increasingly being recognized.

It is hoped that the present volume will bring these fascinating new developments to the attention of a wider audience and that, as a consequence, viroid research will be given increased attention by molecular biologists and plant scientists.

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PART I

GENERAL SECTION

CHAPTER 1

Biological Properties

T. O. DIENER

I. INTRODUCTION

Of the plant diseases that are now known to be viroid incited, most were previously believed to be of virus causation. The potato spindle tuber disease, for example, was first described by Martin (1922) and shown to be an infectious disease soon after (Schultz and Folsom, 1923). From this time on until 1967, all investigators tacitly assumed that the disease was caused by a virus. Chrysanthemum stunt, similarly, was universally believed to be a conventional virus disease, ever since Brierley and Smith (1949) had demonstrated its infectious nature.

These examples demonstrate that, phenomenologically, viroid diseases do not greatly differ from virus-incited diseases. Neither their symptoms nor their epidemiological properties suggested that they are caused by anything but conventional plant viruses. Experimentally also, no clear distinctions were apparent. Procedures used with conventional viruses were readily applicable to what later became recognized as viroid diseases. For example, the agents causing potato spindle tuber or chrysanthemum stunt were found to be easily transmissible in crude extracts by procedures routinely used with many plant viruses. Field and greenhouse experiments showed that both diseases are highly contagious—being efficiently spread from plant to plant by contact with farm implements, such as tractors, cultivators, pruning tools, or even human hands. All of these observations recall properties of diseases caused by the more stable plant viruses and give no hint of the free RNA nature of the causative agents.

The mechanical transmissibility of viroids stands in stark contrast to the difficulty one experiences in efforts to sap-transmit so-called de-

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fective plant viruses, i.e., viruses that exist, because of defective or nonexistent coat proteins, exclusively in the form of free RNA (Siegel *et al.*, 1962).

Within infected plants, also, the behavior of viroids more closely resembles that of many competent viruses than that of defective ones. Whereas the latter are restricted to slow cell-to-cell movement (Siegel *et al.*, 1962), viroids, like competent viruses, usually become distributed relatively rapidly throughout the infected plant. Such rapid spread appears possible only if migration takes place through the plant's vascular bundles.

How is it possible for the viroid to display biological properties that are typical of stable viruses and unlike those of free RNA? Why are viroids relatively stable under conditions that rapidly inactivate free RNA? In this chapter, some general biological properties of viroids and viroid diseases will be discussed and an attempt will be made to present some likely, if tentative, answers to the questions raised. No attempt will be made, however, to achieve an encyclopedic coverage of the topic. For a detailed description of the biological properties of viroids, the reader is referred to Part II of this book, as well as to an extensive earlier treatment (Diener, 1979).

II. SYMPTOMATOLOGY

A. Macroscopic Symptoms

As has been noted before (Diener, 1983), most of the types of symptoms observed with viral diseases also occur with one or other viroid disease. These symptoms include stunting, epinasty (the downward curling of a leaf caused by more vigorous growth of the upper surface), veinal discolorations, leaf distortions, vein clearing, localized chlorotic or necrotic spots, mottling of leaves, necrosis of leaves, and death of whole plants. Although stunting is a predominant macroscopic symptom of most viroid diseases, stunting also occurs as a consequence of infection of plants by various conventional viruses.

These similarities suggest that viroids and viruses affect the same or similar metabolic pathways in infected cells. Present knowledge does not permit specification, in molecular terms, of the metabolic aberrations that eventually result in particular macroscopic symptoms except in broad terms. For example, many of the symptoms caused by viroid infection suggest viroid-induced disturbances in the metabolism of growth substances. As discussed below, changes in growth hormone concentrations have been found in viroid-infected plants; and it is probable that these aberrations are responsible for the reduced growth of infected plants. Similar aberrations probably cause the reduced capacity of viroid-infected plants for root formation (Horst *et al.*, 1977; Flores and Rodriguez, 1981).

In the causal chain that leads from the infecting viroid to the macroscopic symptoms, growth hormone abnormalities, however, are probably late events and give no clue as to the initiating factors that precede them.

One interesting corollary of the fact that viroids can induce a range of symptoms that are familiar from virus-infected plants has to do with the unique properties of viroids. Because viroids do not serve as mRNAs (see Chapter 5, this volume), it follows that whatever abnormalities occur as a consequence of viroid infection must be due to direct interaction of the viroid (or its complement) with cellular constituents and cannot be due to indirect effects, such as via a polypeptide translated from the viroid RNA. It is possible, therefore, that some of the symptoms observed in virus-infected plants similarly are a consequence of direct interaction of the viral RNAs (or DNAs) with cellular constituents and not, as is widely held, by interaction of virus-specific proteins with host constituents.

B. Cytopathic Effects

In addition to macroscopic symptoms, cytopathic effects of viroid infection have been observed.

1. Plasmalemmasomes

Infection of *Gynura aurantiaca* plants with CEV has been reported to result in the appearance of membranous structures of the cellular membranes, so-called "plasmalemmasomes" or "paramural bodies" (Semancik and Vanderwoude, 1976). These bodies vary in size, internal structure, and shape, and their origin and function are not yet entirely understood. Appearance of these paramural bodies has been regarded as the primary cytopathic effect of viroid infection and, in view of the claimed association of CEV with plasma membranes (Semancik *et al.*, 1976), their appearance has been regarded as suggesting a direct causal relationship with the pathogenic RNA.

In another study of cytopathic effects of viroid infection, however, plasmalemmasomes were found to be present at equal frequency in CEV-infected and healthy *G. aurantiaca* plants (Wahn *et al.*, 1980), indicating that they could not be the primary cytopathic effect of viroid infection. Viroid infection, nevertheless, was shown to affect the structure of plasmalemmasomes. In healthy plants, plasmalemmasomes with vesicular or tubular internal structures were found, whereas in CEV-infected plants, the vesicular type exhibited pronounced irregularities in size and shape and contained malformed internal vesicles (Wahn *et al.*, 1980). Hari (1980), on the other hand, did not observe paramural bodies in uninfected tomato leaf cells, but plasmalemmasomes of the type described in CEV-infected *Gynura* developed after infection with PSTV. According to da Graca and Martin (1981), healthy avocado leaves contain only a few plasmalem-

masomes, whereas many more are found in cells of the yellow regions of ASBV-infected leaves.

In still another study, healthy and CEV-infected tomato leaf cells, as well as healthy and HSV-infected hop leaf cells have been reported to contain equal numbers of plasmalemmasomes (Momma and Takahashi, 1982). Both tubular and vesicular structures were observed, but viroid infection did not cause any significant change in their structural characteristics (Momma and Takahashi, 1982). Similar results have recently been reported with healthy and CEV-, PSTV-, HSV-, and CPFV-infected tomato leaf cells, as well as with healthy and HSV- and CPFV-infected cucumber leaf cells (Kojima *et al.*, 1983). Plasmalemmasomes were identified in both healthy and viroid-infected cells; and viroid infection resulted neither in an increase in number, nor in structural changes of the plasmalemmasomes.

It is not clear why different investigators arrived at diametrically opposed conclusions regarding the presence of plasmalemmasomes in healthy and viroid-infected cells. Some of these discrepancies may only be apparent: different host species and cultivars may normally harbor different numbers of plasmalemmasomes. Also, environmental conditions may be important and individual viroids may affect the number and morphology of these structures in different ways.

2. Chloroplast Abnormalities

Disturbances of chloroplast structure, particularly thylakoid membrane abnormalities and paucity of grana, have been reported for the following viroid–host combinations: PSTV in tomato (Hari, 1980; Kojima *et al.*, 1983), ASBV in avocado (da Graca and Martin, 1981), CEV in tomato and HSV in hop (Momma and Takahashi, 1982), and finally HSV and CPFV in cucumber and tomato (Kojima *et al.*, 1983). Interestingly, in some cases, chloroplast disintegration may occur in host plants that do not display visible signs of viroid infection, as is the case with HSV and CPFV in tomato (Kojima *et al.*, 1983). It appears, therefore, that chloroplast abnormalities are a much more general consequence of viroid infection than was previously suspected.

3. Distortion of Cell Walls

Wahn *et al.* (1980) first noticed pronounced cell wall abnormalities in viroid-infected cells (CEV in *G. aurantiaca*), with their profiles appearing corrugated and of irregular thickness. Similar cell wall distortions were later observed in CEV-infected tomato and HSV-infected hop (Momma and Takahashi, 1982), in CEV- and PSTV-infected tomato, and in HSV- and CPFV-infected tomato and cucumber (Kojima *et al.*, 1983). An ultrastructural study of the apical meristem of healthy and HSV-infected hop

shoots revealed no structural changes in the 0.2-mm-thick zone of the short tip containing the apical dome and two pairs of leaf primordia, but undulating cell walls of varying thickness in third-leaf primordia of infected, but not healthy shoots (Momma and Takahashi, 1983).

4. Electron-Dense Deposits

An accumulation of electron-dense deposits in viroid-infected plants has been observed in CSV-infected chrysanthemum leaves and in PSTV-infected *Scopolia sinensis*. In chrysanthemum, these inclusions were detected in chloroplasts of infected plants; they are suspected of being phytoferritin (Lawson and Hearon, 1971). In systemically infected *Scopolia* leaves, a marked proliferation of cytoplasmic membranes was noted and these membranes, as well as the tonoplasts, were studded with electron-dense deposits (Paliwal and Singh, 1981). The chemical nature of these deposits was not investigated.

C. Biochemical Disturbances

1. Nucleic Acids

Aside from the presence of viroid-specific RNAs (monomeric and oligomeric forms of viroids and their complements) in viroid-infected cells, no obvious qualitative or quantitative changes of major nucleic acid constituent of host cells have been reported, indicating that viroid infection does not entail gross disturbances in the synthesis or degradation of host nucleic acids.

2. Proteins

In contrast, viroid-induced quantitative changes in host proteins have been observed by several investigators. For example, in PSTV-infected tomato leaves, two proteins of M_r about 155K and 195K accumulate (Zaitlin and Hariharasubramanian, 1972). Interestingly, the sizes of these proteins are similar to those of two proteins that are induced by infection of plants with tobacco mosaic virus. In the case of PSTV infection, these proteins evidently must be host proteins, because their sizes far exceed the (theoretical) coding capacity of the viroid.

In *G. aurantiaca*, two low-molecular-weight proteins (M_r 15K and 18K) accumulate after infection with CEV (Conejero and Semancik, 1977). Further study (Flores *et al.*, 1978; Conejero *et al.*, 1979) revealed that these proteins are host, not viroid-coded, proteins. This followed most clearly from the demonstration that, in each of several host species (*G. aurantiaca*, Etrog citron, potato, and tomato), infection with CEV results

in drastic increases in the concentrations of two low-molecular-weight proteins, but that the molecular weights of these proteins differ significantly from one host species to another (Conejero *et al.*, 1979).

Similar results were obtained by Camacho Henriquez and Sänger (1982a), who showed that in CEV-infected leaves of celery, citron, tomato, and *Gynura*, a phenol-soluble polypeptide in the M_r range of 12K–15K accumulates and that the apparent molecular weight differs depending on the host species. In each case, traces of what appear to be the same proteins also are detectable in preparations from healthy leaves, indicating that the proteins are host, not viroid, specific. In tomato plants infected with different viroids (PSTV, CEV, CSV, CPFV), a protein of M_r 14K (P14) accumulates dramatically (Camacho Henriquez and Sänger, 1982a). Induction of this protein also takes place after viral and fungal infections and the rate of its accumulation is directly related to symptom severity. The authors concluded that induction of P14 is a general response of tomato plants to infection.

In a further study (Camacho Henriquez and Sänger, 1982b), alterations in the patterns of acid-extractable tomato leaf proteins after infection with PSTV, two viruses, and a fungus were determined. Aside from pathogen-specific proteins (such as viral coat proteins), increases and decreases in the concentrations of several proteins were detected, among which a dramatic increase of P14 was the most obvious in each case (Camacho Henriquez and Sänger, 1982b). Recently, P14 from PSTV-infected tomato leaves has been purified and characterized. It was shown to be a basic protein (isoelectric point about 10.7) that probably assumes in solution an elongated rather than a globular shape (Camacho Henriquez and Sänger,1984). These properties seem to clearly distinguish P14 from the disease-associated proteins in CEV-infected *Gynura* described earlier (Conejero *et al.*, 1979), one of which was reported to be a slightly acidic protein (isoelectric point 5.21) (Flores *et al.*, 1978). Indeed, a detailed comparison of several "pathogenesis-related" (so-called b) proteins from tobacco and other plants has shown that P14 from tomato differs from all others (van Loon *et al.*, 1983). Furthermore, determination of the complete amino acid sequence of P14 and comparison with 3061 published protein sequences has failed to reveal homologies with any other protein—thus characterizing P14 as a structurally novel type (Lucas *et al.*, 1985).

Another protein that accumulates in viroid-infected tomato leaves (Galindo *et al.*, 1984) differs in several respects from all of the disease-associated proteins described to date. This protein has a relatively high molecular weight (140K, apparently consisting of two 70K subunits) and, in contrast to P14, was found to accumulate only as a consequence of infection by a viroid or viral satellite RNA, and not as a consequence of infection by two conventional plant viruses. Serological evidence indicated that accumulation of the 14K protein occurs after infection of plants with any one of several viroids—provided, however, that infection results

TABLE I. Presence or Absence of Symptoms and 140K Protein in Various Pathogen–Host Plant Combinations

Pathogen	Host plant	Symptoms	PM antigen[a]
–	Tomato (cv. Rutgers)	–	–
TPMV	Tomato (cv. Rutgers)	+	+
PSTV	Tomato (cv. Rutgers)	+	+
TASV	Tomato (cv. Rutgers)	+	+
CLV	Tomato (cv. Rutgers)	+	+
CSV	Tomato (cv. Rutgers)	–	–
PSTV	Potato	+	–
PSTV	*Gynura aurantiaca*	+	–
CSV	Chrysanthemum (cv. Mistletoe)	+	–
CLV	*Columnea erythrophae*	–	–
Tobacco mosaic virus	Tomato (cv. Rutgers)	+	–
Cucumber mosaic virus (CMV)	Tomato (cv. Rutgers)	+	–
CMV + CARNA 5	Tomato (cv. Rutgers)	+	+

[a] Determined serologically.

in symptom development in the infected plants (Table I). Infection of host plants other than tomato with several of the same viroids does not lead to accumulation, at detectable levels, of a protein that reacts with antiserum to the 140K protein from tomato. Electrophoretic and immunological evidence showed that the protein occurs in trace amounts in healthy leaves (Galindo A. *et al.*, 1984).

The biological significance of any of the disease-associated proteins described remains to be determined. In particular, it is not clear whether accumulation of these proteins is a product of metabolic aberrations occurring in diseased tissue or whether, on the contrary, their accumulation causes such aberrations.

Induction of P14 and presumably certain other "pathogenesis-related" proteins seems to be a general reaction of plants to infection by a variety of pathogens; and it is possible that subjection of plants to other stresses may similarly trigger their induction. If so, these proteins may belong to the class of proteins, variously called heat shock or stress proteins, that can be identified in many organisms exposed to a variety of stressful conditions (Schlesinger *et al.*, 1982a). The 140K protein, on the other hand, does not readily fit into this category. Not only does its accumulation seem restricted to plants in which a symptom-producing low-molecular-weight RNA is replicating, but, in contrast to stress proteins, which are well conserved throughout nature (Kelley and Schlesinger, 1982; Bardwell and Craig, 1984), the 140K protein could not be identified by immunological means in symptom-bearing viroid-infected plants of host species other than tomato (Galindo A. *et al.*, 1984).

It is worth noting, nevertheless, that one of the major heat shock

proteins of chicken has a molecular weight of 70K and that antibodies to this protein cross-react with proteins of similar molecular weights from heat-shocked *Drosophila*, yeast, corn seedling roots, humans, and many other organisms (Schlesinger *et al.*, 1982b). Furthermore, this protein elutes from Sepharose as an oligomer of 165K (Schlesinger *et al.*, 1982b). Thus, as is the case with the disease-associated protein identified in viroid-infected tomato (Galindo A. *et al.*, 1984), the chicken 70K heat-shock protein may exist *in vivo* as a dimer.

3. Growth Substances

Many of the symptoms caused by viroid infection, such as stunting of plants, epinasty, curling, and deformation of leaves, suggest viroid-induced disturbances in the metabolism of growth substances. Indeed, in a comparison of the concentrations of some plant growth substances in healthy and CEV-infected *G. aurantiaca* plants, an auxinlike substance of unknown chemical nature was found to be formed as a consequence of viroid infection (Rodriguez *et al.*, 1978). Also, a significant decrease in the levels of endogenous gibberellins (probably GA_3 and/or GA_1) was observed in viroid-infected as compared with healthy plants, but no changes in the levels of abscisic or indoleacetic acids were detectable (Rodriguez *et al.*, 1978).

Significantly lower levels of endogenous indoleacetic acid were, however, detected in HSV-infected as compared with uninfected cucumber seedlings. These abnormalities were detectable already 10 days after inoculation, i.e., several days before the appearance of first detectable symptoms (Yaguchi and Takahashi, 1985).

III. ECOLOGY AND EPIDEMIOLOGY

In contrast to the extensive knowledge that has been gained regarding physical–chemical properties of viroids, our knowledge of ecological and epidemiological aspects of viroids and viroid diseases is still rudimentary.

A. Origin of Viroid Diseases

It has been pointed out (Diener, 1979) that, in contrast to many diseases of cultivated plants caused by viruses, the diseases now known to be viroid incited have come to the attention of plant pathologists only recently. Thus, the first report of potato spindle tuber disease goes back only to 1922, of tomato bunchy top to 1931, of citrus exocortis to 1948, of chrysanthemum stunt to 1947, of chrysanthemum chlorotic mottle to 1969, of cucumber pale fruit to 1974, and of coconut cadang-cadang to 1937 (Diener, 1979). One wonders whether the diseases in question had been present previously for a long time and simply were not recognized

or whether they developed as agricultural problems in the recent past only. The former alternative is possible in the case of potato spindle tuber disease, which, according to Schultz and Folsom (1923), has been recognized by growers for many years and constituted one aspect of the then ill-defined "running out" problem of cultivated potato. Similarly, citrus exocortis disease was probably present as far back as the early 1920s in California and South Africa.

With some other viroid diseases, however, it is far more difficult to maintain that they had existed for a long time and simply were overlooked by growers and plant pathologists alike. Thus, chrysanthemum stunt disease has only been recognized since 1945 (Dimock, 1947); and it appears unlikely that the disease could have long gone unrecognized because, only 1 year later, in 1946, the disease had become generally prevalent in the United States and Canada (Brierley and Smith, 1949). Chrysanthemum chlorotic mottle disease was first seen in 1967 and described as a "new" disease 2 years later (Dimock and Geissinger, 1969). Equally brief is the recorded history of cucumber pale fruit disease, which was first observed in 1963 in two glasshouses in the western part of the Netherlands and which has since been observed in different places of that country (Van Dorst and Peters, 1974). In light of these observations, it appears reasonable to postulate that viroid diseases of cultivated plants are of recent origin.

In fact, none of the presently recognized viroid diseases has been known to exist before the 20th century. Why did they appear now and not in earlier centuries? It seems likely that some aspect of modern agricultural practices has favored their appearance. Whereas, in earlier times, chance transfers of viroids from wild to cultivated plants may have occurred as today, the small fields and varying genetic composition of crop plants may have made it impossible for the viroid to maintain itself in the cultivated plants. With today's large-scale monoculture of genetically identical plants, however, the opportunity for a viroid to cause serious problems has dramatically increased. Also, the commercial propagation and distribution phases of production of certain cultivated plants have become highly centralized, thus favoring rapid distribution of pathogens once they are established in the propagating stocks. With chrysanthemum, for example, one firm was supplying a majority of the cuttings distributed annually in the United States already in the 1940s (Keller, 1953). This fact undoubtedly accounts for the epidemic proportions the chrysanthemum stunt disease was able to assume only 2 to 3 years after it had been seen for the first time (Keller, 1953).

It follows that, essentially, viroid diseases of crop plants are iatrogenic diseases—a conclusion that is supported by the fact that most viroids are transmitted from plant to plant by man and his tools and not by arthropod vectors, as is the case with most plant viruses (see below).

Although these ideas may explain why viroid diseases are a recent phenomenon, they do not account for the source of the pathogens involved. Conceivably, viroids could have originated in the cultivated plant

species themselves—e.g., by mutation of normal cellular RNAs. If so, the recent origin of viroid diseases is difficult to understand. Far more plausible, it appears, is the assumption that viroid reservoirs exist in species of wild plants and that viroid diseases originate by chance transfer of a viroid from a wild carrier species to a susceptible cultivated plant species.

This hypothesis is supported by the demonstration that perfectly healthy-looking plants of the species *Columnea erythrophae* often are infected by a viroid that is distinct from all other known viroids in its primary structure, yet causes a disease similar to, but more severe than, that caused by PSTV in potato (Owens *et al.*, 1978). Furthermore, the experimental host ranges of several viroids include many wild species (Diener, 1979), and most of these species tolerate viroid replication without the appearance of recognizable disease symptoms. Thus, as a rule, viroids cause disease mainly in cultivated plant species and only rarely in wild species (presumably their natural hosts).

In this view, viroid diseases usually originate as the result of a chance encounter of the viroid with a susceptible cultivar introduced into the geographic area where the particular viroid is endemic. The potato spindle tuber disease serves as an example. Despite many efforts, no useful resistance factors to PSTV have been identified in potato cultivars (Diener, 1979), indicating that PSTV and the potato did not evolve together. This may be concluded because had they evolved together, gene-for-gene-type vertical resistance should be identifiable, as is the case in other coevolved pathogen–host systems (Buddenhagen, 1983). Also, despite considerable efforts, PSTV could not be identified in wild potatoes in the Peruvian Andes, the ancestral home of the potato (L. F. Salazar, personal communication). It is, therefore, more likely that the potato spindle tuber disease originated as a consequence of man's moving of potato cultivars into many areas of the world, where at an unknown location a chance transfer of PSTV to potato took place from a wild species endemically infected with the viroid.

Recent evidence with the tomato planta macho disease lends credence to these speculations. This disease, which is characterized by severe stunting of affected plants and strong epinasty of leaves and leaflets (see Chapter 18, this volume), was observed for the first time in 1969 in the state of Morelos, Mexico (J. Galindo A., personal communication) and was first described by Belalcazar and Galindo A. (1974). Later, it was shown to be caused by a viroid (Galindo A. *et al.*, 1982), TPMV, which belongs to the PSTV group, but is distinct in its nucleotide sequence from other members of the group (Kiefer *et al.*, 1983).

Occurrence of the disease is highly variable and infected plants often are seemingly scattered at random in tomato fields. In some cases, however, infected plants have been observed along the edges of plantations near wild vegetation (J. Galindo A., personal communication), suggesting spread of the viroid from a putative wild plant reservoir into tomato plants.

Indeed, a concerted search for naturally viroid-infected wild plants has revealed that, in Morelos, four wild solanaceous plant species, *Solanum nigrescens, S. torvum, Physalis* aff. *foetens,* and *Jaltomata procumbens,* harbor a viroid with incidences ranging from 0.2 to 12% (Orozco Vargas, 1983). In tomato, this viroid incites symptoms indistinguishable from those of TPMV and recent evidence obtained by nucleic acid hybridization indicates that the viroid is indeed TPMV (Diener and Smith, unpublished). Of the naturally infected wild species, *P.* aff. *foetens* and *J. procumbens* showed clear signs of viroid infection; the others displayed only mild symptoms or none at all (Orozco Vargas, 1983). These results strongly suggest that the tomato planta macho disease originated by chance transfer of the viroid from one of its naturally infected wild host plants to cultivated tomatoes.

B. Environmental Factors

Most viroids replicate best at relatively high temperatures (30–33°C); at "normal" greenhouse temperatures of 18–20°C (Sänger and Ramm, 1975), some viroids hardly replicate at all (Diener, 1979; Takahashi and Takusari, 1979). Symptom expression, also, is usually favored by growth of plants at relatively high temperature.

The high temperature optima of most viroids reflect the fact that viroids primarily affect crops grown under tropical or subtropical conditions or else in areas with hot growing seasons (continental climates) or in greenhouses. That this is not an invariable rule is evident from the two chrysanthemum viroids (CSV and CCMV), which replicate and show symptoms best at 20–21°C (Hollings and Stone, 1973; Dimock *et al.,* 1971). With all viroids studied, high titer and early symptom expression are favored by growing plants under high light intensity (Hollings and Stone, 1973; Raymer *et al.,* 1964; Takahashi and Takusari, 1979) and sufficient fertilizer to ensure vigorous growth of plants (O'Brien and Raymer, 1964). Recent studies with PSTV confirmed the necessity of high temperature and light intensity for rapid symptom expression and titer buildup (Harris and Browning, 1980; Grasmick and Slack, 1985).

One interesting effect of an environmental factor on symptom expression was observed by Yang and Hooker (1977), who showed that PSTV-infected tomato plants develop albinism in the new growth when exposed to continuous light and that the viroid titer is from 3 to 10 times greater in white portions of infected plants than in green portions.

C. Natural Transmission

As mentioned above, most viroids are transmitted from plant to plant by man and his tools. Thus, simply brushing healthy potato plants with

foliage from PSTV-infected plants or driving contaminated tractor wheels over the foliage of healthy plants resulted in high percentages of transmission (Merriam and Bonde, 1954). With CEV, transmission occurs primarily through contaminated budding knives (Garnsey and Jones, 1967); and HSV is believed to be transmitted by hands, sickles, scissors, and bine contact during dressing, pulling, and picking operations (Sasaki and Shikata, 1980). With HSV, the viroid can overwinter in roots of infected plants; no evidence for wild-plant viroid reservoirs could be obtained (Yaguchi and Takahashi, 1984).

Despite many attempts to identify arthropod vectors of viroids, few positive results have been obtained. Walter (1981) presented evidence of TASV transmission by one of three aphid species investigated, but with low frequency only; and DeBokx and Piron (1981) reported transmission of PSTV by one of three tested aphid species in a nonpersistent manner. However, in view of the easy mechanical transmissibility of viroids by tools and the like, it is doubtful that transmission by arthropods is of great consequence in the natural transmission of viroids.

PSTV is transmitted through both seed and pollen of infected plants (Singh, 1970a) but, in view of the almost universal vegetative propagation of potatoes, this mode of transmission is unlikely to be significant in the natural spread of the disease. Vertical transmission has been demonstrated also with ASBV (Wallace and Drake, 1962), but not with other viroids.

The ready transmissibility of most viroids by mechanical means and the contagious nature of viroid diseases appear, *a priori*, incompatible with the concept of a free RNA pathogen. It must be remembered, however, that in infected cells, the great majority of viroid molecules are present in the nucleus of infected cells (Diener, 1971) or, more precisely, in the nucleolus (see Chapter 4, this volume) and that, *in situ*, the viroid is mostly associated with host constituents—probably proteins (Wolff *et al.*, 1985). While present in these complexes, for example in extracts made with low-ionic-strength buffers, viroids are relatively resistant to attack by nucleases (Diener and Raymer, 1969; Sasaki and Shikata, 1978). Presumably, under natural conditions, viroids are not transmitted as free RNAs, but in the form of viroid-host constituent complexes. Thus, although viroids lack a protective protein coat, they are nevertheless relatively well protected from attack by nucleases by virtue of their association with host constituents. Furthermore, even when liberated from these complexes (by high-salt buffers, for example), viroids are significantly less sensitive to hydrolysis by nucleases than are single-stranded viral RNAs, particularly under conditions of high ionic strength (Diener and Raymer, 1969).

These properties of viroids readily account for their mechanical transmissibility, as well as for the contagious nature of the diseases they cause. They also explain why viroids, in general, do not require specific associations with arthropod vectors. In cases where mechanical transmission of a viroid by man and his tools appears unlikely, such as in postulated

chance transfers from wild into cultivated plants, almost any moving object may suffice. Thus, goats have been suspected of having spread PSTV in one case (K. H. Fernow, personal communication). Vectorless spread of certain plant viruses is well known and, with potato virus X, it has been shown that the virus can be transmitted by contact with contaminated fur of rabbits or dogs (Todd, 1958). No reason exists why viroids could not spread, on occasion, by similar means.

D. Economic Importance and Control Measures

Several viroid-incited plant diseases are of considerable economic importance (see Part II, this volume) and all pose a potential threat to agriculture.

Several efforts have been made to eliminate viroids from infected plants by heat treatment, followed by meristem-tip culture. A small percentage of viroid-free regenerated plants was obtained in experiments with PSTV in potato (Stace-Smith and Mellor, 1970) and CSV in chrysanthemum (Hollings and Stone, 1970; Bachelier *et al.*, 1976). A different approach was taken by Lizarraga *et al.* (1980), who showed that PSTV can be eliminated by prolonged cold treatment (5–8°C) of infected plants, followed by meristem culture. Of the regenerated plants, about one-half proved to be viroid free. In view of the generally high temperature optimum of viroid replication, cold treatment of infected plants would appear to be a more promising approach than heat treatment.

Despite these therapeutic successes, most control measures are based on prevention rather than cure. No chemicals are known that will eliminate viroids (or viruses, for that matter) from infected plants, but Singh *et al.* (1975) have reported that piperonyl butoxide, the active ingredient of certain commercial insecticide formulations, is a potent inhibitor of PSTV lesion formation in *Scopolia sinensis*, provided that the compound is sprayed onto the leaves before or no later than 4 days after inoculation with PSTV. The compound also inhibits infection of potato plants with PSTV, but in an experiment designed to simulate field conditions, some infection occurred despite weekly spraying with the compound (Singh, 1977).

With many plant viruses, incorporation of genetic resistance factors into the genomes of commercially desirable cultivars has long been the preferred method of control. So far, this approach has not been successful with viroid diseases—apparently because of the absence or paucity of resistance factors identifiable in viroid host plants. Manzer *et al.* (1964), for example, tested 2037 seedlings from 24 family lines, 274 seedling selections, and 34 named cultivars of *Solanum tuberosum* for resistance to PSTV. Of these, only nine showed a high level of resistance after two inoculations and results of reinoculation tests on selections surviving initial exposure revealed that field resistance and not true immunity was involved.

More recently, Singh (1985) tested 17 plant introductions of *Solanum berthaultii* for resistance to PSTV. Two clones were resistant to both a mild and a severe strain of PSTV when inoculated mechanically, but not when graft inoculated. Even after graft inoculation, however, plants remained symptomless and the viroid was present in small amounts only—as shown by the failure to detect it by polyacrylamide gel electrophoresis (Singh, 1985).

As shown by Pfannenstiel and Slack (1980), individual potato cultivars respond to infection with a PSTV strain that causes severe symptoms in tomato with symptoms ranging from mild to severe. Three cultivars were tolerant—suggesting that such plants may be important in the perpetuation of PSTV in the field (Pfannenstiel and Slack, 1980).

Preventive measures used in agricultural practice include growing of crops from viroid-free seeds or planting stock and measures to stop these pathogens from entering and spreading through crops. Separation of viroid-infected seeds or propagation material from healthy ones requires diagnostic tests of adequate sensitivity, specificity, and rapidity. Diagnostic methods that are used for viroid detection are described below.

IV. EXPERIMENTAL BIOLOGY

A. Experimental Transmission

Because all viroids are transmissible by mechanical means, some method of mechanical transmission has been used almost exclusively in modern experimental work. With most viroids, the standard leaf abrasion method, used with many plant viruses and viral nucleic acids, is suitable. In this process, leaves are usually dusted with 500- or 600-mesh carborundum and are then lightly rubbed with cotton-tipped applicators or cheesecloth pads soaked with inoculum, followed by washing with a spray of water. With some viroids, this standard method of inoculation is inefficient or fails completely. With CEV and ASBV, a "razor slashing" method, consisting of making a number of cuts into the stems or petioles of receptor plants with a razor blade previously dipped into the inoculum, proved to be more efficient than leaf abrasion procedures (Garnsey and Whidden, 1970; Desjardins *et al.*, 1980) and with CCCV, transmission by high-pressure injection of inoculum into coconut palm seedlings has been used (Randles *et al.*, 1977). For details of these procedures see Part II of this volume.

B. Experimental Host Range

Host range studies have shown that individual viroids greatly vary in their capacity to infect and replicate in different plant species. Whereas

PSTV, for example, has been shown to multiply in about 160 species (mostly Solanaceae, but also a few species scattered among 10 other families) (Singh, 1970b), no host plants other than two species of *Chrysanthemum* have been identified for CCMV (Horst and Romaine, 1975), only two species belonging to the family Lauraceae for ASBV (da Graca and van Vuuren, 1980), but a wide range of cucurbitaceous species for CPFV (Van Dorst and Peters, 1974) and (in addition to hops and certain solanaceous species) HSV (Sasaki and Shikata, 1980). In part, this wide variety of host range may reflect the uneven attention given to different viroids and additional host species undoubtedly will come to light upon further investigation. It is evident, however, that individual viroids have clearly defined host ranges and that these differ from one viroid to another. Elucidation of the molecular mechanisms that permit a viroid to replicate in one host species but not in another, often closely related, species is one of the most important challenges of viroid research. Lists of viroid host ranges have been published (Diener, 1979; Boccardo *et al.*, 1981; Sasaki and Shikata, 1980).

C. Propagation

1. In Intact Plants

Although the source of a viroid necessary for the study of its properties could consist of infected plants of the same species in which the viroid is found in nature, this approach sometimes is not practical because of long incubation times, relatively low titers of the viroid, or undesirable plant constituents. Because of this, efforts have been made to find more suitable propagation hosts and, as a corollary, a number of new experimental viroid hosts have been detected. Also, various attempts have been made to propagate viroids in tissue and cell cultures, as well as in protoplasts.

The most commonly used propagation host for PSTV, TPMV, and TASV is tomato (*Lycopersicon esculentum* Mill.) cv. Rutgers (Raymer and O'Brien, 1962). CSV and CCMV are usually propagated in florists' chrysanthemum (*Chrysanthemum morifolium* [Ramat.] Hemsl.), cv. Mistletoe (Keller, 1951), or Deep Ridge (Romaine and Horst, 1975), respectively, whereas CEV is often propagated in *Gynura aurantiaca* plants (Weathers and Greer, 1972). Other viroids are propagated in their natural host plants: ASBV in avocado (Palukaitis *et al.*, 1979), CCCV in coconut (Randles, 1975), CPFV in cucumber (Van Dorst and Peters, 1974), and HSV in hop (Sasaki and Shikata, 1977).

2. In Tissue or Cell Culture

In principle, propagation of viroids in plant tissue culture systems is preferable to propagation in intact plants. Plant tissue culture systems,

however, still pose some problems and few investigators have used them for the propagation of viroids.

Mühlbach *et al.* (1977) have isolated viable protoplasts from leaves of healthy and viroid-infected tomato plants by a two-step enzyme treatment. Such protoplasts incorporated radioactive uridine into all cellular RNA species. In protoplasts from tomato leaves systemically infected with CEV, a band of radioactive viroid could be detected 30 hr after adding the [^{3}H]uridine, indicating that although viroid synthesis occurs in these protoplasts, its rate is low as compared with that in intact plants (Mühlbach *et al.*, 1977). In other experiments, protoplasts from three uninfected tomato cultivars have been inoculated with CEV, PSTV, or CPFV. Bioassay, as well as [^{3}H]uridine incorporation, indicated that CPFV replicated in protoplasts of the cultivar Hilda 72, but not in the other cultivars tested (Mühlbach and Sänger, 1977).

In callus cultures from PSTV-infected wild-type potato (*Solanum demissum* L.) and tomato (*Lycopersicon peruvianum* L. Mill.), in cell suspensions derived from potato (*Solanum tuberosum* L.), and in protoplasts that were inoculated *in vitro* with PSTV, continuous replication of viroid occurred over a prolonged period (Mühlbach and Sänger, 1981). The rate of ^{32}P incorporation into the viroid was about 2–3% of that into tRNA, indicating that the rate of viroid replication in these cells was comparable to that observed in whole tomato plantlets under optimal conditions within 5 days (Sänger and Ramm, 1975). Long-term replication of cell suspension cultures has also been reported by Zelcer *et al.* (1981) and Marton *et al.* (1982).

Although intact plants are still universally used for the propagation of viroids, cell culture systems have been used successfully in studies on the mechanisms of viroid replication (see Chapter 5, this volume).

D. Identification

Until some years ago, viroids were identified routinely by inoculation of suitable indicator plants. It has become evident, however, that determination of the identity of a viroid on the basis of biological properties is not feasible. A case in point is CEV, which has a host range that is almost identical with that of PSTV and induces in most of its hosts symptoms that are indistinguishable from those induced by PSTV. Because of these biological similarities, some investigators in the past have considered PSTV and CEV to be independent isolates of one and the same pathogen (Semancik and Weathers, 1972; Singh and Clark, 1973; Semancik *et al.*, 1973), yet RNA fingerprint analyses (Dickson *et al.*, 1975) and later nucleotide sequence determinations unequivocally showed that the two viroids possess different primary structures.

Clearly, identification of a viroid must be based on its nucleotide sequence, and not on its biological properties.

E. Quantitation

At a time when viroids could not be recognized as physical entities, but only by virtue of their biological activity, titer estimates could be obtained only by suitable bioassay and, indeed, many of the physicochemical properties of viroids have been elucidated using titer estimates obtained in this fashion. Today, however, far more accurate methods for the determination of viroid concentration are available. These methods have largely supplanted bioassay. They include gel electrophoresis of nucleic acid preparations from infected tissue and various techniques of molecular hybridization with viroid-specific probes. Because these modern techniques are equally useful for diagnostic purposes, they are described in Section G.

F. Purification

Viroids constitute only a very small fraction of the total RNA extractable from infected tissue. For this reason, their purification poses a formidable separation problem. In addition, contrary to viruses, which usually have sedimentation characteristics different from those of any subcellular particles (the "virus window" that permits purification by differential centrifugation), viroids must be separated from host constituents that do not differ greatly in molecular weight but occur in far larger amounts than the viroid.

All viroid purification schemes are adaptations of procedures widely used for the isolation and separation of nucleic acids; they involve initial extraction of the plant's nucleic acids, usually in biphasic systems of which phenol (and often chloroform), as well as a strong detergent (often sodium dodecyl sulfate), are components. High-molecular-weight RNAs are removed by precipitation in 2 M LiCl, DNA is removed by incubation with deoxyribonuclease, and the bulk of tRNA can be removed by permeation chromatography. Invariably, final purification steps consist of two or more cycles of polyacrylamide gel electrophoresis, usually with one cycle performed under strongly denaturing conditions. Many variations of this general scheme for viroid purification have been described (Diener *et al.*, 1977; Diener, 1979; Dickson, 1979; Randles, 1975; Palukaitis and Symons, 1980; Niblett *et al.*, 1980; Yoshikawa and Takahashi, 1982; among others). The reader is referred to these publications and Part II for detailed protocols.

G. Diagnostic Procedures

Suitable diagnostic tests for the rapid, specific, and reliable detection of viroids are of paramount importance both in viroid research and in

efforts to control viroid diseases in practical agriculture. Because of their unique properties, certain procedures that are widely used with plant viruses are not readily applicable to viroids. Not surprisingly, serological procedures [such as the enzyme-linked immunosorbent assay (ELISA), which today is the method of choice for the detection of many plant viruses] have not been reported. Lack of antigenic coat proteins and of virionlike particles renders procedures that are based on immunological principles or on electron microscopic visualization difficult, if not impossible, to apply. Three kinds of test have been widely used for the detection of viroids: bioassay on suitable indicator hosts, gel electrophoretic detection, and detection by hybridization with specific molecular probes.

1. Bioassay

Although, as stated above, symptom expression in diagnostic hosts does not permit definitive conclusions regarding the identity of the viroid in question, it constitutes adequate evidence for viroid infection of the tested plant and is, therefore, a valuable tool in the control of viroid diseases in the field. With PSTV, it was particularly important to find a suitable indicator host, because symptoms in some potato cultivars are difficult to recognize and are usually nonexistent in a first-year infection—making it difficult to exclude PSTV-infected tubers from next year's "seed" stock (Diener, 1981).

Although indexing on Rutgers tomato (Raymer and O'Brien, 1962) greatly facilitated this task, the existence of PSTV strains that result in only mild or no symptoms on tomato, caused problems (Fernow, 1967).

Use of suitable indicator hosts was instrumental in shortening the time required for completion of a bioassay. This is well illustrated in the case of CEV. Originally, trifoliate orange was one of the few sensitive indicator hosts known for the detection of CEV in symptomless citrus trees. This method required $1\frac{1}{2}$ to more than 5 years from grafting to symptom expression (Calavan and Weathers, 1961). Later, Calavan *et al.* (1964) used selections of Etrog citron for indexing with which a reaction was obtained within 1 to 5 months. Finally, the use of mechanical inoculation of *G. aurantiaca* plants further shortened the incubation period to about 14 days (Weathers and Greer, 1972). Today, however, tests on physical–chemical properties of viroids have largely supplanted biological detection of viroids.

2. Gel Electrophoretic Detection (Polyacrylamide Gel Electrophoresis)

Because viroids constitute only a very small portion of the host's total nucleic acid complement, tests based on the electrophoretic mobility of viroids require that they be at least partially separated from host constituents. Many variations of extraction and gel electrophoretic procedures for the detection of viroids have been described. Usually these

are simplified versions of viroid purification schemes (Morris and Wright, 1975; Morris and Smith, 1977; Schumann *et al.*, 1978; Pfannenstiel *et al.*, 1980; Singh, 1982; Van Gelder and Treur, 1982), but Schumacher *et al.* (1983) have developed a novel electrophoretic technique specifically designed for the rapid and sensitive detection of circular viroids. With these two-dimensional and bidirectional procedures, viroid concentrations as low as 60 ng/g tissue can be detected unambiguously and without the need for radioactive materials or highly specialized laboratory equipment.

3. Detection by Molecular Hybridization

For the analysis of large numbers of samples, as is required in agricultural applications, both bioassay and PAGE procedures have serious drawbacks. Inherently, in view of the slow replication cycle of viroids, bioassay procedures are slow. They also require large amounts of greenhouse space and are unreliable under certain environmental conditions. PAGE, on the other hand, is laborious and expensive.

One alternative diagnostic method is hybridization of highly radioactive, viroid-complementary DNA with the viroid bound to a solid support and autoradiographic detection of the resulting DNA–RNA hybrids. Hybridization in solution has been used to detect the presence of viroids in purified RNA preparations (Owens, 1978; Palukaitis and Symons, 1978; Randles and Palukaitis, 1979; Allen and Dale, 1981; Palukaitis *et al.*, 1981). Two conditions had to be fulfilled before hybridization with viroid cDNA could be developed into a practical diagnostic test: (1) the viroid cDNA had to be available in unlimited quantities and at high specific radioactivity, requiring cloning of the cDNA by recombinant DNA technology and nick-translation of the DNA; and (2) to expedite and simplify sample preparation, clarified sap rather than purified nucleic acid had to be suitable as the viroid source. With PSTV in potato, this proved possible by judicious choice of extracting buffers designed to inhibit enzymatic oxidation of polyphenolic compounds and to liberate the viroid from cellular constituents. Thus, a practical test for the detection of PSTV in large numbers of potato "seed" samples based on nucleic acid hybridization has been developed (Owens and Diener, 1981). Detailed evaluation showed that the hybridization method is adequate to detect PSTV in potato tissue without prior purification of the viroid and that the test is highly specific and at least ten times as sensitive as PAGE (Owens and Diener, 1981). Further evaluation with potato selections that had been tested previously by bioassay and PAGE revealed that the nucleic acid hybridization test often gave positive results with samples that had tested negative by bioassay and/or PAGE (Salazar *et al.*, 1983). Follow-up of these results indicated that the plants from which these samples had been obtained were indeed PSTV-infected, but with the viroid present at too low a concentration to be detectable either by PAGE or by simple bioassay (Salazar *et al.*, 1983).

Both in viroid research and in practical agriculture, diagnostic procedures based on nucleic acid hybridization have become widely used and several variations of the procedure have been developed (van Wezenbeek *et al.*, 1982; Mohamed and Imperial, 1984; Macquaire *et al.*, 1984; Barker *et al.*, 1985; among others). Noteworthy improvements are the use of formaldehyde-denatured viroid-containing samples in the dot blot assay, which increases sensitivity tenfold (Skrzeczkowski *et al.*, 1985), and efforts to use nonradioactive hybridization probes (Forster *et al.*, 1985).

Diagnostic tests are most urgently needed with vegetatively propagated perennial crops that are susceptible to viroid-induced diseases, such as chrysanthemum (CSV and CCMV), hops (HSV), citrus (CEV), and avocado (ASBV). In each case, development of a nucleic acid spot hybridization test requires isolation and purification of the respective viroid, preparation of specific cDNA, and cloning of the cDNA. Also, use of crude extracts from the various hosts as viroid sources may require modification of the extraction buffer because these host plants, in contrast to tomato and potato, may contain substances that interfere with the hybridization reaction.

Nucleic acid spot hybridization tests may also become useful for the early detection of viroid infection in young seedlings. One case in point is the coconut cadang-cadang disease. Because of the great sensitivity of the hybridization test, it is possible that routine testing of seedlings in new plantations could identify infected palm trees long before symptoms appear or before the cadang-cadang viroid would become detectable by PAGE analysis. Early roguing of infected palm trees might materially diminish tree-to-tree spread of the viroid in these plantations. Similar considerations may apply to avocado plantations and their protection from the ASBV.

Nucleic acid spot hybridization tests are already widely used also for the diagnosis of infection by certain conventional viruses (Maule *et al.*, 1983). For example, immunological assays are not practical with viruses that produce labile virions or whose coat proteins are only weakly antigenic. In such cases, tests based on hybridization of the viral genome with specific cloned recombinant DNA may be more advantageous than conventional tests, although preparation of the tissue extracts probably would be more cumbersome than with viroids. Presumably, the viral genome, to be reactive in the test, would have to be liberated from virions, which would require some sort of nucleic acid isolation procedure. Conceivably, however, nonencapsidated viral nucleic acid, such as RF or RI, might be present in sufficient quantity to react with the cDNA probe.

H. Interaction with Other Pathogens

Viroids share with viruses the phenomenon of cross protection, in which infection of plants with a mild strain of a viroid protects the plants

from the effects of superinfection with a severe strain of the same viroid (Fernow *et al.*, 1969; Niblett *et al.*, 1978). As with viruses, cross protection is believed to occur only if the mild and superinfecting pathogens are related. Thus, in tomato, mild strains of PSTV protect against the effects of infection with a severe strain of PSTV (Fernow *et al.*, 1969), but also against CEV (Niblett *et al.*, 1978). In chrysanthemum, CEV and mild and severe strains of PSTV protect against CEV, but CCMV does not protect against PSTV, CEV, or CSV (Niblett *et al.*, 1978). Mild strains of PSTV afford only partial protection against TPMV (Galindo *et al.*, 1982).

The molecular mechanism of cross protection is unknown, but in two cases it could be shown that in doubly infected plants, both the protecting and the challenge viroids were able to replicate (Niblett *et al.*, 1978). Thus, protection seems to be due to a symptom-repressing effect rather than to interference with the replication of the superinfecting strain. This conclusion, however, may not be justified, because the importance of quantitative differences in viroid replication in "protected," as compared with "unprotected," plants has not been ruled out.

Very little is known regarding possible interference of other pathogens with viroids. In doubly infected tomato plants, tobacco mosaic virus and PSTV replicate independently from one another, and the characteristic symptoms of both pathogens are expressed as well as in singly infected plants (T. O. Diener, unpublished observations). On the other hand, in chrysanthemum (cv. Sunfire) plants doubly infected with tomato aspermy virus and CSV, symptoms of the latter appeared earlier than in plants singly infected with CSV (Bachelier *et al.*, 1976).

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CHAPTER 2

PHYSICAL–CHEMICAL PROPERTIES
Molecular Structure (Primary and Secondary)

PAUL KEESE AND ROBERT H. SYMONS

I. INTRODUCTION

Essential to understanding how viroids function is the determination of their nucleotide sequences. Within each viroid sequence is all the information to allow it to function *in vivo*. Our major problem is to relate this sequence to the secondary and tertiary structure of the viroid and the way in which this information is encoded, interpreted, and expressed in the plant cell.

In this chapter, we describe methods that have been used for the purification of viroids, procedures that have been used in the sequencing of viroids, and some characteristics of primary and proposed secondary structures.

II. PURIFICATION OF VIROIDS

In infected plants, viroids exist mostly in the circular monomeric form with only low or negligible levels of linear monomers. Low levels of monomeric minus (complementary) forms and of oligomeric plus and minus forms have been detected by hybridization analysis after fractionation of tissue extracts by gel electrophoresis (Branch *et al.*, 1981; Owens and Diener, 1982; Mühlbach *et al.*, 1983; Ishikawa *et al.*, 1984; Hutchins *et al.*, 1985). One exception is CCCV where the concentration of dimeric

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plus CCCV in coconut palms can increase to levels similar to those of the monomer late in infection (Imperial *et al.*, 1981; Mohamed *et al.*, 1982). Of the other viroids, dimers of ASBV can be detected by staining after gel electrophoresis of plant extracts (Bruening *et al.*, 1982).

A. Preparation of Plant Extracts

A commonly used approach has been to prepare a partially purified nucleic acid extract of infected plant tissue and then to purify the circular and linear forms of the viroid by gel electrophoresis. Many of the earlier methods for the preparation of the plant extracts contained a number of steps and required several days for completion (Diener *et al.*, 1977; Semancik *et al.*, 1975; Niblett *et al.*, 1980). However, more recent methods (Palukaitis and Symons, 1980; Bruening *et al.*, 1982) are simpler and faster and nucleic acid extracts can be readily prepared from 500-g quantities of plant material in about 1 day.

During the preparation of the partially purified extract, phenol is still the best deproteinizing agent and it also has the advantage of removing a considerable amount of colored material. In order to circumvent the use of large volumes of phenol when 500-g quantities or more of plant material are used, we developed a two-step extraction procedure (Palukaitis and Symons, 1980; Bruening *et al.*, 1982) that has proven very effective for ASBV, CSV, CEV, and PSTV. In the first step, plant material is homogenized in the presence of sodium dodecyl sulfate (SDS), a range of compounds to inhibit color development, and $MgCl_2$ to inhibit the solubilization of pectins. Addition of NaCl (to 0.5 M) causes precipitation of proteins as an SDS complex (Kay *et al.*, 1952). The nucleic acids are concentrated by ethanol precipitation and further deproteinized with phenol–$CHCl_3$ in a volume of about 10% of the original aqueous extract. After dialysis overnight and a further ethanol precipitation, the partially purified extract is ready for fractionation by gel electrophoresis. An alternative to the dialysis step that involves precipitation of nucleic acids with 6 M LiCl was found to be very useful when a number of smaller samples of about 25 g of plant material are being extracted (Barker *et al.*, 1985).

When only a few grams of plant material are to be extracted, a procedure modified from that of Laulhere and Rozier (1976) gives rapid and efficient extraction of viroids (Hutchins *et al.*, 1985) and has proven effective in the extraction of CCCV from coconut palm leaves (M. E. Keese, unpublished findings). In this procedure, the plant material is thoroughly homogenized with 0.5 M sodium acetate, 10 mM $MgCl_2$, 20% ethanol, 3% SDS, and an equal volume of water-saturated phenol. After a second phenol–$CHCl_3$ extraction of the aqueous phase, nucleic acids are recovered by ethanol precipitation and are ready for gel electrophoresis.

Although leaf material is usually chosen for viroid extraction because

of its ease of harvesting and homogenization, other parts of the plant can be used. For example, Colpan *et al.* (1983) found, on a weight basis, concentrations of PSTV in stems and roots of tomato plants that were nearly as high as those in leaves. M. W. Schwinghamer (personal communication) has used bark from the main trunk of citrus trees since it contains a higher concentration of CEV than leaves; extraction was done after pulverizing the bark in liquid nitrogen. In the case of avocados, flowers contain a higher concentration of ASBV than do the leaves of infected trees (da Graca and Mason, 1983). CCCV was isolated from coconut palm roots, nut stalks, coconut husk, and the inflorescence, while high levels were found in pollen; only the coconut meat and milk and the trunk contained low concentrations (J. S. Imperial, unpublished findings).

B. Purification

Slab PAGE is a very efficient method for the fractionation of nucleic acids in partially purified plant extracts. In a two-step procedure that we have used extensively for viroids (Palukaitis and Symons, 1980; Bruening *et al.*, 1982; Visvader *et al.*, 1982) nucleic acid extracts are first run on a nondenaturing 5% polyacrylamide slab gel to separate the viroid (circular and linear forms comigrate) from host high- and low-molecular-weight nucleic acids and colored material; extract from 100–125 g of plant material is loaded on a 0.65 × 15 × 15-cm gel. After location of the viroid band by brief staining, the band is excised and recovered from the gel by electroelution followed by ethanol precipitation. In the second step, the viroid is further purified and fractionated on a 5% polyacrylamide slab gel containing 7 M urea, under which conditions the circular RNA molecules are separated from the faster-migrating linear forms. In this second step, the partially purified viroid from 1 to 2 kg of plant material is electrophoresed on one slab gel. The circular and linear molecules are again located by brief staining and recovered by electroelution and ethanol precipitation. Yields of viroids have varied from about 20 μg/kg tomato leaves for CEV up to about 1 mg/kg avocado leaves for ASBV (J. E. Visvader, J. M. Barker, and R. H. Symons, unpublished findings).

For the rapid purification of circular viroid, small volumes of plant extract are electrophoresed directly on a 7 M urea, 4% polyacrylamide slab gel and the viroid band eluted after location by staining. RNA isolated in this way has proven to be pure enough for many purposes, e.g., for direct RNA sequencing (see below) or for the preparation of cDNA for cloning (J. S. Visvader and P. Keese, unpublished findings). When gels less than 1 mm thick were used, the viroid band was eluted with high recovery simply by soaking the intact gel band overnight at room temperature in 0.5 M ammonium acetate, 10 mM EDTA, pH 8, 0.1% SDS, and recovering the RNA by ethanol precipitation.

A novel approach for the rapid, large-scale purification of viroids using Cs_2SO_4 gradient centrifugation and high-performance liquid chromatography (HPLC) has been described by Colpan *et al.* (1983). The procedure involves the preparation of a partially purified nucleic acid extract of infected plants, and banding of the viroid and other low-molecular-weight RNA by equilibrium density gradient centrifugation in Cs_2SO_4 and the final purification of the viroid by HPLC on a weak anion-exchange resin in a specially modified machine. Electrophoretically pure PSTV was obtained in yields of about 0.5 to 1 mg/kg tomato leaves. Unlike gel electrophoresis in the presence of 7 M urea, HPLC did not separate the circular and linear forms of the viroid.

All preparations of viroids contain linear as well as circular molecules, with the linear molecules varying from almost undetectable levels up to about 10% of the circular forms. We have found this range for three viroids (CSV, ASBV, CEV) in numerous preparations over several years. It has proven difficult to determine whether these linear molecules exist as such *in vivo* or arise, at least in part, by nicking of circular molecules during the extraction and purification procedures. The other unresolved problem is whether any linear molecules that exist in intact cells are the result of adventitious nicking by intracellular RNases or are the product of a step in the replication cycle. Some reports showed the linear PSTV (Owens *et al.*, 1977) and CSV (Palukaitis and Symons, 1980) to be infectious; presumably the linear RNAs were first ligated to the circular form before being replicated. Further discussion can be found in Sänger (1982).

III. SEQUENCE DETERMINATION OF VIROIDS

The usual starting material for sequence analysis is the purified, infectious circular viroid. Although the linear monomeric forms of PSTV and CSV are infectious when isolated from infected plants (Sänger, 1982; Palukaitis and Symons, 1980), the ends are heterogeneous, possibly because of nonspecific cleavage of the circular molecules. This makes sequencing difficult by the direct method, which requires a unique 5′- and 3′-end (see Section III.B). There are, at present, four approaches to compare viroids and to determine their complete sequences.

A. Fingerprint Analysis

Two-dimensional fingerprinting is a rapid and sensitive method for comparing two or more RNAs and was used extensively in the initial characterization of viroids (Gross *et al.*, 1977, 1978, 1981; Dickson *et al.*, 1975, 1978, 1979; Mohamed *et al.*, 1982). Purified RNA was digested to

completion with a base-specific RNase such as RNase T_1, specific for guanine; RNase U_2, specific for adenine at pH 3.5; or pancreatic RNase A, specific for pyrimidines. Since all these RNases cleave to leave a 5′-hydroxyl terminal nucleotide, the mixture of RNA fragments was usually 5′-labeled with ^{32}P using [γ-^{32}P]-ATP and phage T4 polynucleotide kinase. Alternatively, viroids were sometimes labeled at C nucleotides with ^{125}I prior to enzymatic digestion. The labeled oligonucleotides were then separated on the basis of size and charge by two-dimensional paper chromatography (Gross *et al.*, 1978) or by two-dimensional PAGE (Frisby, 1977; de Wachter and Fiers, 1972) to give a characteristic fingerprint. The method is sensitive for the detection of minor differences between viroids since even a single base change can lead to the disappearance of one oligonucleotide spot and the appearance of another.

Each oligonucleotide after purification can be sequenced as originally performed for PSTV (Gross *et al.*, 1978) with the complete sequence of the viroid being determined from the sequence overlap of many fragments. Although this approach is very tedious for the accumulation of sequence data, fingerprinting can still play a role in rapid diagnosis as, for example, in the confirmation of the identity of the dimeric forms of ASBV (Bruening *et al.*, 1982) and of CCCV (Mohamed *et al.*, 1982). Fingerprint analysis provided the first conclusive test to distinguish CEV and PSTV, which share similar sizes and biological properties (Dickson *et al.*, 1975).

B. Direct RNA Sequencing

This technique is used after prior partial cleavage of the RNA with base-specific RNases followed by 5′-end labeling with [γ-^{32}P]-ATP and T4 polynucleotide kinase or 3′-end labeling with T4 RNA ligase and [^{32}P]-pCp (England and Uhlenbeck, 1978). Ideally, the partial cleavage should produce a series of overlapping fragments that are representative of the whole molecule. After end labeling, the fragments are separated by PAGE, located by autoradiography, eluted from the gel, and individual fragments sequenced directly with base-specific enzymes (Donis-Keller *et al.*, 1977; Haseloff and Symons, 1981) or by specific chemical cleavage in the case of the 3′-labeled fragments (Peattie, 1979).

There are two difficulties with the use of base-specific enzymes for RNA sequencing. First, although purines can be readily distinguished using RNase T_1 and RNase U_2 at pH 3.5 (Donis-Keller *et al.*, 1977; Simoncsits *et al.*, 1977), there are no enzymes completely satisfactory for deciphering pyrimidines. Enzymes that have been commonly used include RNase PhyI (Pilly *et al.*, 1978), RNase PhyM (Donis-Keller, 1980), extracellular RNase from *Bacillus cereus* (Lockard *et al.*, 1978), and chicken liver RNase (Boguski *et al.*, 1980). However, none of these except possibly RNase PhyM provide digestions uniform enough to allow unambiguous

distinction between C and U nucleotides. Second, internal secondary structure of fragments from G:C-rich RNAs such as viroids can be stable enough so that fragments are difficult to resolve on sequencing gels due to band compression (Kramer and Mills, 1978), even in the presence of 7 M urea. The use of a stronger denaturant such as 98% formamide (Haseloff and Symons, 1981) or 40% formamide and 5 M urea (J. E. Visvader, unpublished findings) has aided in overcoming this problem.

The chemical method of sequencing (Peattie, 1979) can satisfactorily distinguish all four bases. In addition, after the various chemical treatments, the fragments sequenced by this method no longer form tight secondary structures. However, chemical cleavage of RNA is more laborious than enzymatic sequencing and it can only be used for sequencing fragments that are 3′-labeled. Chemical sequencing of 5′-labeled fragments does not produce readable sequencing gels.

In practice, direct enzymatic sequencing is rapid and can provide about 80% or more of the sequence of a viroid in a few days starting with only a few micrograms of RNA. However, finalizing the sequence involves considerably more effort with repeated reactions under a variety of conditions to resolve ambiguities between C and U nucleotides and band compressions. In addition, any region of the RNA molecule that is highly susceptible to RNase digestion can be poorly represented in the partial RNase digests (e.g., Symons, 1981). The complete sequence of the circular viroid is finally obtained from overlaps of the sequences of a number of fragments.

C. Primer-Directed Sequence Analysis

An approach with considerable potential for the rapid examination of a number of isolates of the same viroid is to use an oligonucleotide primer for the synthesis of cDNA with reverse transcriptase. Sequencing of the DNA can be done by the chemical method of Maxam and Gilbert (1977) starting with a 5′-^{32}P-labeled primer or by the dideoxynucleotide chain termination technique (Zimmern and Kaesberg, 1978; Symons, 1979). This approach has been used for the rapid examination of a number of different PSTV isolates with three chemically synthesized oligonucleotide primers that hybridize to different parts of the PSTV molecule (Sänger, 1984). In the case of CEV, the use of synthetic primers for dideoxy sequencing has provided useful initial data but extensive band compression has prevented its use for complete sequence determination (J. E. Visvader, unpublished observations). In the selection of DNA primers for this approach, it is important to make use of regions of the viroid molecule containing a minimum of secondary structure and to have primers of sufficient length (preferably at least 15 residues) to ensure a stable hybrid for initiation of cDNA synthesis. DNA primers can also be isolated by

restriction enzyme digestion of recombinant cDNA clones of viroids and have been used for the confirmation of part of the sequence of ASBV (Symons, 1981) and CEV (Visvader *et al.*, 1982).

D. Sequencing of Viroids Using Cloned cDNA

The recognition that naturally occurring isolates of CEV obtained from a single citrus tree can be complex mixtures of a number of sequence variants, some with up to 29 nucleotide differences (Visvader and Symons, 1983, 1985), has important implications for sequence analysis. Direct RNA sequencing and primer-directed sequencing cannot fully resolve mixtures of two or more sequence variants and will not detect minor amounts of sequence variants in the presence of a single dominant variant (Visvader *et al.*, 1982; Visvader and Symons, 1983). The most feasible approach to overcome these difficulties is to sequence a number of DNA clones constructed from full-length cDNA transcripts synthesized from individual RNA molecules present in the purified isolate of the viroid. The complexity of the isolate will determine the number of clones that have to be sequenced to give a measure of the number of sequence variants and their relative distribution in the isolate.

Both partial-length and full-length cDNA clones of viroids have been reported (Owens and Cress, 1980; van Wezenbeek *et al.*, 1982; Cress *et al.*, 1983; Barker *et al.*, 1985; Visvader and Symons, 1983, 1985; Tabler and Sänger, 1984). Cloning into the ssDNA of bacteriophage M13 vectors allows rapid sequence determination by the dideoxy chain termination procedure (Sanger *et al.*, 1980). The preparation of full-length clones of viroids (Ohno *et al.*, 1983; Visvader and Symons, 1985) has the distinct advantage of being able to identify individual RNA variants and has been successfully used to resolve the highly complex mixtures of two CEV isolates (Visvader and Symons, 1985).

In the preparation of full-length cDNA clones of HSV, Ohno *et al.* (1983) started with linear molecules and used the procedure of Okayama and Berg (1982), which does not require prior knowledge of the viroid sequence. We have found an alternative approach, which requires at least partial knowledge of the viroid sequence, to be very successful for the rapid cloning of CEV using only 0.5–2 μg of the circular viroid (Visvader and Symons, 1985). The procedure for the cloning of CEV in the phage M13mp93 vector is outlined in Fig. 1. It requires the sequential use of two synthetic oligonucleotide primers to prepare full-length cDNA, which is then blunt-end ligated into the replicative form of the vector. Infection of bacteria produces clones of progeny M13 phage containing either plus (viroid) or minus (complementary) sequences, each of which can usually be sequenced fully in a single reaction by the dideoxy nucleotide chain termination technique (Sanger *et al.*, 1980).

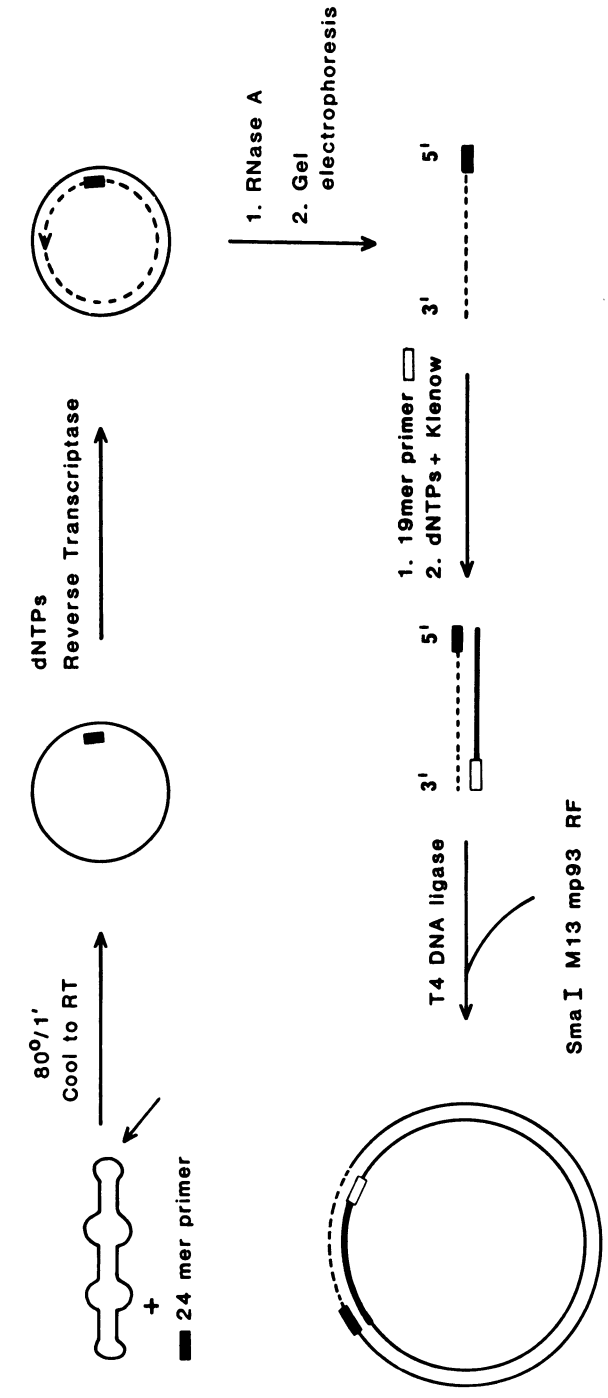


FIGURE 1. Outline of the procedure used in the construction of full-length cDNA clones of CEV [Visvader and Symons, 1985]. The site of priming of the 24-nucleotide DNA primer at the right-hand end of the predicted native structure of CEV is indicated by an arrow.

IV. STRUCTURE OF VIROIDS

A. Circular Structure of Viroids

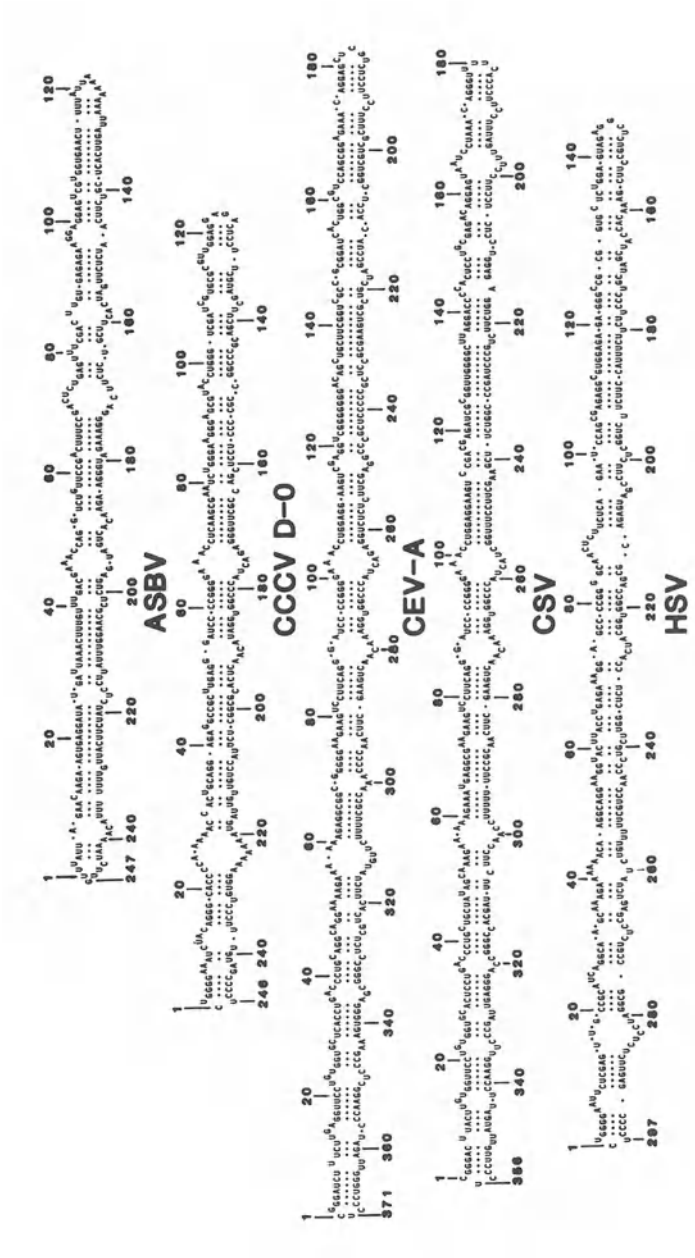
Although the circularity of viroids was clearly shown by sequence analysis, initial evidence for most viroids was provided by observation in the electron microscope. PSTV appears under native conditions as rodlike molecules of an average length of 37 ± 6 nm (Sänger *et al.*, 1976; Riesner *et al.*, 1979). From the molecular weight of PSTV and its sedimentation coefficient determined independently in solution, an axial ratio of 20:1 was calculated, a value in good agreement with the electron microscopic data. When PSTV was spread under denaturing conditions for electron microscopy, it was visible as covalently closed, single-stranded circles with a contour length of 100 ± 6 nm (Sänger *et al.*, 1976; McClements and Kaesberg, 1977; Riesner *et al.*, 1979), a size consistent with its length of 359 residues as determined by sequencing (Gross *et al.*, 1978).

Similar, but less extensive, analysis by electron microscopy of CSV (Palukaitis and Symons, 1980), CEV and CPFV (Sänger *et al.*, 1976), CCCV (Randles and Hatta, 1979), HSV (Ohno *et al.*, 1982), and ASBV (Palukaitis *et al.*, 1979) has shown that they are similar to PSTV in being single-stranded, covalently closed RNA molecules. The lengths of these molecules when spread under denaturing conditions were consistent with the exact sizes subsequently determined by sequencing.

B. Sequence and Structure of Viroids

In the nine viroids that have been sequenced (Table I, Fig. 2), it has been possible to predict a secondary structure for each RNA involving intramolecular base-paired regions separated by small internal loops to give a linear, rodlike structure (Fig. 2). In practice, the quickest way to arrive at the possible secondary structure is to maximize the number of base pairs and to compare the structure with that of other related viroids. The sequence of PSTV was determined in 1978 and its secondary structure predicted, aided by evidence indicating the sites of single-stranded regions that were susceptible to single-strand-specific RNases and to bisulfite modification (Gross *et al.*,1978). It was then straightforward to rapidly derive a secondary structure for related viroids on the basis of sequence homology and the maximization of the number of base pairs. However, in the case of the unrelated ASBV (Symons, 1981) it was necessary to use the base pairing matrix procedure of Tinoco *et al.* (1971) as a first step in the development of a secondary structure.

Calculation of the thermodynamically most stable structure can be used to add refinements to the predicted structure. The lowest free energy (ΔG) has been calculated for each structure using a set of stability constants, reaction enthalpies and entropies for base pair formation (Riesner


ASBV
CCCV D–0
CEV–A
CSV
HSV

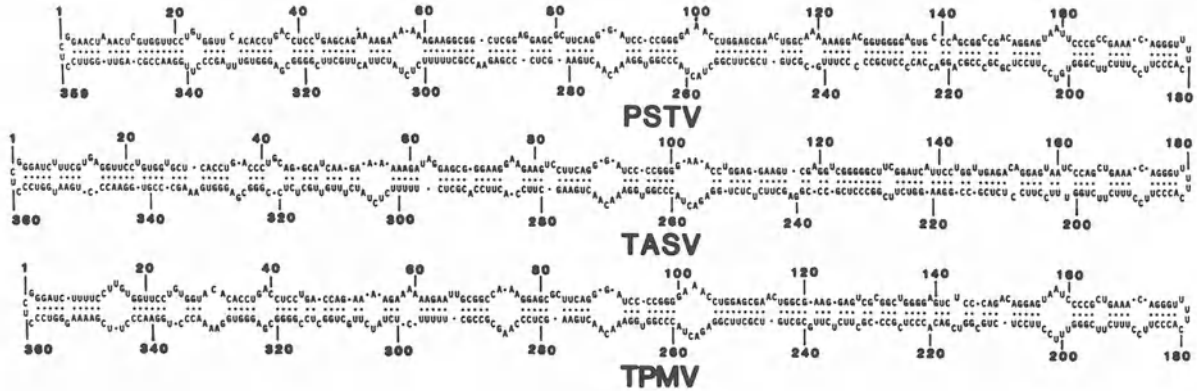


FIGURE 2. Sequences and proposed secondary structures of ASBV (Symons, 1981), CCCV D-O, the basic 246-nucleotide species (Haseloff *et al.*, 1982), CEV-A (Visvader *et al.*, 1982), CSV (Haseloff and Symons, 1981), HSV (Ohno *et al.*, 1983), PSTV (Gross *et al.*, 1978), TASV (Kiefer *et al.*, 1983), and TPMV (Kiefer *et al.*, 1983). CPFV (Sano *et al.*, 1984) is a sequence variant of HSV (Table I). Sequences and structures of other isolates of CEV and CSV are in Gross *et al.* (1982), of CEV in Visvader and Symons (1983, 1985), and of PSTV in Sänger (1984).

TABLE I. Viroids That Have Been Sequenced

Viroid	Abbreviation	Number of nucleotides	References
Avocado sunblotch viroid	ASBV	247	Palukaitis *et al.* (1979), Symons (1981)
Chrysanthemum stunt viroid	CSV	356, 354	Haseloff and Symons (1981), Gross *et al.* (1982)
Citrus exocortis viroid	CEV	370–375	Gross *et al.* (1982), Visvader *et al.* (1982), Visvader and Symons (1983, 1985)
Coconut cadang-cadang viroid[a]	CCCV	246, 247	Haseloff *et al.* (1982)
Coconut tinangaja viroid	CTV	253	P. Keese, M. O. Keese, and R. H. Symons (unpublished findings)
Cucumber pale fruit viroid[b]	CPFV	303	Sano *et al.* (1984)
Hop stunt viroid	HSV	297	Ohno *et al.* (1983)
Potato spindle tuber viroid	PSTV	359	Gross *et al.* (1978), Sänger (1984)
Tomato apical stunt viroid	TASV	360	Kiefer *et al.* (1983)
Tomato planta macho viroid	TPMV	360	Kiefer *et al.* (1983)

[a] CCCV infections produce four major RNA components, all derived from the infectious monomeric small form (D-0 of 246 or 247 nucleotides). These include monomers and dimers of both the D-O form and any of a set of larger forms (D-41, D-50, or D-55; see Fig. 5) containing a duplication involving 41, 50, or 55 nucleotides.

[b] CPFV is a sequence variant of HSV since the two viroids have 95% sequence homology.

et al., 1979, 1983; Steger *et al.*, 1984). The values used by different authors have varied but they depend on the type of base pair formed and the type of neighboring base pair. In addition to the usual A:U and G:C base pairs, the G:U base pair is taken into account since its stability is similar to that of A:U base pairs (Riesner *et al.*, 1979; Steger *et al.*, 1984). Furthermore, in the case of the first base pairs on each side of a loop, the parameters depend on the size and kind of the loop. Computer programs are being developed that make use of a given set of parameters to calculate the most thermodynamically stable structure (Nussinov and Jacobson, 1980; Zuker and Stiegler, 1981; Steger *et al.*, 1984; Comay *et al.*, 1984). Further discussion on secondary structure can be found in Chapter 3, this volume.

C. Structural Domains of Viroids

The viroids examined to date range in size from 246 to 375 nucleotides (Table I). On the basis of sequence and structural homology, the sequenced viroids can be divided into two classes. One class consists only of ASBV while the remaining viroids constitute a second class since they are all related to the best characterized viroid, PSTV. Although ASBV possesses a predicted rodlike structure (Fig. 2) with similar properties to other viroids, it has a higher A:U content (Table II) and lower sequence homology (20%) than any other viroid (Symons, 1981).

The PSTV-like viroids (Table I, Fig. 1) reveal at least 35% sequence homology between any two members, and all contain a conserved, central U-bulged helix

$$\begin{array}{l} \text{CC CCGG} \\ \text{GGUGGCC} \end{array}$$

and an oligoadenosine sequence that occurs in the same relative position about 35 residues 5′ of the central conserved sequence

$$\text{GGA}\begin{matrix}\text{U}\\\text{G}\end{matrix}\text{CCCCGGGG}\begin{matrix}\text{A}\\\text{C}\end{matrix}\text{AAC}$$

(Figs. 2 and 3).

From an examination of sequence homology among more than 40 PSTV-like viroid variants, a model of viroid structure applicable to all viroids, except ASBV, has been developed (Keese and Symons, 1985) in which five structural domains are distinguished (Fig. 3, Table III). From this model it is possible to examine evolutionary relationships and draw functional inferences.

TABLE II. Properties of Proposed Secondary Structures of Viroids

Viroid	No. of nucleotides	Nucleotides				% Nucleotides base paired	Base pairs as % of total			References
		A	U	G	C		A:U	G:C	G:U	
ASBV	247	68	85	51	43	67	51	34	14	Symons (1981)
CSV	356	75	93	89	99	70	35	52	13	Haseloff and Symons (1981)
CEV-A	371	72	75	112	112	69	28	56	16	Visvader and Symons (1983)
CCCV D-0	246/7	53	47	73	73/4	65	24	69	8	Haseloff *et al.* (1982)
CPFV	303	64	70	81	88	69	31	65	4	Sano *et al.* (1984)
HSV	297	61	69	79	88	67	29	64	7	Ohno *et al.* (1983)
PSTV	359	73	77	101	108	70	29	58	13	Gross *et al.* (1978)
TASV	360	70	90	101	99	73	32	57	11	Kiefer *et al.* (1983)
TPMV	360	72	81	99	108	68	31	60	9	Kiefer *et al.* (1983)

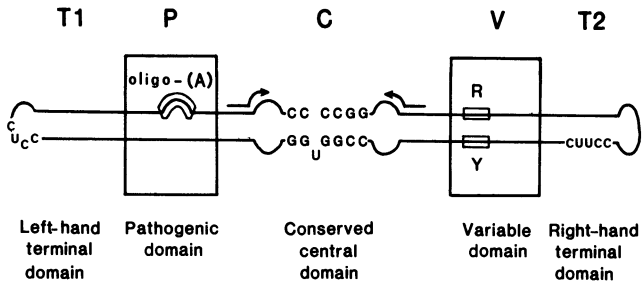


FIGURE 3. Model of five viroid domains (T1, P, C, V, T2) determined from sequence homologies among viroids (Keese and Symons, 1985). The arrows depict an inverted repeat sequence that can form the stem loop depicted in Fig. 4A. R, Y: a short oligopurine, oligopyrimidine helix.

1. C Domain

The central domain of viroids (Fig. 3) is the most highly conserved region among viroids (Table IV) (Haseloff and Symons, 1981; Haseloff *et al.*, 1982; Visvader *et al.*, 1982; Gross *et al.*, 1982; Kiefer *et al.*, 1983). It consists of about 95 nucleotides rather than 44 or 56 nucleotides as originally 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 TASV and CEV-A in the C domain although showing only 73% overall sequence homology (Table IV). The C domain of CCCV D-0, which constitutes 40% of the molecule, shows 70% sequence ho-

TABLE III. Location of Domains in Viroids[a]

	Nucleotide numbers at boundaries of domains				
	T1	P	C	V	T2
PSTV	1–46	47–73	74–120	121–148	149–212
	359–315	314–287	286–241	240–213	
TPMV	1–46	47–75	76–122	123–147	148–209
	360–311	310–285	284–237	236–210	
CEV-A	1–46	47–75	76–122	123–150	151–214
	371–326	325–294	293–244	243–215	
TASV	1–46	47–76	77–123	124–149	150–211
	360–316	315–285	284–235	234–212	
CSV	1–44	45–72	73–119	120–154	155–206
	356–312	311–286	285–238	237–207	
CCCV D-0	1–12	13–40	41–87	88–102	103–143
	246–236	235–205	204–157	156–144	
HSV	1–31	32–59	60–107	108–125	126–172
	297–272	271–241	240–192	191–173	

[a] Derived from sequences and structures of Fig. 2.

mology 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 strongly support the association of CCCV D-0 with the PSTV-like group of viroids.

The boundaries of the C domain were determined from pairwise sequence comparisons of viroids containing highly homologous central domains (e.g., between PSTV, TPMV and CCCV, or between CEV, TASV, and CSV). High sequence and structural homology of the partially base-paired helical domain extends to about 5 to 9 nucleotides 5′ and about 7 to 15 nucleotides 3′ of a nine-nucleotide inverted repeat (Fig. 3, Table III). This inverted repeat can form a stem loop corresponding to stem loop I observed during the thermal denaturation of PSTV, CEV, CSV, and CCCV (Fig. 4A) (Henco *et al.*, 1979; Riesner *et al.*, 1979, 1983; see Chapter 3, this volume). It flanks a strictly conserved bulged helix

CC CCGG
GGUGGCC

postulated to be present in the purified native state (Figs. 1 and 4B). Both the inverted repeat and the bulged helix, although highly conserved among viroids, are mutually exclusive. On this basis, it has been proposed that the two alternative structures control separate functions (Keese and Symons, 1985). One function postulated involves the processing of viroid replicative intermediates since only intact cDNA clones of PSTV and

TABLE IV. Sequence Homology between Domains of Different Viroids[a]

Viroids used for pairwise comparison		% Sequence homology					
		Domains					
1	2	T1	P	C	V	T2	Overall
TASV	CEV-A	91	54	99	49	46	73
	PSTV	67	59	65	30	90	64
TPMV	PSTV	67	73	94	42	95	76
	CEV-A	80	70	69	29	37	60
CCCV D-0	PSTV	25	14	70	37	27	38
	HSV	52	33	42	31	50	39
HSV	PSTV	23	58	35	37	28	35
CSV	CEV-A	77	42	82	28	38	59
	PSTV	69	49	71	31	81	61
CEV-A	PSTV	62	71	65	31	38	55

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

$$\% \text{ sequence homology} = \frac{\text{number of matching nucleotides in both sequences}}{\text{total number of nucleotides in both sequences}} \times 100$$

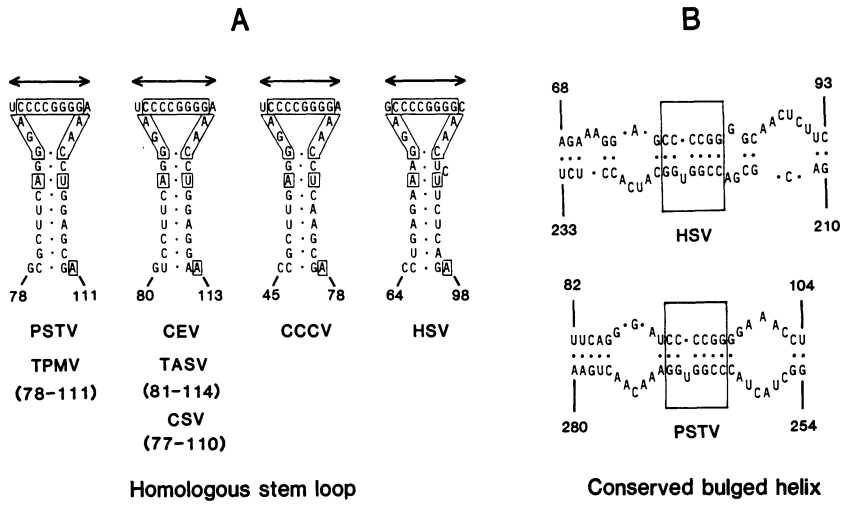


FIGURE 4. Two alternative structures that may form in the C domain of PSTV-like viroids. (A) Stem loop as described by Henco *et al.* (1979) with a nine-base-pair stem and ten-nucleotide self-complementary sequence in the loop. The same structure can form for all PSTV-like viroids except for HSV where there is a non-base-paired C nucleotide in the stem (Keese and Symons, 1985). Conserved nucleotides are boxed. (B) Conserved bulged helix postulated for the native structure of purified viroids. The upper strand corresponds to the self-complementary loop predicted in A.

CEV containing repeats of at least some part of the C domain are highly infectious (Tabler and Sänger, 1984; Visvader *et al.*, 1985). Processing *in vivo* within these repeats of RNA transcripts generated from DNA clones either *in vivo* or *in vitro* would allow for the generation of infectious monomeric viroid.

2. P Domain

The P domains of all PSTV-like viroids except CCCV include a long adenine-dominated oligopurine sequence of 15 to 17 nucleotides in one strand and an oligo(U_{4-7}) sequence in the opposite strand. The more distantly related viroid HSV shares these features and shows relatively high sequence homology with the P domain, in contrast to 35% overall sequence homology with PSTV (Table IV). CCCV, however, retains only an oligo(A_5) sequence in the P domain. This may reflect the distinctive host range of CCCV, which appears to be restricted to members of the palm family (Imperial *et al.*, 1985), whereas the other PSTV-like viroids, including HSV, share an overlapping host range, infecting members of several dicotyledonous families (e.g., tomato, chrysanthemum, and cucumber) (Runia and Peters, 1980).

The boundaries of the P domain (Table III) have been based on se-

TABLE V. Location of Sequence Differences in the Domains of 17 Sequence Variants of CEV and 6 Sequence Variants of PSTV[a]

	Number of nucleotides that vary in viroid domain				
Viroid	T1	P	C	V	T2
CEV	2 (92)	16 (61)	5 (97)	20 (57)	0 (64)
PSTV	0 (91)	12 (55)	0 (93)	2 (56)	0 (64)

[a] Number of nucleotides in each domain (Table III) is given in parentheses. See Fig. 3. Data for CEV are given relative to CEV-A (371 nucleotides) and taken from Visvader and Symons (1985). Data for PSTV are taken from Sänger (1984).

quence homologies between the P domain of HSV and other viroids such as PSTV and by certain pairwise comparisons, e.g., CEV-A and TASV, in which there is a significant change from relatively low sequence homology in the P domain to higher sequence homology in the adjacent T1 and C domains. The P domain incorporates the virulence modulating domain of PSTV (Sänger, 1984) that was based on the correlation of variation of sequence within this domain of PSTV sequence variants with different symptoms when propagated on the same variety of tomato (Dickson *et al.*, 1979; Gross *et al.*, 1981; Sänger, 1984). Further support for the P domain is provided by the assignment of 17 sequence variants of CEV into two sequence classes that differ mainly in the P and V domains (Table V) by a minimum of 23 nucleotides between any two sequence variants from each class (Visvader and Symons, 1985). The two sequence classes of CEV correlate with differences in symptom expression when propagated on tomato; one is associated with mild symptoms and the other induces severe stunting and leaf curling (Visvader and Symons, 1985). Results obtained from the use of chimeric constructs of mild and severe variants of CEV show that only sequence differences in the P domain can be correlated with changes in symptom expression (Visvader and Symons, 1986).

3. V Domain

This is the most variable region and shows less than 50% sequence homology between otherwise closely related viroids, such as between TASV and CEV-A, or TPMV and PSTV (Table IV). Similarly, the 17 sequence variants of CEV show up to 29 nucleotide differences in this region (Table V) (Visvader and Symons, 1985). It is the V domain, rather than the more highly conserved T2 domain (Table IV), that is responsible for the low sequence conservation reported for the right-half portions of PSTV, CEV, CSV, TASV, and TPMV (Sänger, 1984; Riesner and Gross, 1985). The only significant sequence relationship between viroids in the V region appears to be the presence of an oligopurine:oligopyrimidine helix, usu-

ally with a minimum of three G:C base pairs (Fig. 3). The boundaries (Table III) have been defined by a change from low sequence homology in the adjacent C and T2 domains.

4. T Domains

The most notable sequence homologies between PSTV-like viroids in the T domains include a strictly conserved CCUC sequence in the end loop of the T1 domain and a CCUUC sequence in the T2 domain (Fig. 2). These sequences may facilitate the binding of DNA-dependent RNA polymerase II. This enzyme, which has been proposed as an *in vivo* replicase of viroids (see Chapter 5, this volume) has been shown by electron microscopy to bind to the T domains of PSTV (Goodman *et al.*, 1984).

Sequence data show that three viroids (TASV, TPMV, and CCCV) exhibit unusual relationships with respect to their terminal sequences. For example, TASV shares 73% overall sequence homology with CEV-A but the T2 domains are only 46% homologous (Table IV). 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 PSTV-like viroid and all but the T2 domain of a CEV-like viroid. Similarly, TPMV is a potential recombinant between the T1 domains of CEV and all but the T1 domain of PSTV. CCCV shows two potential sets of terminal domain rearrangements. In one, the left half of the T1 domain of CCCV is almost identical to HSV but the C domain is more homologous to PSTV (70%) than to HSV (42%). In the second, a partial duplication of the T2 domain plus part of the V domain has been found in different isolates of CCCV (Haseloff *et al.*, 1982). Three forms (D-41, D-50, and D-55; Fig. 5), in which 41 (X = 21, Y = 20), 50 (X = 26, Y = 24), or 55 (X = 28, Y = 27) nucleotides are duplicated, arise during infection of the small CCCV D-0 form but only one of the three T2 duplications has been found in any individual coconut palm (Haseloff *et al.*, 1982). It has been suggested that the postulated T domain rearrangements of TASV, TPMV, and CCCV D-0, together with the terminal duplications of CCCV D-0 (Fig. 5), have arisen by a common mechanism of RNA polymerase "jumping" during viroid replication (Keese and Symons, 1985).

D. Structure of ASBV

ASBV, which only infects members of the family Lauraceae (da Graca and van Vuuren, 1980), shows a number of sequence and structural differences with other viroids. The A + U nucleotide composition of ASBV is 62% of the total nucleotides in comparison with 40–44% for the PSTV-like viroids (Table II). This is further reflected in the distribution of base

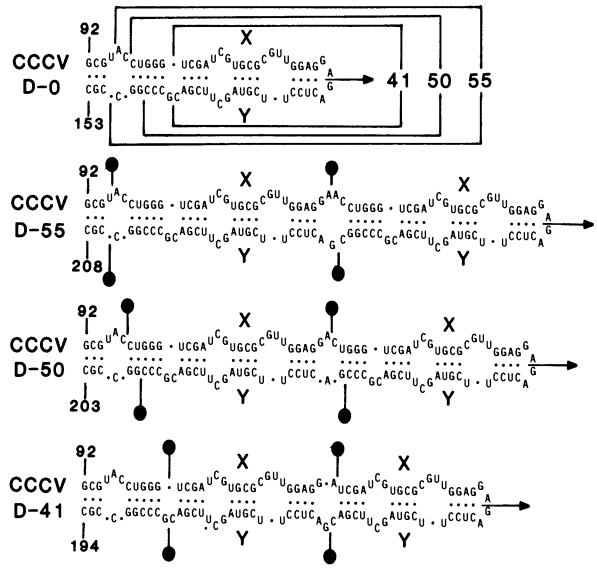


FIGURE 5. Partial sequence duplications of the small form of CCCV (CCCV D-0) in which two adjacent sequences (X, Y) of variable size are repeated, leading to a double duplication of either 41, 50, or 55 nucleotides (D-41, D-50, D-55) (Haseloff *et al.*, 1982). The arrows depict the boundary of the X and Y sequences. The solid circles mark the boundaries of the duplicated sequences.

pairs in the predicted secondary structures (Fig. 2) in which A:U plus G:U base pairs in ASBV constitute 65% of total base pairs as compared to 32–48% for the PSTV-like viroids (Table II). This difference most likely explains the lower T_m of ASBV as compared to that of other viroids (Steger *et al.*, 1984; Riesner and Gross, 1985; Chapter 3, this volume). Second, although a rodlike secondary structure was originally proposed for ASBV (Symons, 1981), an alternative structure based on theoretically deduced thermodynamic principles has been proposed that allows for small bifurcations in ASBV (Steger *et al.*, 1984). Finally, ASBV shows limited sequence homology with other viroids, lacking in particular the central conserved bulged helix and the flanking nine-nucleotide inverted repeat (Fig. 3) common to PSTV-like viroids. These differences may indicate a polyphyletic origin for viroids.

Infection of avocado trees with ASBV is associated with greater-than-unit-length viroid (plus) and viroid complementary (minus) RNA forms (Bruening *et al.*, 1982; Hutchins *et al.*, 1985), a feature common to other viroids in other host plants (Hutchins *et al.*, 1985; Branch *et al.*, 1981; Owens and Diener, 1982). It is generally accepted that these higher-molecular-weight forms arise by some form of RNA-dependent rolling circle replication followed by cleavage and ligation to produce the monomeric plus and/or minus forms (see Chapter 5, this volume). This is supported

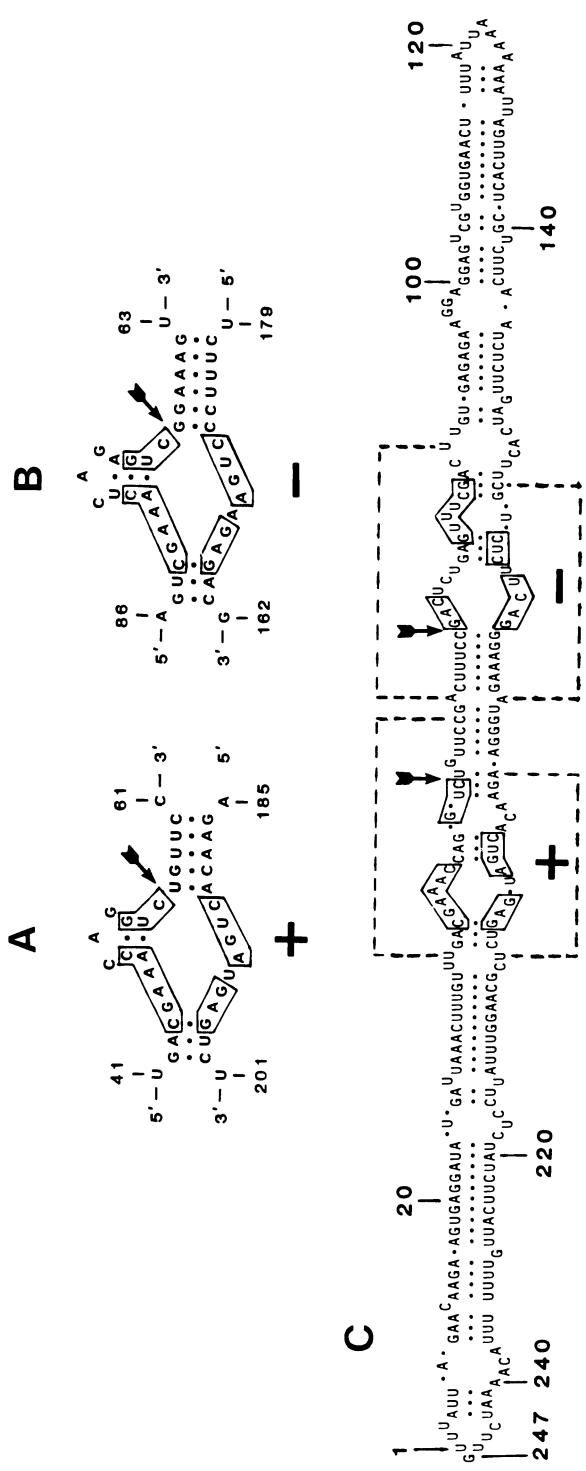


FIGURE 6. Secondary structures of ASBV postulated to be involved in signaling self-processing of ASBV plus (A) and minus (B) RNA species showing homologous three-stem structures and sequences (boxed nucleotides) that are common to virusoids and the satellite RNA of tobacco ringspot virus (Hutchins *et al.*, 1986; Keese and Symons, 1986; G. Bruening, personal communication). The locations of the sequences relative to the proposed native structure of ASBV (C) (Symons, 1981) are delineated by dashed boxes. The arrows depict self-processing sites in ASBV plus and minus RNA. The sequence numbers of ASBV minus RNA (B) are the same as those of the plus RNA and, therefore, read in the 3′-to-5′ direction.

by the ability of RNA transcripts generated *in vitro* from cDNA clones of ASBV to process at specific sites in the absence of enzymes but in the presence of a divalent cation such as Mg^{2+} or Ca^{2+} (Hutchins *et al.*, 1986). On the basis of the single site of processing in the plus and minus transcripts, models of secondary structure have been postulated (Fig. 6A,B) that may contribute to the formation of an active tertiary complex that allows bond cleavage at a specific site (Hutchins *et al.*, 1986). These structures show striking sequence and structural homologies with those predicted around the processing sites of the precursors of virusoids (see Chapter 8, this volume) and the satellite RNA of tobacco ringspot virus (sTRSV) (Hutchins *et al.*, 1986; Keese and Symons, 1986; A. C. Forster and R. H. Symons, unpublished findings). The processing sites of these RNAs, first proposed for three virusoids by Haseloff (1983) (see Symons *et al.*, 1985), were determined from the nonenzymatic processing of naturally occurring dimers of satellite TRSV (Prody *et al.*, 1986), the presence of a 2'-phosphate at C-49 of the virusoid of *Solanum nodiflorum* mottle virus (Kiberstis *et al.*, 1985), and the *in vitro* processing of plus and minus transcripts of cDNA clones of the virusoid of lucerne transient streak virus (A. C. Forster and R. H. Symons, unpublished findings). It remains to be seen whether or not a similar mechanism of nonenzymatic RNA cleavage occurs in the PSTV-like viroids.

V. CONCLUSION

The sequences of more than 40 viroid isolates have been determined to date. Together with extensive structural analysis of certain viroids such as PSTV, we now have a wealth of comparative data suitable for functional analysis. Since viroids appear to lack mRNA activity and their small size argues for limited genetic capacity, the RNA itself must provide the necessary sequence and structural signals for viroid function. This is reflected by the model presented of five structural domains, applicable to all viroids except ASBV, that may also represent functional domains. Sequence variation in one of these domains, the P domain, is associated with the modulation of symptom expression. The T domains have been identified as binding sites for DNA-dependent RNA polymerase II (Goodman *et al.*, 1984), an enzyme possibly involved in viroid replication (see Chapter 5, this volume). Finally, the C domain may signal RNA processing of replicative intermediates (Visvader *et al.*, 1985). In the case of ASBV, where RNA processing has been demonstrated to occur at specific sites (Hutchins *et al.*, 1986), as much as one-third of the molecule may be devoted to regulating this function (Fig. 6C).

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CHAPTER 3

PHYSICAL–CHEMICAL PROPERTIES
Structure Formation

DETLEV RIESNER

I. INTRODUCTION

Among the nucleic acids, the single-stranded RNA molecules have the highest potential to form a large variety of structures and to undergo quite different structural transitions. The determination of their structure and structural transitions is a prerequisite for understanding their function in replication, transcription, translation, and regulation. In many cases the problem of RNA structure is particularly difficult, for in addition to the secondary structure formed by Watson–Crick base pairs and wobble base pairs, a complicated tertiary structure may exist that cannot be described by a few prototypes of interactions. These general statements hold for viroids as well as for transfer RNA, ribosomal RNA, small nuclear RNA, messenger RNA, and viral RNA. To date, the structure and structural transitions are best understood with tRNA, to a great deal as a consequence of x-ray analysis, whereas only incomplete data exist on the other RNAs. In this chapter it will be shown that, with the exception of tRNA, the structure formation of viroid RNA is best understood among all RNAs. Furthermore, these results have gained some relevance for the understanding of the function of viroids.

In terms of the history of viroid research, physicochemical studies started right after pure viroid material had become available. Many features of viroid structure and structure formation became known even before the first sequence of a viroid had been determined. At present, however, the features of viroids will be discussed, of course, on the basis of the known sequences. Viroid research has gone the more classical way, in the sense that study of the biology of viroids was followed by that of

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the physical chemistry, before detailed studies on the molecular biology of viroids were initiated.

The process of structure formation of RNA is always intimately correlated to the structure itself. If a transition from the native to the completely denatured state of a viroid is discussed, *a priori* only the structure of the denatured state, i.e., that of the random coil, is known. Therefore, assumptions or results on the process of structure formation inevitably imply features of the native structure. In this chapter the main emphasis is on the mechanism of structure formation, whereas the native structure itself is the main subject of Chapter 2, this volume.

II. EXPERIMENTAL ANALYSIS

The process of structure formation was studied mainly by physicochemical methods. More chemically or biochemically oriented methods, such as chemical modification, limited enzymatic digestion, or ligand binding, were used to study the native structure but cannot be applied to follow structural transitions. Since transitions are induced by a change in temperature or solvent condition, the method of interest has to be applicable under quite different temperatures and/or solvent conditions. A further restriction is the limited availability of purified viroid material.

A. Optical Melting Curves

The transition of nucleic acids from the native state, which is formed by internal base pairs, to the denatured state, where all base pairs are disrupted, can be induced by an increase in temperature, and it is associated with a 30–40% increase in UV absorption at 260 nm. The transition curves, commonly called melting curves, can now be recorded with computer-controlled spectrophotometers, using microcuvettes of 50-μl sample volume and 1-cm optical path length (Henco *et al.*, 1980; Brosius *et al.*, 1984). Consequently, less than a microgram of nucleic acid is needed to obtain a melting curve. In Fig. 1, melting curves of PSTV and other types of RNA and DNA are presented. Melting curves are commonly prepared in the differentiated form, as in Fig. 1, because the appearance of more than one transition may be recognized more clearly, and evaluation of the midpoint temperature T_m and the half-width $\Delta T_{1/2}$ is easier.

As is obvious from Fig. 1, the melting curve is a characteristic feature of a nucleic acid. tRNA shows the well-known curve of several broad transitions at a fairly low, average T_m-value. The other extreme is the denaturation of homogeneously double-stranded RNA from reovirus, which occurs in several very sharp transitions at high temperatures. DNA from phage λ shows a multistep curve at somewhat lower temperatures than reovirus RNA. An extraordinary situation is found with PSTV in that the

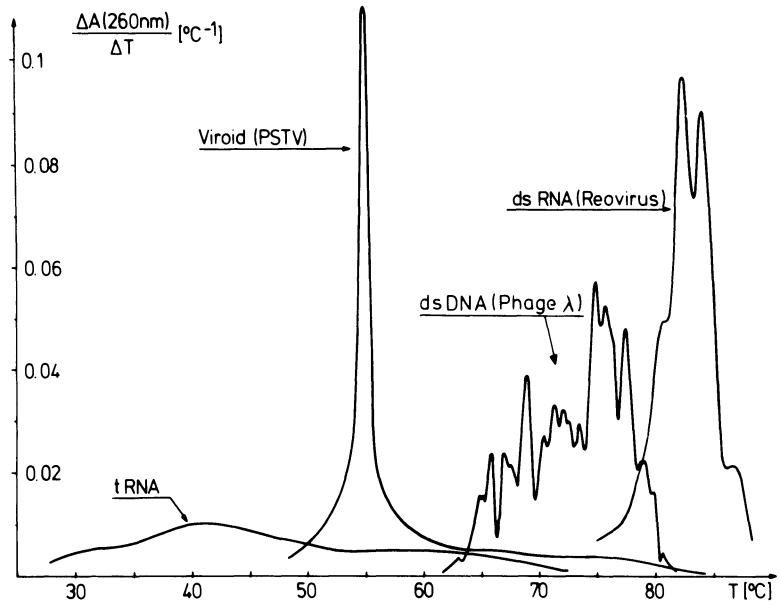


FIGURE 1. Optical melting curves of different nucleic acids. The curves are presented in the differentiated form. Buffer conditions: 20 mM NaCl, 0.1 mM EDTA, 1 mM sodium-cacodylate, pH 6.8; concentration of the nucleic acid: A_{260} (20°C) = 1. From Riesner *et al.* (1983) with permission.

sharpness of the transition of a double-stranded nucleic acid is combined with the low thermal stability of single-stranded nucleic acids, such as tRNA, 5 S RNA, and mRNA. It may be used as a qualitative argument—and has been shown quantitatively without assuming a particular viroid sequence—that viroid structure combines indeed typical features of double-stranded and single-stranded nucleic acids (Langowski *et al.*, 1978). The sharpness of the transition, which is otherwise typical for double strands, is a consequence of the completely unbranched arrangement of all double helices; and the relatively low T_m value, which is similar to that of single-stranded tRNA, is due to the small number of base pairs in any one helical stretch and the many internal loops. $\Delta T_{1/2}$ is a measure of the van't Hoff enthalpy ΔH of the transition. $\Delta H_{\text{van't Hoff}}$ may either be determined from the approximative formula (Riesner and Römer, 1973):

$$\Delta H_{\text{van't Hoff}} = 4 \cdot RT_m^2 \cdot \Delta T_{1/2}^{-1} \quad (1)$$

or be evaluated from a van't Hoff plot of ln K versus T^{-1} according to:

$$d \ln K/d(1/T) = -\Delta H_{\text{van't Hoff}}/R \quad (2)$$

where K is the equilibrium constant

$$(K = \text{molecules in the helix state/molecules in the coil state}) \quad (3)$$

R is the gas constant, and T is the absolute temperature. $\Delta H_{\text{van't Hoff}}$ is the reaction enthalpy of the total transition if it is interpreted as an all-or-none process. In an all-or-none process, either all or no base pairs are intact; intermediate states are not present in measurable concentrations. If, however, the all-or-none process does not hold exactly, which means that intermediate conformations are also present in measurable concentration, then $\Delta H_{\text{van't Hoff}}$ would be lower than the sum of the ΔH values of all base pairs involved in the transition. One may compare $\Delta H_{\text{van't Hoff}}$ with the value calculated for the sum of all base pairs ΔH_{total} (cf. Section III.A) and use it as a measure of cooperativity:

$$\text{cooperativity} = \Delta H_{\text{van't Hoff}}/\Delta H_{\text{total}} \tag{4}$$

In the few cases (see Section II.B), ΔH_{total} could be determined experimentally by calorimetric measurements.

At temperatures above the main, very sharp transition, low values of hypochromicity were measured up to 80°C (cf. Fig. 1). This contribution was resolved into separate transitions by applying fast kinetic techniques as discussed in detail in Section II.E. At this point, suffice it to say that it was possible to resolve the overall melting curve into different processes that are described by their characteristic time courses. As summarized

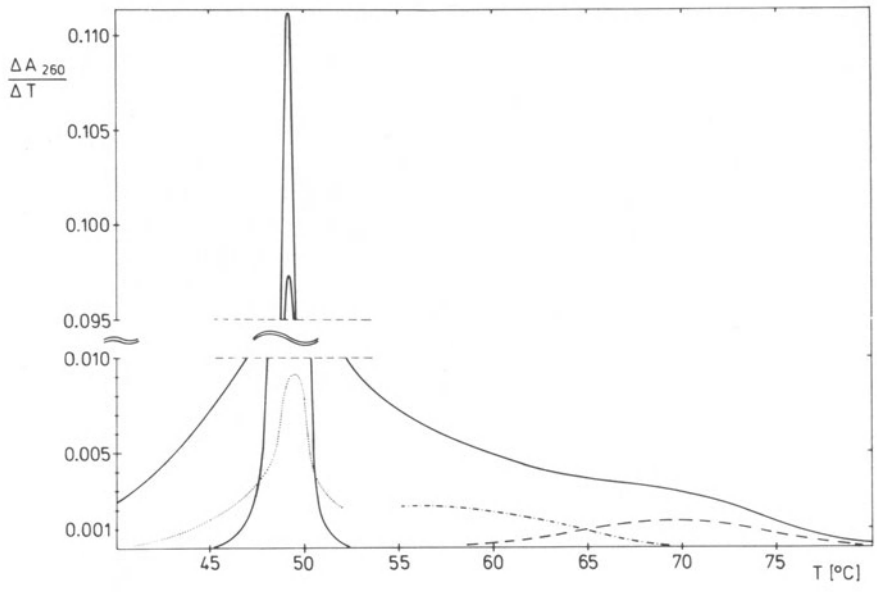


FIGURE 2. Composite melting curve of PSTV. Buffer conditions: 8 mM sodium-cacodylate, 0.1 mM EDTA, pH 6.8. The dashed and dotted lines refer to transitions that occur in the time range of milliseconds and were studied with the temperature-jump technique. From Henco *et al.* (1979) with permission.

in Fig. 2, PSTV melting occurs in one highly cooperative main transition and two broader transitions of lower amplitudes at higher temperatures.

If the spectrum of the hypochromicity of a transition is determined, the relative amounts of A:U and G:C base pairs dissociating in that transition may be calculated. This is because the spectrum is different for dissociating A:U and G:C base pairs, and the measured spectrum—normally characterized by the two values at 260 and 280 nm—is a superposition of those of pure A:U and G:C base pairs, respectively.

PSTV is the viroid most intensively studied by physicochemical methods. This came from its early date of biological characterization and from the fact that it was available early as purified RNA (Diener, 1972; Sänger *et al.*, 1976). For the same reasons, it was the first viroid to be sequenced (Gross *et al.*, 1978). CEV was also studied fairly early with physical methods (Semancik *et al.*, 1975; Sänger *et al.*, 1976). Today, most thermodynamic parameters discussed so far have been determined for a series of other viroids. In Table I the thermodynamic parameters of the main transition are listed. The corresponding values of the transition at higher temperatures were evaluated mainly from kinetic measurements and therefore are listed together with the kinetic parameters in the corresponding section (IV.3).

B. Calorimetry

From optically recorded melting curves, van't Hoff enthalpies were determined. If the all-or-none model is not an adequate description of a transition, $\Delta H_{\text{van't Hoff}}$ is lower than ΔH_{total}. For mechanistic considerations (see Section IV), it is relevant to determine ΔH_{total} and thereby the cooperativity [cf. equation (4)] experimentally.

Calorimetric measurements may provide ΔH_{total} without model assumptions. The molar concentration of the biopolymer is the only additional information needed. ΔH_{total} is evaluated from the additional heat capacity Δc_p due to the transition of interest according to the following equation:

$$\Delta H_{\text{total}} = \int_{T_1}^{T_2} \Delta c_p \, dT \tag{5}$$

where T_1 and T_2 are temperatures below and above the transition range. Using a scanning microcalorimeter for liquid samples, total reaction enthalpies were determined for the main transition of PSTV and CEV (Klump *et al.*, 1978). The scanning curves, Δc_p versus T, were very similar to optical melting curves in shape and position on the temperature scale. The results are also given in Table I. They show that ΔH_{total} is remarkably higher than $\Delta H_{\text{van't Hoff}}$.

TABLE I. Experimentally Determined Properties of Main Transition[a]

Viroid	N	BP/N %	T_m (°C)[b]	Hypochromicity (%)	f_{GC} (%)[c]	$\Delta T_{1/2}$ (°C)	$\Delta H_{van't\ Hoff}$ (kJ/mole)	ΔH_{total} (kJ/mole)	Cooperativity (%)	References
PSTV	359	69	49.5	14	66 ± 2	0.9	3890 ± 240	3930 ± 150[d]	99 ± 10	Langowski *et al.* (1978)
CEV	371	68	50.5	14	65 ± 2	1.0	3490 ± 200	4200 ± 150	83 ± 8	Henco *et al.* (1979)
CSV	354	70	48.5	18	59 ± 4	1.1	3130 ± 150			Klump *et al.* (1978) Steger *et al.* (1984)
CCCV[e]										
1 small	246/7	62	48.8	15	70 ± 2	1.4	2470 ± 100			Randles *et al.* (1982)
large	287–303	62	48.1	15.5	73 ± 2	1.2	2880 ± 130			Steger *et al.* (1984)
2 small	492/4	62	45.8	12	73 ± 2	2.5				
large	574–606	62	45.7	14.5	74 ± 2	1.8				
ASBV	247	67	37.5	18	37 ± 2	1.5	2140 ± 130			Steger *et al.* (1984)

[a] The number of nucleotides N and the degree of base pairing (BP/N) are taken from the secondary structure (see Chapter 2, this volume). All experimental values refer to 10 mM NaCl, 0.1 mM EDTA, 1 mM sodium-cacodylate, pH 6.8.

[b] The values differ slightly from the values originally published. Steger (1984) carefully reevaluated all existing data and eliminated small variations from the use of different buffer conditions and different instruments.

[c] The evaluation did not take into account G:U base pairs. This may be the reason why all values are higher than expected from the sequence.

[d] The value was originally published as measured on CPFV (Klump *et al.*, 1978). The strain was grown on tomato, and the sequence determination later showed that it was actually PSTV (H. L. Sänger, personal communication).

[e] CCCV consists of four different RNAs (see Chapters 2 and 13, this volume). CCCV 2 are exact dimers of CCCV1. The small and large forms are called by other authors "fast" and "slow" forms because of their gel electrophoretic mobility. Strains of CCCV also differ in the size of the large forms.

C. Electron Microscopy

Electron microscopy was originally applied to elucidate the native structure of viroids, and the rodlike conformation of viroids became evident already from the very early studies (Sogo *et al.*, 1973; Sänger *et al.*, 1976). When the parameters of the conformational transitions became known from thermodynamic experiments, they could be used to prepare and observe viroids in different well-defined conformations.

A systematic investigation was carried out with PSTV (Riesner *et al.*, 1979). After heating and slow renaturation to room temperature, the viroids show a rodlike conformation of length 37 ± 6 nm (Fig. 3a). The length is slightly shorter if a viroid is redissolved from its ethanol precipitate without a heating–cooling cycle. The renatured state after a heating–cooling cycle is the thermodynamically most stable state. This is the state that is always depicted by the secondary-structure models. The aspect of reversibility will be discussed later.

Viroids were also studied after denaturation at 65°C in the presence of 3 M urea and subsequent spreading onto water at 40°C. The T_m value

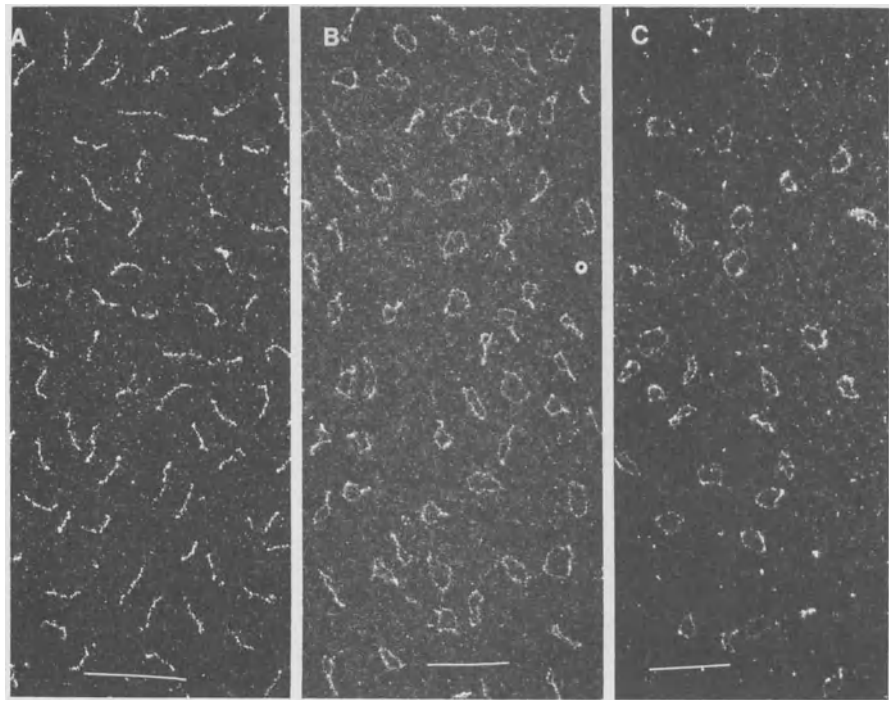


FIGURE 3. Electron micrographs of PSTV in different conformational states. (A) Renatured; (B) intermediate with stable hairpin formation; (C) completely denatured circle. Bars = 100 nm. Preparation of the micrographs is described by Riesner *et al.* (1979). From Dr. G. Klotz, Ulm.

of the main transition, as determined by optical melting curves under those conditions, is 50°C. The micrographs showed distinct classes of molecular conformations (Fig. 3b). Rodlike molecules as well as completely open circles were found. All other structures represent intermediates of the helix–coil transition with possible structural alterations due to the influence of surfactant and preparative interactions. The molecules could be classified as being shaped like either a dumbbell, a tennis racket, or a circle.

Fully denaturing conditions were maintained when preparation and spreading were carried out at 70° C (Fig. 3c). Over 80% of the molecules showed up as single-stranded circles devoid of any obvious base-pairing. Denatured viroids could also be prepared at lower temperatures if a high concentration of denaturant such as formamide or urea was present.

The electron microscopic study of completely denatured viroids was a milestone in the sense that it was the first time a circular RNA was detected in nature (Sänger *et al.*, 1976). This result, found originally with PSTV, has been confirmed with all viroid RNAs studied with this method (McClements and Kaesberg, 1977; Randles and Hatta, 1979; Palukaitis and Symons, 1980; Ohno *et al.*, 1982; Chen *et al.*, 1983).

D. Gel Electrophoresis

The gel electrophoretic properties of viroids have been studied for analytical as well as for preparative purposes. Preparative gel electrophoresis was for long the only way to purify viroids to homogeneity (e.g., Sänger *et al.*, 1976; Palukaitis and Symons, 1980). Nowadays, chromatographic procedures seem to be superior (Colpan *et al.*, 1983). When viroids undergo the conformational transitions discussed above, their electrophoretic mobility changes drastically. This is caused by the denatured structure of the open circle, which migrates much slower than any other RNA of similar molecular weight (Sänger *et al.*, 1979). Consequently, the nicked form of a denatured viroid runs faster than the circular form. The transition may be made visible in a gel if a urea gradient is formed perpendicular to the electrophoretic direction. In Fig. 4, gel electrophoresis of circular and nicked viroids in a urea gradient is shown. The extraordinary difference in the electrophoretic mobility of native and denatured viroids is evident. Furthermore, at least two transitions may be detected in the transition curve of circular viroids. The faster migrating band at high urea concentrations represents denatured nicked molecules.

Schumacher *et al.* (1986) have utilized the extraordinarily low mobility of circular, denatured viroids to devise a simple and sensitive test to detect viroid infections. They combined an electrophoretic run under native conditions with one under denaturing conditions and separated viroids from all other nucleic acids in a very crude extract. Viroid amount as low as 50 pg may be detected in a routine test taking 4–5 hr.

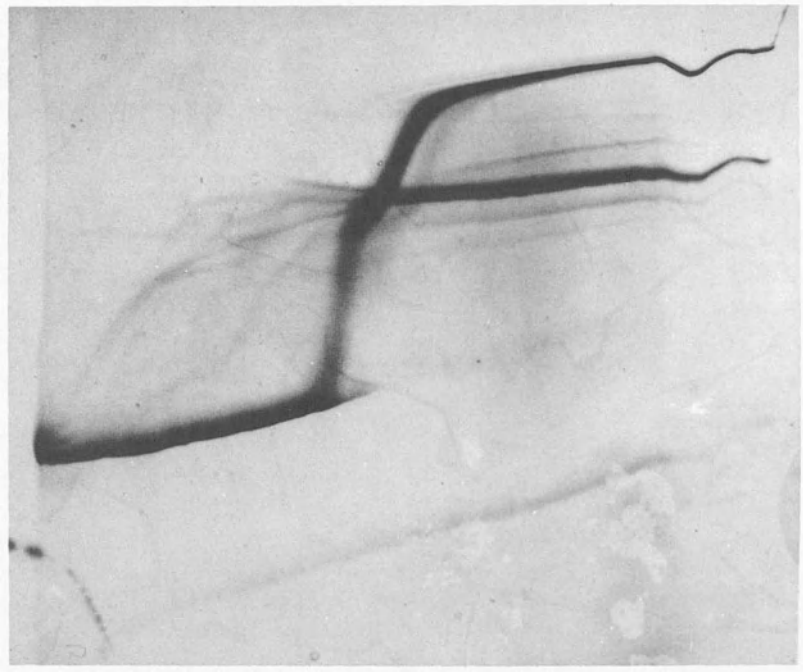


FIGURE 4. Electrophoresis of circular and linear PSTV in a urea-gradient polyacrylamide gel. The urea gradient is from 0 M at the left to 8 M at the right side of the gel. Other conditions of the gel electrophoresis are: 5% polyacrylamide, 17.8 mM tris, 17.8 mM boric acid, 20°C. The circular viroid forms the fast band in low urea, shows two resolved transitions at intermediate urea concentrations, and is the very slow band at high urea concentrations. The band of the linear viroid is homogeneous only after denaturation (fast band at high urea); it is inhomogeneous below the transitions, because of the random site of linearization. From Rosenbaum (1986).

E. Kinetics

In kinetic experiments, a denaturation process is characterized not only by its overall hypochromicity but also by its complete, exponential time course after a stepwise temperature increase or decrease. If more than one process contributes, more than one exponential with its corresponding amplitude is measurable. The method is called *chemical relaxation* because the solvent conditions of the new equilibrium are established faster than the actual concentrations of the chemical system; thus, the actual concentrations deviate from and therefore relax to the new equilibrium concentrations. The "slow" temperature-jump technique, in which the temperature change is induced by switching thermostating baths (Pohl, 1968), as well as the fast technique with temperature jumps resulting from a capacitor discharge (Eigen and De Maeyer, 1968) were applied to cover the time range from microseconds to minutes (Henco *et al.*, 1979; Riesner *et al.*, 1979). Both techniques were optimized

for the requirements of the study on viroids, i.e., high temperatures and exceedingly little RNA material (Henco *et al.*, 1980; Riesner *et al.*, 1982a).

The main transition (around 50°C) proceeds in the time range of seconds to minutes. The kinetics confirmed the high cooperativity observed in the equilibrium melting curve. Eighty to ninety percent of the change in base pairing was found in a single exponential, the remaining contribution in the millisecond range. Single-stranded regions in nucleic acids always show a relaxation effect faster than microseconds. This part is from the stacking–destacking equilibrium of the bases in single-stranded regions and therefore not involved in base-pairing processes. In Fig. 5A the relaxation times together with the corresponding amplitudes are depicted in the form of an Arrhenius plot. For a monomolecular denaturation–renaturation equilibrium:

$$\text{native state} \underset{k_R}{\overset{k_D}{\rightleftharpoons}} \text{denatured state} \tag{6}$$

where k_D is the dissociation rate constant and k_R the recombination rate constant. The equation for the relaxation time τ is the following:

$$1/\tau = k_R + k_D \tag{7}$$

At low temperature $1/\tau$ extrapolates to k_R, at high temperature to k_D as is also evident from Fig. 5A. Applying the Arrhenius relationship

$$d \ln k/d\,(1/T) = -\Delta E^{\#}/R \tag{8}$$

(where $\Delta E^{\#}$ is the activation enthalpy), the activation enthalpy for the recombination process, $\Delta E_R{}^{\#}$, as well as for the denaturation process, $\Delta E_D{}^{\#}$, were obtained. Measurements on different viroids, such as PSTV, CEV, and CSV, gave characteristic values around 3000 kJ/mole for $\Delta E_D{}^{\#}$ and $-$ 600 kJ/mole for $\Delta E_R{}^{\#}$. The difference $\Delta E_D{}^{\#} - \Delta E_R{}^{\#}$ is in good agreement with the reaction enthalpy $\Delta H_{\text{van't Hoff}}$ from equilibrium measurements as expected from the following relationship:

$$-\Delta H_{\text{van't Hoff}} = \Delta E_D{}^{\#} - \Delta E_R{}^{\#} \tag{9}$$

From the mechanistic point of view, one may note that the value of $\Delta E_R{}^{\#}$ is highly negative, which is impossible for an elementary reaction. It demonstrates that a fast preequilibrium comprising 15–20 base pairs has to precede the rate-limiting step (see Riesner and Römer, 1973). This will be discussed later in terms of viroid secondary structure.

Although at temperatures above the main transition the amplitudes decrease drastically (see Fig. 2), in PSTV two additional transitions could be followed quantitatively by fast kinetic methods. In Fig. 5B the relaxation times and amplitudes of the transition with the highest T_m are

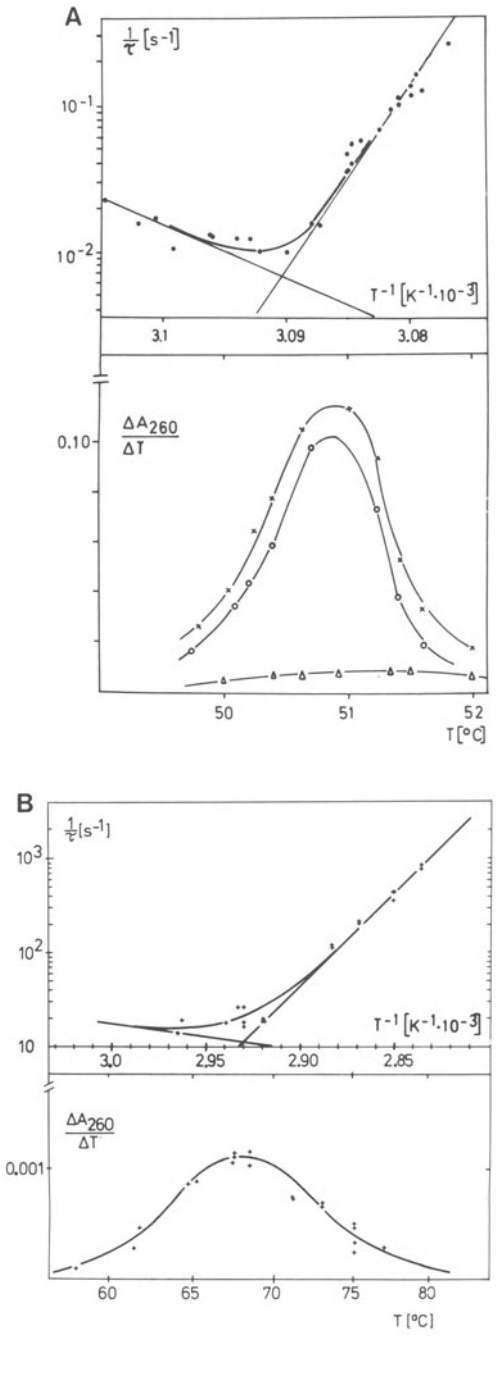


FIGURE 5. Temperature dependence of the relaxation times (upper graphs) and relaxation amplitudes (lower graphs). (A) Main transition; (B) high-temperature transition. The relaxation times are presented in the form of an Arrhenius plot of log $1/\tau$ versus $1/T$. In (A) the contributions of the process in the second range (○) and the millisecond range (△) to the total hypochromicity (×) are shown. From Henco *et al.* (1979) with permission.

shown; both transitions are contained in the composite melting curve of Fig. 2. Similarly, two transitions in addition to the main transition were found in CEV and CSV (Henco *et al.*, 1979) and only one transition in CCCV RNA 1 (Randles *et al.*, 1982). The ΔH values and rate constants were evaluated in the same way as for the main transition from graphs like those of Fig. 5B. From ΔH values the number of base pairs dissociating in the corresponding transition was derived. The G:C content of the dissociating base pairs was estimated from the wavelength dependence of the hypochromicity (Henco *et al.*, 1979; Randles *et al.*, 1982); in kinetic measurements the hypochromicity is measured by the amplitudes of the relaxation curves. As a characteristic result form kinetic experiments, the size of the loop that has to be closed for helix formation may be estimated (Henco *et al.*, 1979). In summary, from kinetic data such as those in Fig. 5B, values for the following were obtained: T_m, G:C content, number of base pairs, size of the loop. As will be discussed in Section IV.B, the transitions could be attributed to well-defined hairpin structures.

As long as chemical relaxation kinetics is followed after small temperature jumps (1–2°C), the results depend only upon the final temperature of the temperature jump. In that case, all reactions are completely reversible and always lead to the state of thermodynamic equilibrium. This is not true for large jumps, particularly if the final temperature is below the transition range of the main transition.

F. Influence of Ionic Strength and Other Solvent Conditions

Quite generally, helix–coil transitions of nucleic acids are shifted to higher temperature by increasing ionic strength, and to lower temperature by addition of several organic solvents. These effects have been elucidated with several nucleic acids and would not have to be repeated for viroids if the quantitative data were not specific for viroids. They are of enormous practical importance for other experiments, such as gel electrophoresis, hybridization, purification procedures, and so on.

Figure 6A shows the dependence of the T_m value of the main transition upon the logarithm of the ionic strength (Langowski *et al.*, 1978). The dependence is nearly linear between 0.005 M and 1 M NaCl, with a slope of 13.2°C/log c_{Na+}. In 1 M NaCl the T_m value is of particular interest, because the theoretical thermodynamic treatment (see next section) refers to this high ionic strength. The cooperativity decreases only slightly with higher ionic strength. An exception in this respect is CCCV1, which is very cooperative at low ionic strength but exhibits three well-resolved transitions in 1 M NaCl (Randles *et al.*, 1982). The ionic strength dependence of 13.2°C/log c_{Na+} is very similar to the ionic strength dependence of homogeneously double-stranded, polymeric RNA of the same G:C content (Steger *et al.*, 1980), whereas isolated hairpins of a few base pairs

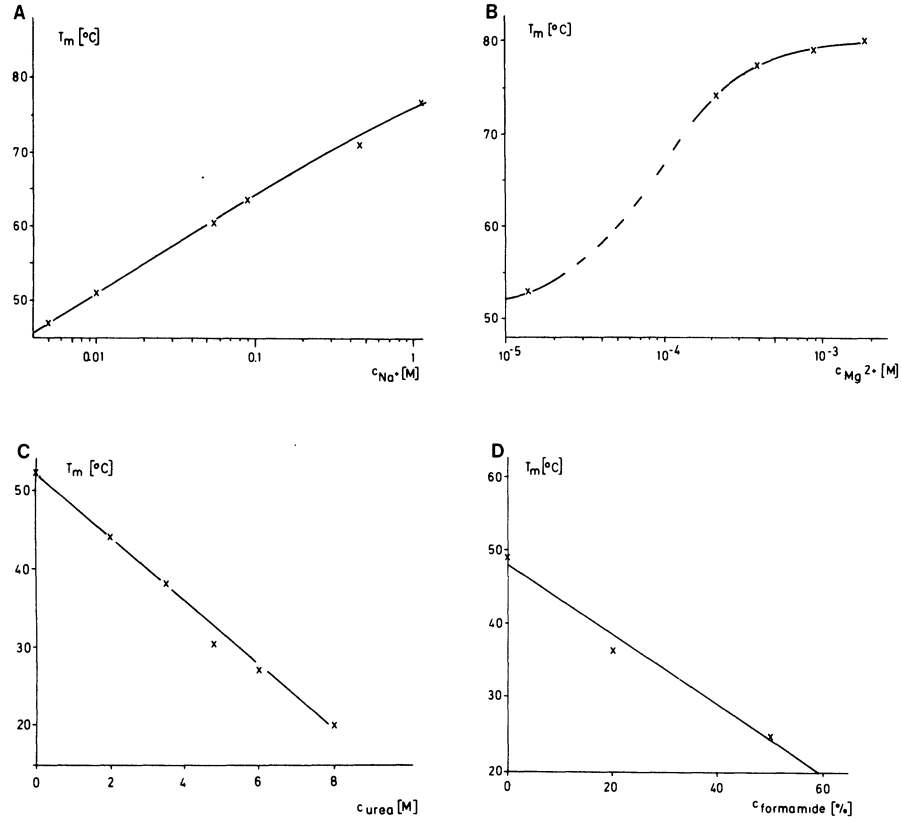


FIGURE 6. Dependence of the T_m value upon solvent conditions. Aside from the varying concentrations of NaCl in (A), $MgCl_2$ in (B), urea in (C), and formamide in (D), buffer conditions were: 1 mM sodium-cacodylate, 0.1 mM EDTA, pH 6.8 for (A), and in addition 10 mM NaCl for (B)–(D). $MgCl_2$ is the free concentration, i.e., the value is corrected for EDTA binding. (A) from Langowski *et al.* (1978); (C) from Henco *et al.* (1977); (B, D) from Rosenbaum (1986) with permission.

have a lower dependence (Coutts, 1971). Thus, the interruption of a homogeneous double strand by internal loops, as is the case with viroids, decreases neither the ionic strength dependence nor the cooperativity of the helix–coil transition.

The conformational transitions of viroids are very sensitive to Mg^2 ions. From Fig. 6B it is evident that at a concentration as low as 10^{-4} M Mg^{2+} ions bind to viroids and raise the T_m value by about 30°C (Rosenbaum, 1986).

The T_m values of the transitions of viroids are lowered by urea (Henco *et al.*, 1977) or formamide (Rosenbaum, 1986). The results, as studied on the main transition of PSTV, are shown in Fig. 6C,D. It should be mentioned that the linear dependence of T_m (c_{urea}) as measured with viroids is more the exception than the rule among nucleic acids. The data of Fig.

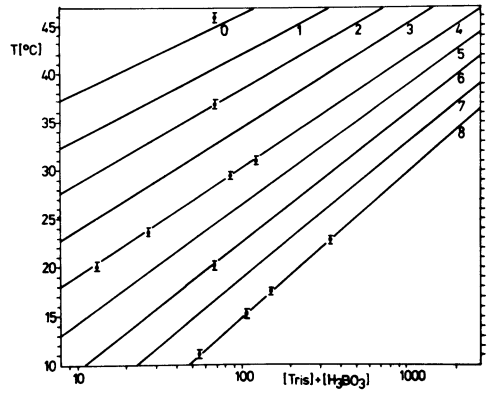


FIGURE 7. Dependence of T_m of the main transition of PSTV upon the conditions of gel electrophoresis. The interpolated straight lines refer to urea concentrations from 0 to 8 M as indicated. From Henco (1979).

6A–D may be used to establish the right conditions of denaturation or close to denaturation in gel electrophoresis and hybridization, respectively.

In gel electrophoresis, the ionic strength, as well as the concentration of denaturing agents, may be varied. In Fig. 7 the dependence of T_m of the main transition of PSTV upon the tris-borate and urea concentration is depicted (Henco, 1979). At every triplet of conditions (temperature, ionic strength, and concentration of denaturant), Fig. 7 shows whether viroids are in the native or the denatured state.

The helix–coil transitions of viroids discussed above are reversible. By chemical modification with formaldehyde or glyoxal, however, the native structure of viroids, as that of other nucleic acids, is destroyed irreversibly. Only at very high temperature (> 80°C) may chemical modification be reversed.

III. THEORETICAL ANALYSIS

A theoretical analysis has to derive viroid properties from our knowledge of the physical chemistry of nucleic acids and from the nucleotide sequence of a particular viroid. The native structure of a viroid is found by searching for the structure of lowest free energy. Intermediate structures during the process of structure formation or denaturation will be found by the same procedure, if the temperature dependence of the structural parameters is incorporated.

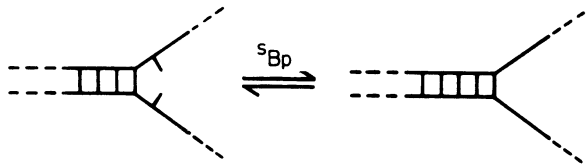
A. Elementary Parameters

Calculation of the free energy of a given base-pairing scheme is carried out in the way that the structure is built up starting from the completely

coiled viroid strand and forming, step by step, all base pairs. Whenever a base pair is formed that is not immediately next to an already existing base pair, the new base pair closes a loop of unpaired nucleotides. Therefore, the stability parameters, i.e., reaction enthalpy ΔH and reaction entropy ΔS, of forming base pairs without loop-closing (helix growth) and with loop-closing (helix nucleation) have to be taken into account. These data may be taken from the literature; they are mostly known from studies on synthetic oligonucleotides and polynucleotides. Because different numbers of the parameters are available from the literature, the data have to be selected critically so as to result in consistent calculations. Steger *et al.* (1984) have carried out extensive calculations and discussed in detail the criteria of selecting parameters from the literature. This discussion will not be repeated here. But, as a summary, the selected set of data will be given here as a recipe for the calculation of the free energy of any desired viroid base-pairing scheme.

1. Helix Growth

The equilibrium constant s is assigned to the formation of a base pair adjacent to an already existing base pair:



$$-RT \ln s = \Delta H_{BP} - T\,\Delta S_{BP} = \Delta G_{BP} \qquad (10)$$

with ΔG, ΔH, and ΔS denoting the changes in Gibbs free energy, enthalpy, and entropy. Those values are listed in Table II for all combinations of neighbors of A:U, G:C, and wobble G:U base pairs. All values refer to 1 M NaCl, absence of $MgCl_2$, neutral pH. These conditions are close to native conditions, i.e., around 0.1 M NaCl, 10 mM $MgCl_2$, neutral pH. The results may be extrapolated to other solvent conditions according to Section II.F. ΔH and ΔS values were assumed to be independent of temperature. It should be mentioned that the values in Table II are particularly suitable to calculate stabilities, i.e., T_m values. The values for the temperature region above 54°C (Table II) differ slightly from those below 54°C. The temperature region above 54°C is more applicable to viroids, if one considers the experimental T_m values at the high ionic strength of 1 M. If emphasis is particularly put on ΔH values, for example to calculate the width of a transition, another set of parameters should be used (see Steger *et al.*, 1984).

TABLE II. Helix Growth Parameters[a]

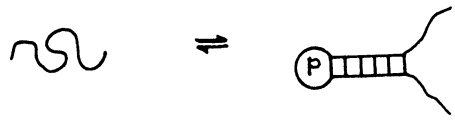
Neighbors of base pairs	$-\Delta G_{25°C}$ (kJ/mole)	$-\Delta H$ (kJ/mole)	$-\Delta S$ (kJ/mole · K)
A:U–A:U G:U–A:U G:U–G:U	6.4	37.7	0.105
G:C–A:U G:C–G:U	7.3	31.2	0.080
G:C–G:C	13.0	53.0	0.134

[a] Values of ΔH are from Gralla and Crothers (1973a); ΔS values were corrected according to Steger *et al.* (1980); values hold for $T > 54°C$.

2. Helix Nucleation

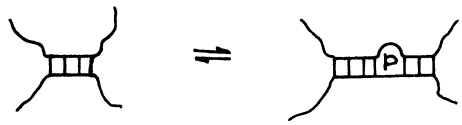
The first base pair of a new helix may form different types of loops. These are:

hairpin loops:

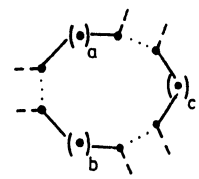


internal loops:

bulge loops:



bifurcations:



with p, a, b, c, numbers of unpaired nucleotides. The values of ΔG (at 25°C) and ΔS are listed in Table III.

TABLE III. Helix Nucleation Parameters[a]

	Hairpin loop		Internal loop		Bulge loop	
Loop size	ΔG	$-\Delta S$	ΔG	$-\Delta S$	ΔG	$-\Delta S$
1	—	—	—	—	11.7	0.0394
2	—	—	0.42	0.0014	16.4	0.0549
3	35.3	0.1185	3.9	0.0129	19.3	0.0649
4	24.6	0.0825	6.7	0.0225	21.0	0.0703
5	17.0	0.0569	8.9	0.0299	21.6	0.0724
6	17.9	0.0599	10.5	0.0351	22.2	0.0745
7	18.6	0.0624	11.7	0.0392	22.8	0.0766
8	19.1	0.0641	12.5	0.0421	23.5	0.0787
9	19.9	0.0666	13.4	0.0449		

[a] Values are from Gralla and Crothers (1973a,b), Fink and Krakauer (1975), Fink and Crothers (1972), and Steger *et al.* (1984). Odd numbers are interpolated from experimental values. Free energies are for 25°C in kJ/mole, entropies in kJ/mole · K. ΔH is taken as zero.

- Formation of hairpin loops:

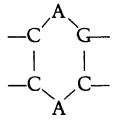
  for larger loops:
  $\Delta S = \{R \cdot \ln [0.003 \cdot 0.319 \cdot (p + 1)^{-1.5} \exp(-1.086/(p + 1))] + 20.9\}/1000$ with $R = 8.314$ J/mole · K; closing base pair = G:C or A:U or G:U

- Formation of internal loops:

  for larger loops:
  $\Delta S = \{R \cdot \ln [0.003 \cdot 0.319 \cdot (p + 1)^{-1.5} \exp(-1.086/(p + 1))] + 40.9\}/1000$; closing base pair = G:C
  correction for AU pairs:

$$\Delta S \quad \begin{matrix} \overset{\frown}{\text{A A}} \\ \text{p} \\ \underset{\smile}{\text{U U}} \end{matrix} \quad e = \Delta S \quad \begin{matrix} \overset{\frown}{\text{A G}} \\ \text{p} \\ \underset{\smile}{\text{U C}} \end{matrix} \quad - 0.0119 \text{ kJ/mole} \cdot \text{K} = \Delta S \quad \begin{matrix} \overset{\frown}{\text{G G}} \\ \text{p} \\ \underset{\smile}{\text{C C}} \end{matrix} \quad - 0.0239 \text{ kJ/mole} \cdot \text{K}$$

  correction for purines in small loops ($p \leq 3$); ΔS/purine = -0.023 kJ/mole · K according to Riesner *et al.* (1979) example:



$$-\Delta S = 0.0014 + 2 \cdot 0.023$$

- Formation of bulge loops:

  for larger loops:
  $\Delta S = \{R \cdot \ln [0.003 \cdot 0.319 \cdot (p + 1)^{-1.5} \exp(-1.086/(p + 1))] + 7.56\}/1000$

- Formation of bifurcations:

  (i) $a = 0$ or $b = 0$ or $c = 0$: bulge loop with $p = a + b + c + 4$

  (ii) $a \neq 0$, $b \neq 0$, $c \neq 0$: internal loop with $p = a + b + c + 4$

  Each additional helix in the loop is counted as four single-stranded bases.

B. Mechanistic Models

Structure and structural transitions are calculated similarly. The free energy for the formation of the double-helical stretch m with k base pairs is:

$$\Delta G_{\mathrm{m}} = \sum_{i=2}^{k} \Delta H_i - T\left(\sum_{i=2}^{k} \Delta S_i - \Delta S_1\right) \tag{11}$$

with ΔH_i and ΔS_i, respectively, for the ith base pair and the loop entropy ΔS_1 due to the formation of the first base pair. ΔG of a total secondary structure sums up all helices m, the entropy of the remaining loop ΔS_{r}, and in the case of a circular nucleic acid the entropy of the open circle without a base pair, ΔS_{o}:

$$\Delta G_{\text{secondary structure}} = \sum_{m=1}^{\text{number of helices}} \Delta G_m + \Delta S_{\mathrm{r}} - \Delta S_{\mathrm{o}} \tag{12}$$

The hypochromicity of a structural transition is the sum of the hypochromicities of all base pairs involved. The relative hypochromicities Hy are: Hy (A:U, 260 nm) = 1; Hy (G:C, 260 nm) = 0.396; Hy (A:U, 280 nm) = 0.037; and Hy (G:C, 280 nm) = 0.683; and Hy (G:C) = (A:U).

The method to calculate free energies and the experimental elementary parameters hold for every given structure. The mechanistic models discussed below differ only in the structures taken into account, not in the way to calculate free energies, hypochromicities, and other physicochemical properties.

1. All-or-None Model

Only the initial and the final structure are taken into account according to equation (3). The cooperativity according to equation (4), in which ΔH_{total} is measured calorimetrically or calculated as described above, is a quantitative measure for the validity of the model.

2. "Cooperative Helix" Approximation

As outlined by Langowski *et al.* (1978), this model is an essential improvement over the all-or-none model. It is assumed that cooperativity does not include all helical stretches involved in a helix–coil transition. Only every single helix present in the secondary-structure model, opens or closes in an all-or-none manner, and ensembles of open and closed

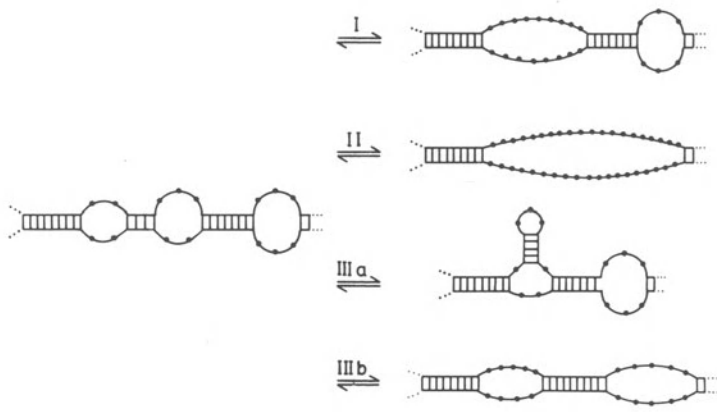


FIGURE 8. Possible transitions of a helix that are calculated with the model of "cooperative helices." I, denaturation; II, cooperative denaturation of neighboring helices; III, rearrangement of one or several helices with formation of new helices. From Steger *et al.* (1984) with permission.

helices are taken into account. The most probable order of melting of the individual helices is then obtained by calculating their T_m values, and for a particular helix the conformational state of the neighboring region is taken into consideration. As dissociation of a helix results in an increase of the loop size, the neighboring helices are destabilized. Cooperative T_m values for neighboring helices are obtained in many cases. For example, if the stability of transition I in Fig. 8 is estimated, possibilities for transition II (dissociation of neighboring helix) and for transition IIIa and IIIb (rearrangements) have to be checked.

3. Energy Minimization Algorithm

At present the most accurate method available for calculating structural transitions is based on an exact algorithm to find the secondary structure of lowest free energy. The algorithm was originally developed by Nussinov and Jacobson (1980), a well applicable program was reported by Zuker and Stiegler (1981), and the particular requirements for the application to the viroid structure, i.e., allowing for circular strands, were introduced by Steger and Hofmann and their colleagues (Steger *et al.*, 1984). With this method, secondary structures of lowest free energy were determined at varying temperatures and the hypochromicity was calculated as the difference in absorption between the structures at different temperatures. *A priori*, rearrangements such as processes III in Fig. 8 are taken into account. As in the other models, only the secondary structure of lowest free energy was considered at every temperature and not the total partition function, which would also include the less probable structures.

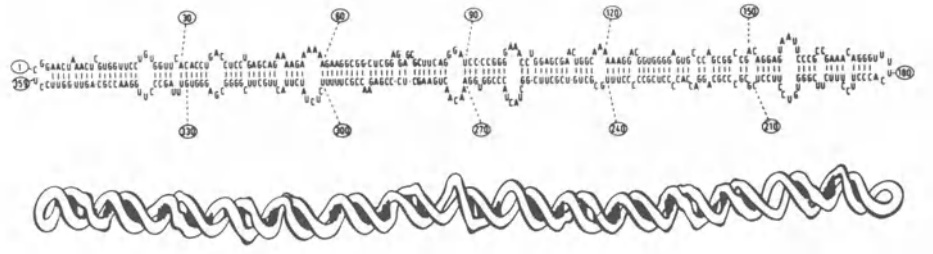


FIGURE 9. Structure of PSTV. (Top) Base-pairing scheme; (bottom) three-dimensional representation. From Gross and Riesner (1980) with permission.

IV. MECHANISM

The complete mechanism of viroid structure formation was derived from experiments as well as from theoretical studies. For the interpretation of the results, it was particularly favorable that viroid structure is formed solely by Watson–Crick and wobble base-pairing. Because PSTV will be discussed in greatest detail, the secondary structure of this viroid is shown in Fig. 9. Especially from studies on tRNAs and rRNAs, it is well known that single-stranded RNAs may form higher-order structures beyond simply two dimensions. In that case the calculations would become much more complicated. Viroids, however, are exceptional among single-stranded RNAs. The two-dimensional, rodlike representation of the structure in Fig. 9 is supported by electron micrographs (Sogo *et al.*, 1973; Sänger *et al.*, 1976), by accessibility of the loops for oligonucleotide binding (Wild *et al.*, 1980) and of the double helices for dye binding (Riesner *et al.*, 1979), and by hydrodynamic studies of viroids in solutions (Riesner *et al.*, 1982b). No evidence exists that viroids form a more globular tertiary structure.

A. Premelting Regions

If the stabilities of all helices present in the native viroid are calculated at increasing temperatures, two or in some cases three characteristic regions show lowest stability (Steger *et al.*, 1984; Gross *et al.*, 1981). These premelting regions have not yet been accessible to experimental studies but are deduced solely from stability calculations. They are shown in Fig. 10 for CEV, CSV, and PSTV, the viroids of highest sequence homology. It is evident that the premelting regions are in parts of the molecules with highly conserved sequences, as may be seen from the different symbols for variable and conserved nucleotides. The left premelting region contains the oligopurine sequence, which plays a role in the virulence of viroids as discussed later. The second premelting region is close to the middle of the molecule and adjacent to one of the most stable hel-

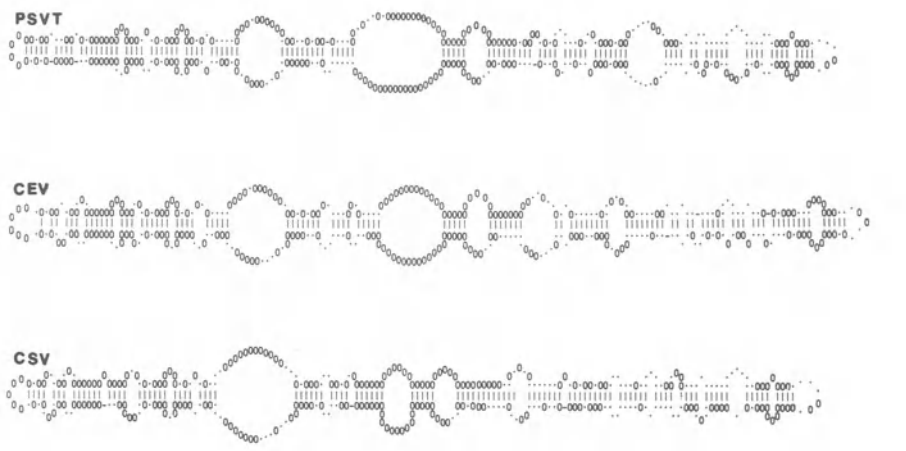


FIGURE 10. Premelting regions in PSTV, CEV, and CSV. The opening of the premelting loops was calculated at about 70°C, i.e., a few degrees below the main transition, applying the "cooperative helix" model. Nucleotides that are conserved in all three viroids are shown as circles, others as points. From Steger *et al.* (1984) with permission.

ices, which divides the molecule into a less stable left half and a more stable right half (Riesner *et al.*, 1979). The difference in the stabilities of both parts when calculated as independent entities is, however, not expressed in two thermal transitions with different T_m values. The more detailed discussion of the total denaturation mechanism (next section) will show a unique type of coupling between the left and the right half.

B. Main Transition and Formation of Stable Hairpins

The conformational transitions following premelting have been followed by thermodynamic, kinetic, and electron microscopic studies as described in the experimental section. In the main transition (see Fig. 2) all base pairs dissociate except those that dissociate in the transitions at higher temperatures. Therefore, the most straightforward manner was to derive the region of highest stability and then interpret the structural change involved in the main transition.

The high-temperature transitions have been studied experimentally in PSTV, CEV, CSV (Henco *et al.*, 1979; Riesner *et al.*, 1979), and CCCV 1 (Randles *et al.*, 1982). T_m values, number of base pairs, content of G:C base pairs, and loop size were derived theoretically for these double helices and compared with the experimental values (see Section II.E). This comparison is shown in Table IV. The agreement between theoretical and experimental values supports strongly the assumption that these hairpins are present in the temperature range between the main and the high-temperature transitions. Also, in electron micrographs taken under these

TABLE IV. Properties of the Stable Hairpins[a]

Hairpin	T_m (°C)		f_{GC} (%)		Loop size		Number of base pairs	
	Theory	Exp.	Theory	Exp.	Theory	Exp.	Theory	Exp.
PSTV								
I	86	80–85	69	77 ± 5	14	20 ± 10	9	6[b]
(III)	86		85		28		7	
II	92	93	90	90 ± 5	82	>40	10	10 ± 1

CEV									
	239 251 CCCUCGCCCGGAG :: : : : : : : : : : : : 79 GGGAGCGGGCCUC 339 327	98	95	91	90 ± 5	79	>40	11	10 ± 1
II									
CSV									
	223 233 CCCUAGCCCGG : : : : : : : : : : : 76 GGGAUCGGGCC 322 312	92	97	82	95 ± 5	76	>40	11	9 ± 1
II									
CCCV1-large									
	46 54 CGCUUGAGG : : : : : : : : : 14 GCGAACUCC 77 69	85	87	67	71 ± 7	14	20 ± 10	9	10 ± 1
I									

[a] The theoretical values are calculated from the sequences and the base-pairing schemes. T_m values correspond to 1 M ionic strength. The experimental values were evaluated from temperature-jump experiments. The experimental T_m values were extrapolated to 1 M ionic strength, assuming $dT_m/d \log c_{Na^+} = 12.5°C$. The numbers of base pairs were evaluated from the reaction enthalpies. The roman numerals indicate corresponding segments in the different viroids. The experimental evidence for hairpin III in PSTV is not as strong as for the other hairpins. Hairpin I is also present in CEV and CSV but was not followed quantitatively.

[b] Because of the superposition of both transitions, only a lower limit could be evaluated.

conditions (see Fig. 3B), intermediate structures with one or in a few cases with two hairpins were visible.

The hairpins found at high temperatures are not part of the native secondary structure. This was a surprising result, but has to be concluded unequivocally from the thermodynamic and kinetic results. Also, the electron micrographs (see Fig. 3B) showing two hairpins demonstrate that the hairpins cannot be the left and right end of the native structure, because the two single-stranded regions that connect the hairpins are not of the same length.

In summary, in the main transition of viroids all base pairs of the native structure are disrupted and one to three—depending on the viroid species—stable hairpins are newly formed. At higher temperatures these hairpins dissociate. The complete denaturation scheme is depicted in Fig. 11.

The formation of stable hairpins increases the cooperativity of the main transition. In Fig. 11 a transient state (b) that is not present in measurable concentrations is also shown. If the left half, i.e., the half of lower stability (see above), is dissociated, the unpaired nucleotides can form the stable hairpins. They do so by combining with their complementary sequences, which are still involved in the native structure of the right half of the molecule. The strong tendency to form the stable hairpins (see arrows in Fig. 11) is inevitably a strong driving force for the dissociation of the right half of the molecule and thereby results in a cooperative melting of the left and right half.

The mechanism described above has been tested experimentally in PSTV, CEV, CSV, and CCCV1, but has to be expected also for TPMV and TASV and with less expressed hairpin formation for HSV and CPFV. Because hairpin I (see Table IV) is partly formed by the highly conserved central region, it is the hairpin that has been found in all viroids exept ASBV.

C. Reversibility

All structural transitions described so far are totally reversible. Starting from the completely unpaired circle, first the stable hairpins are formed and with further decrease of the temperature the hairpins dissociate in favor of the native rodlike structure. The activation enthalpy of the renaturation process as studied systematically in the temperature range of the main transition (see Fig. 5A) is highly negative, i.e., the renaturation proceeds much faster at lower temperatures. The value of about -600 kJ/mole (CEV) means that fast preequilibrium with $\Delta H = -600$ kJ/mole precedes the rate-limiting step. The structure formed in the preequilibrium is unstable at $T = T_m$. As soon, however, as the consecutive helical section is formed, the structure involved in the preequilibrium is stabilized and the renaturation proceeds further. A quantitative discussion

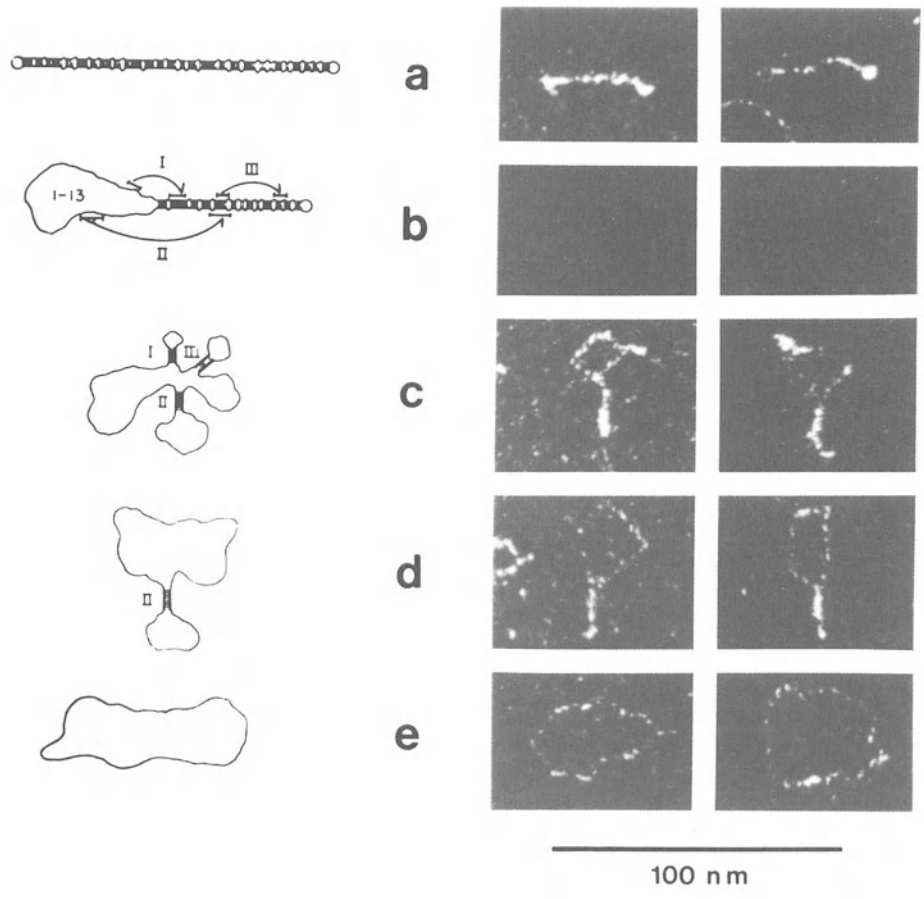


FIGURE 11. Denaturation mechanism of PSTV. The molecule undergoes the transition from (a) to (e) with increasing temperature. On the right are shown electron micrographs of PSTV in the corresponding conformations (courtesy of Dr. G. Klotz, Ulm). Because conformation (b) is a kinetic intermediate, it is not observable in electron micrographs.

(Henco *et al.*, 1979) shows that the activation barrier has been overcome after a structure close to state b in Fig. 11 has been formed. The mechanism of renaturation described so far holds only at temperatures close to the transition range.

Experimental conditions have also been described, under which the viroids assume metastable conformations. These are base-pairing schemes that are stable only below the temperature of the main transition and deviate clearly from the native secondary structure. They may be obtained after redissolving ethanol precipitates or in the presence of other organic solvents or may be trapped during a particular heating and cooling cycle. It was shown by gel electrophoresis that a redissolved ethanol precipitate exists in different conformation and may even form dimers (Riesner *et*

al., 1979). Dimers of viroids were also found, when viroids at high concentrations were heated in the presence of formamide (Colpan, 1983). All denatured conformations or dimers could be transferred into the native conformation if brought into aqueous buffer and heated close to the main transition.

The native structure is formed in the main transition in seconds or minutes depending upon the temperature. With a particular temperature-jump technique, the temperature may be lowered from a temperature above the transition to one several degrees below the transition range. This large, negative temperature jump may be carried out faster than the time needed for structure formation. In that case, several structures deviating from the native structure are formed within seconds. These metastable structures need minutes or at low temperature hours to undergo the transition to the native conformation (Riesner *et al.*, 1979).

It has also been shown (Henco *et al.*, 1979) that the native structure is established fastest, if the stable hairpins were already present. Following the process of structure formation from the completely unpaired circle to the native structure (see Fig. 11), the most effective way is to form the stable hairpins first and redissolve them in favor of the base-pairing scheme of the native structure. When the "stable hairpin" state was left out by a fast cooling process from a high temperature down to the T_m of the main transition, a slower formation of the native structure was observed.

D. Experiment and Theory

The complete mechanism of conformational transitions of viroids was deduced originally from experiments and from semiempirical calculations. The theoretical description had to be restricted to the condition of high ionic strength because the elementary parameters (see Tables II and III) were determined at 1 M NaCl. This is not a serious restriction for viroids such as PSTV that exhibit at high and at low ionic strength nearly the same cooperativity. The "cooperative helix" approximation (Langowski *et al.*, 1978; Riesner *et al.*, 1979; Steger *et al.*, 1984) or the more exact energy minimization algorithm (Steger *et al.*, 1984) were able to describe the transitions with good agreement in T_m and $\Delta T_{1/2}$. A comparison of the experimental curve with the calculated curves according to both models is presented in Fig. 12. The results from the calculations on several viroids are listed in Table V. The conformational switch from the extended native structure to the branched structure was obtained without any assumptions on the mechanisms; on the basis of the elementary parameters of Tables II and III, the extraordinary denaturation mechanism of viroids was deduced in a straightforward manner from the viroid sequence and circularity.

Although all viroids (including CCCV) studied to date have shown very similar cooperativity and stability, the theoretical description failed for CCCV. Experiments resulted in high cooperativity at 10 mM ionic

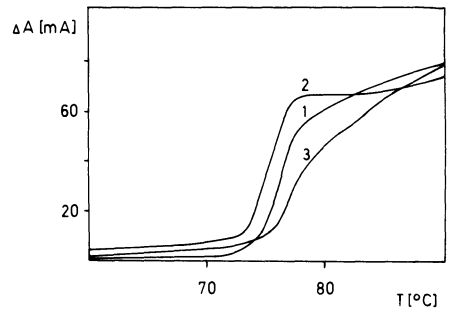


FIGURE 12. Experimental and theoretical denaturation curves of PSTV. (1) Measured in 1 M NaCl, 1 mM sodium-cacodylate, 0.1 mM EDTA, pH 6.8; (2) calculated with the model of "cooperative helices"; (3) calculated with the Zuker–Nussinov algorithm. From Steger *et al.* (1984) with permission.

strength, but theory predicted low cooperativity. This, however, was a consequence of ionic strength dependence because, when carried out at high ionic strength, experiments showed three broad transitions in accordance with the calculations (Randles *et al.*, 1982). Steger *et al.* (1984) have pointed out that the stability of small hairpins depends less upon ionic strength than that of internal helices, and therefore the stabilities of different parts of the viroid molecule may shift against each other with varying ionic strength, resulting in lower cooperativity.

V. STRUCTURE FORMATION AND FUNCTION

Although the mechanism of viroid structure formation is quite different from that known from other RNA species, it has been found in all viroids studied. From this point of view, one has to expect a direct functional relevance of the structural and dynamic features. Only a few aspects will be outlined in this section, particularly those where dynamic features may be correlated with experimental results of viroid replication and pathogenesis. It has also been shown that the physicochemical properties of the structural transitions can be utilized as a simple and sensitive aid in the diagnosis of viroid infection (Schumacher *et al.*, 1983, 1986).

TABLE V. Theoretical and Experimental Results in 1 M NaCl[a]

		T_m (°C)		Number of base of pairs N	
Viroid	Transition	Theory	Exp.	Theory	Exp.
PSTV	Main	76.3	77	67	60 ± 5
CEV	Main	74.8	77	85	88 ± 5[b]
CSV	Main	74.5	75	81	82 ± 5[b]
CCCV1-large	1	69	72	28	16
	2 (see text)	82	76	24	17
	3	86	86	21	12

[a] Theoretical values are calculated with the "cooperative helix" approximation.
[b] These numbers have been determined experimentally only in low ionic strength and are assumed to be identical in low and high ionic strength.

A. Viroids: A Dynamic Principle

As mentioned above, the thermodynamic behavior of viroids is remarkably different from that of other single-stranded RNAs. None of the latter shows a comparably high cooperativity of thermal denaturation. Double-stranded RNAs, on the other hand, combine high cooperativity with a thermal stability of an approximately 30°C higher denaturation temperature (Steger *et al.*, 1980). The characteristics of viroid denaturation—high cooperativity and low stability—could be derived from oligonucleotide parameters and the viroid sequences. The successful use of oligonucleotide parameters is certainly facilitated by the fact that viroids evidently do not contain modified nucleotides and that no tertiary structure folding interferes with the calculations of base-pairing schemes (see above).

The mechanistic details of structure formation in viroids are also unique as compared to other nucleic acids. The main transition of viroids is not a mere dissociation of base pairs, but involves the concerted opening of all base pairs and formation of 9–28 new base pairs depending on the viroid species. Similar refolding mechanisms have been discussed for other nucleic acids, for example tRNA, but could not be interpreted in terms of detailed base-pairing schemes as in the case of viroids (Hilbers *et al.*, 1976).

The similarity of all viroids with respect to their dynamic features is not a consequence of high sequence homology, because the homology between PSTV and CCCV, for example, is only 11%. On the other hand, only very specific nucleotide sequences may guarantee the dynamic features of viroids. This is best seen if viroid properties are compared with those of "viroidlike" RNA molecules, such as virusoids (see Chapter 8, this volume), and circular RNAs of similar size and GC content but random, i.e., computer-generated, sequence (Riesner *et al.*, 1979; Steger *et al.*, 1984). In Table VI their thermodynamic parameters are listed and compared to those of a typical viroid. Furthermore, calculation of the secondary structures of the two virusoids (Randles *et al.*, 1981; Haseloff and Symons, 1982; Chapter 8, this volume) with the same set of parameters as used in the base-pairing schemes of viroids (Steger *et al.*, 1984) results in degrees of base pairing of 66 and 64% for the virusoids and 59% as a mean value of five different random sequences. This shows that the degree of base pairing in some viroids, e.g., 64% in CCCV, is not much higher than in virusoids or random sequences. The absence of bifurcations, however, is a characteristic feature that is found only in viroids. A characteristic feature can also be seen in the thermodynamic stability, either expressed by $\Delta G/N$ or by the T_m values. The cooperativity represented by $\Delta T_{1/2}$ is high only in viroid transitions, and the formation of stable hairpins during the highly cooperative transition occurs only in viroids.

From the thermodynamic point of view, viroid structure may be

TABLE VI. Comparison of Viroids, Virusoids, and Random Sequences[a]

RNA	BP/N (%)	Bifurcations	ΔG/N (kJ/mole)	T_m (°C)	$\Delta T_{1/2}$ (°C)	Stable hairpin formation
PSTV	69	0	1.67	49.5	0.9	+
SNMV2	66	1	1.43	38.2	2.8	–
VTMoV2	64	1 or 2	1.32	38.1	2.0	–
Random sequence	59 ± 1	3 ± 1	1.27 ± 0.06	36	5	–

[a] Viroids are represented by PSTV; SNMV2 and VTMoV2 are the virusoid RNAs from *Solanum nodiflorum* mottle virus and from velvet tobacco mottle virus; for random sequences, number of base pairs and A, U, G, and C content are taken from PSTV. ΔG/N is the difference in free energy per nucleotide during forming the native structure from the completely coiled circle. The values for random sequences are calculated and averaged for five different sequences. T_m and $\Delta T_{1/2}$ values refer to an ionic strength of 0.011 Na^+, pH 6.8. For the sequences of SNMV2 and VTMoV2, see Chapter 8, this volume.

described as an optimal compromise between stability and flexibility. On one hand the native structure is completely intact at temperatures below the transitions, including the premelting one; on the other hand, the opening of the structure is much easier to induce in viroids than, for example, in a completely double-stranded RNA. The fact that viroid denaturation starts at particular points, the so-called "premelting regions," and that stable hairpins are formed, may be correlated with detailed biological features as shown in the next two sections.

ASBV is clearly different from the unique class of the other viroids in that its nucleotide sequence exhibits almost no homology with other viroids (Symons, 1981). Although its secondary structure and cooperativity of denaturation are similar to those of the other viroids, its T_m value is about 10°C lower and the formation of stable hairpins could not be proven experimentally (Steger *et al.*, 1984). Symons (1981) has argued that ASBV may belong to another class of viroids that have evolved separately from the others.

B. Premelting and Virulence

Different strains of PSTV have been isolated and their sequences determined (Schnölzer *et al.*, 1985). Symptoms of tomato plants infected with different strains of PSTV were classified from mild to lethal. The corresponding variation in the sequence (see Fig. 9) is restricted predominantly to the segment formed by nucleotides 42–60 and its opposite segment in the secondary structure 300–319 and to a minor part around nucleotides 115–125 (Sänger, 1984; Schnölzer *et al.*, 1985). The stretch 42–60 overlaps with the polypurine stretch 48–65. As described in Section IV.A, a region of lowest stability, so-called premelting region, is located between nucleotides 42 and 60 in the upper strand and 319 and 300 in the lower strand. The stability and the base-pairing scheme of the pre-

melting region have been estimated for all sequence variations. In Fig. 13 the virulence-modulating region (VM region) and the region of the premelting (PM) loop 1 are depicted for the different strains. Sänger and his colleagues (Schnölzer *et al.*, 1985) found that decreasing stability, i.e., easier opening, of PM loop 1 correlates with increasing virulence. Quantitatively, this was expressed by the ΔG values for the opening of PM loop 1 at 25°C; these values are also shown in Fig. 13. The authors suggested from these results that it is the virulence-modulating region in its at least partially dissociated state, PM loop 1, that interacts with host

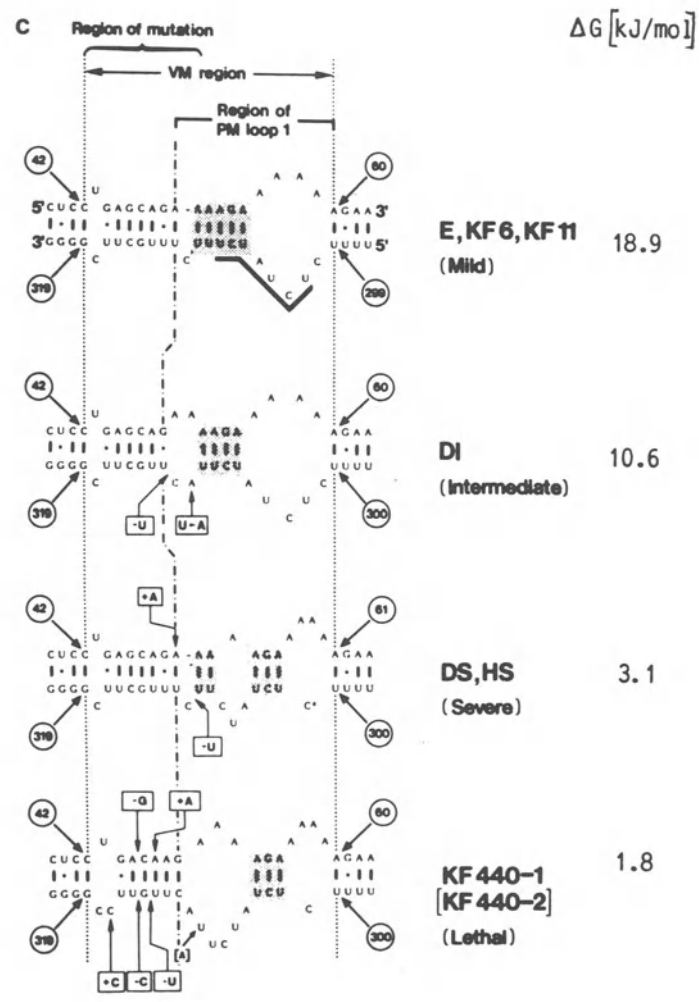


FIGURE 13. Primary and secondary structures of PSTV isolates of different virulence. Only the main region of the variation in the sequence (see text) is shown. The ΔG values of forming the open PM loop 1 are listed on the right. From Schnölzer *et al.* (1985) with permission.

factors and that this interaction leads to the expression of symptoms. The model would also explain why viroids are more virulent at higher temperature.

In CEV also, the sequence variations are located predominantly in two regions, and one region overlaps with the premelting region (Visvader and Symons, 1985). In direct contrast to PSTV, however, the stability of the premelting region was calculated and found to be considerably higher in sequence variants with severe symptoms than in those with mild symptoms. In both viroid species, the premelting region seems to be related to pathogenicity, but a consistent correlation with the process of premelting was found only in PSTV.

C. Relevance of Stable Hairpins

The formation of stable hairpins, together with their location in the highly conserved region has given rise to several hypotheses on their functional relevance. Because of a striking sequence homology between the lower part of the conserved region (254–277 in PSTV) and the 5′ end of eukaryotic snRNA U1, it has been suggested that viroids interfere with the normal splicing process (Diener, 1981; Gross *et al.*, 1982). The interaction of the viroid, instead of the snRNA U1, with the splicing site of the unprocessed mRNA would be facilitated if the opposite part in the viroid structure would switch over into the stable hairpin leaving the lower part with less internal base pairing (Riesner *et al.*, 1983). There is, however, no experimental evidence for these models. Keese and Symons (1985) discussed the switch from the native to the hairpin-containing structure quite generally as a switch between different functions. For example, the native structure could resemble protein-binding sites, possibly for replication, and the hairpin could be generated transiently during replication and serve as a favorable structure for cutting oligomeric intermediates to monomeric species.

An experimental indication for the involvement of the stable hairpin region in viroid maturation was presented by Meshi *et al.* (1985). First, they and others (see Chapter 6, this volume) have shown that plasmids carrying two cDNA units of viroids tandemly repeated are highly infectious, whereas plasmids carrying only a monomeric viroid cDNA unit are not infectious. Meshi *et al.* (1985) found that the minimal entity that had to be present twice was the region of the stable hairpin I. They argued that after the viroid precursor-RNA was transcribed from the plasmid DNA, the region of the stable hairpin is relevant to cut the viroid precursor in that region to the exact monomeric length, probably by cellular endonucleases. These results are in accordance with the finding of Tabler and Sänger (1984) that M13-cloned single-stranded PSTV DNA is infectious if an 11-nucleotide-long sequence from the conserved region is present twice.

VI. ADDENDUM

Between submitting and printing of this chapter, significant progress was made in understanding the relevance of stable hairpin formation (see Table IV and Fig. 11). Transcripts of (+) and (−) polarity, from unit up to sixfold length, were synthesized from DNA clones of PSTV with the SP6 transcription system (Tabler and Sänger, 1985). The oligomeric transcripts served as models for natural replicative intermediates. It could be concluded from a series of physicochemical experiments and from stability calculations (Steger *et al.*, 1986; Riesner *et al.*, 1987) that the secondary structure of lowest free energy for unit-length and oligomeric transcripts is a rodlike structure similar to that of the mature circular viroids. At higher temperatures, however, a change of the rodlike secondary structure of (+)-strand transcripts has been observed; the central conserved region, which is present in all viroids and is involved in the formation of hairpin I in mature viroids, forms together with the same region of another viroid unit of the oligomer a helical region of three stable helices. This rearrangement leads to a branched structure of oligomeric replicative intermediates. After the branched structure has been formed once, it remains in a state of metastability also under physiological conditions. The metastable branched structure of a trimeric PSTV intermediate together with the sequence of the rearranged segments and their position in the mature viroid is shown in Fig. 14. A corresponding metastable structure of (−)-strand intermediate transcripts is of much lower stability, indicating a clear difference between (+)- and (−)-strand intermediates.

The rearrangement of the extended structure into a branched structure was found originally with mature circular viroids (see Section IV.B) only at elevated temperature. In contrast to mature viroids, in replicative intermediates the branched structure described above is metastable under physiological conditions and may have direct functional relevance for the replication mechanism. It has been suggested (Diener, 1986) that the region containing the three stable helices as shown in Fig. 14 is the site at which detachment of monomeric viroids from oligomers occurs. The hypothesis was put forward first solely on the basis of infectivity studies. For instance, in the shortest longer-than-unit-length segment of HSV that was found still infectious, the complete region of the three helices may be formed (Meshi *et al.*, 1985). In the case of PSTV, although only 11 nucleotides were present twice, the branched structure was still thermodynamically more favorable than the extended structure. If, however, only 5 complementary nucleotides of the conserved region are doubled, the extended arrangement becomes more favorable in relation to a complete loss of infectivity (Tabler and Sänger, 1984). In accordance with the infectivity data, the physicochemical experiments have demonstrated the stability of the corresponding structure under physiological conditions

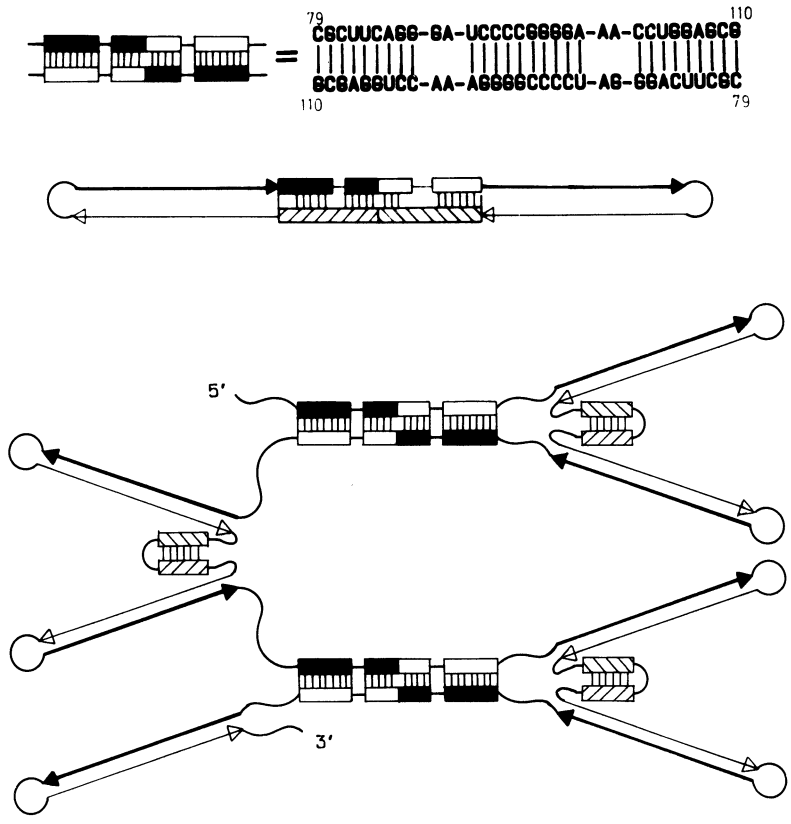


FIGURE 14. Structure of a trimeric PSTV replicative intermediate. For comparison the sequence (upper graph) and the position in the mature circular viroid (middle graph) of the segment, which forms the three stable hairpins in the intermediate, are depicted.

(Steger *et al.*, 1986; Riesner *et al.*, 1987). As may be seen from the model in Fig. 14, an appropriate cut in the region of the three stable helices releases a unit viroid molecule. Ligation of both ends of the unit viroid results in the circular mature viroid; ligation of the open ends left at the oligomer (due to the detachment of the monomer) leaves an oligomer containing one unit less than the original molecule. Obviously, the whole process may repeat, producing further circular unit viroid molecules.

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CHAPTER 4

VIROID FUNCTION
Subcellular Location and *in Situ* Association with Cellular Components

DETLEV RIESNER

I. INTRODUCTION

Viroids differ in two essential structural features from viruses. They are much smaller, and they are not encapsidated. Because of their small size, they possibly do not underlie all topical barriers inside the cell. Because of their infectivity as naked RNA one may speculate that also inside the cell they are present as dissolved molecules and not part of tight complexes. Such a simplified view would actually be in accordance with the very early experiments that led to the concept of viroids as a new class of infectious agents (see Diener, 1979). In those experiments, it was shown that viroid infectivity in a tissue homogenate was unprotected against RNase digestion, and that viruslike particles were not visible in infected tissue. Other early experiments indicated, however, that viroids are not ubiquitous but rather located in organelles, and even molecular viroid complexes have been suspected (Diener, 1971; Sänger, 1972).

From a mechanistic point of view, one has to assume specific complexes of viroids with components of the host cell. Those complexes are required for protection against degradation, for replication, for the expression of symptoms, and possibly also for the spreading of the systemic infection.

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In this chapter mainly three topics will be discussed: (1) where viroids are located, (2) how many viroids are present at a defined site, and (3) what is known about the nature of viroid complexes. The starting point for the quantitative consideration is the finding that as an average, 10,000 viroid molecules per cell are present in highly infected tissue. This number was determined in leaves from PSTV-infected tomato plants (Schumacher *et al.*, 1983a).

II. SUBCELLULAR LOCATION

In order to identify the subcellular location of viroids, the components of the cell have been fractionated and analyzed for their viroid content. This type of work has been carried out by several investigators; the studies differ in the purity obtained with subcellular fractions and in the sensitivity and method for viroid detection.

A. Nuclei

In early investigations carried out on PSTV-infected tomato plants (Diener, 1971) and CEV-infected citrus plants (Sänger, 1972, Semancik *et al.*, 1976), mainly a series of differential centrifugations was applied for purifying nuclei; the analysis of viroid content was based on infectivity tests. Viroids were found in the nucleus-rich fractions. An association with membranes was also reported (Semancik *et al.*, 1976). Because the purification procedures could not be sufficiently stringent at that time and the analysis of viroids was not quantitative, no quantitative conclusion about relative distribution and copy numbers of viroids could be drawn. Takahashi *et al.* (1981) studied the subcellular location of HSV in leaves from infected hop, cucumber, and tomato and found the viroid associated with the nucleus fraction.

An essential progress in the purification method of nuclei was made when Percoll density gradients were introduced as the final step. Schumacher *et al.* (1983a) obtained preparations of highly purified, intact nuclei from tomato leaf tissue. The purity may be seen from micrographs in Fig. 1A and B, where by comparing phase-contrast and fluorescence graphs it could be proven that all particles show the brilliant yellow-red fluorescence typical for nuclei. The higher magnification of Fig. 1C shows that the nuclei are intact and that in most of them nucleoli are visible.

Isolated nuclei were analyzed for the presence of viroids by applying the method of bidirectional gel electrophoresis. This technique is a combination of electrophoretic runs under native and denaturing conditions by which the particular thermodynamic and hydrodynamic features of the structural transitions of viroids are utilized in order to separate viroids

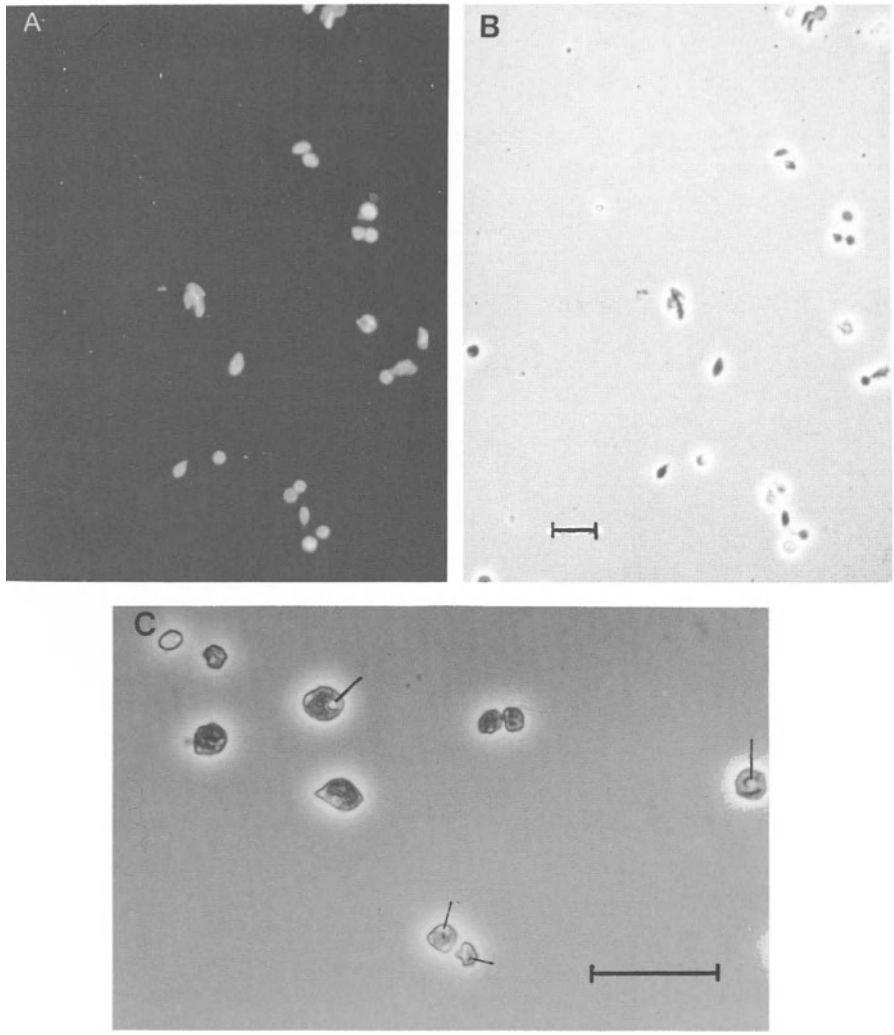

FIGURE 1. Micrographs of ethidium-stained nuclei, isolated from PSTV-infected tomato leaf tissue under fluorescence (A) and under phase contrast (B). Identical areas are shown in both micrographs indicating the purity of the sample. At higher magnification (C), nucleoli (arrows) are clearly visible. Bars = 20 μm. From Schumacher *et al.* (1983a) with permission.

from all other components of the crude RNA extract. The details are described elsewhere (see Chapter 3 and Schumacher *et al.*, 1983b).

From a gel analysis as in Fig. 2, the presence of viroids in isolated nuclei was clearly demonstrated. Furthermore, the copy number could be estimated. Depending upon the progress of the disease, up to 10,000

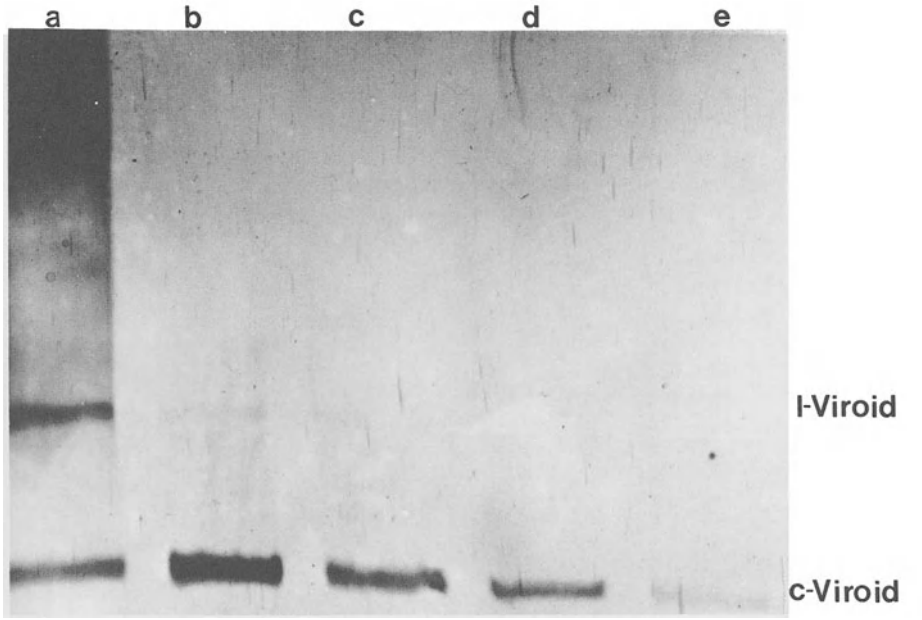

FIGURE 2. Estimation of viroid concentration, in isolated nuclei by bidirectional gel electrophoresis. The silver-stained bands of the circular (c-PSTV) and linear (l-PSTV) viroid RNA obtained from 2 × 10^8 nuclei are visible in lane a. The viroid concentration was estimated by comparison of the intensity of the bands with purified viroid preparations in lane b (60 ng PSTV), lane c (24 ng PSTV), lane d (9.6 ng PSTV), and lane e (3.8 ng PSTV). From this experiment the presence of 2000 viroid molecules per nucleus was calculated. From Schumacher *et al.* (1983a) with permission.

PSTV molecules per nucleus were observed. The sensitivity of the test allows the detection of infection titers as low as 10 copies per nucleus (Schumacher, 1984; Schumacher *et al.*, 1986). Because the copy numbers determined are average values, and an equal distribution of viroids over all cells cannot safely be assumed, it may be possible that in some cells even more than 10,000 viroids are present.

From the fact that the copy number per nucleus is the same as the copy number per cell, one may conclude that all viroids are located inside the nucleus. This conclusion, however, is only true within the limit of the error of the determination of the copy numbers. After repeated experiments, the error limit is around 20%. Therefore, from the experimental results one may only state that the predominant part of the viroids is located in the nuclei.

The subcellular location was also concluded from studies on viroid synthesis in nucleus-rich or highly purified nucleus fractions. Viroid synthesis was found in nuclei from PSTV-infected tomato tissue (Takahashi and Diener, 1975; Spiesmacher *et al.*, 1985) and from CEV-infected citrus tissue (Flores and Semancik, 1982; Semancik and Harper, 1984).

B. Other Organelles

Other components of the host cell have been tested for their content of viroids as well. The results are unequivocal as long as highly purified preparations of intact organelles could be studied. This was the case with chloroplasts from PSTV-infected tomato tissue. The absence of viroids was evident (Schumacher *et al.*, 1983a).

Results are much more difficult to interpret if nonhomogeneous fractions have to be analyzed. One has to take into consideration that during the procedure for preparing highly purified nuclei, 98–99% of all nuclei break. Consequently, their content is discharged into other fractions. If the test for viroids is not sufficiently sensitive, one may find the viroids only in fractions containing the majority of broken nuclei and not in the fraction with the few percent nuclei remaining intact. For these reasons the preliminary reports of Randles *et al.* (1976) about the cytoplasmic location of CCCV and of Mohamed and Thomas (1980) about the association of ASBV with partly purified fractions of chloroplasts and endoplasmic reticulum have to be regarded with skepticism.

III. SUBNUCLEAR LOCATION

Inside the nucleus, viroids may be present as dissolved molecules in the nucleoplasm or they may be associated with one of the nuclear macromolecular aggregates. These are the chromatin, the nucleolus, the ribonucleoprotein particles, or the inner side of the nuclear membrane. In order to differentiate between these possibilities, the subnuclear components had to be fractionated and analyzed for their viroid content.

A. Chromosomal Network

If nuclei are lysed by an osmotic shock, the chromatin of many nuclei forms a macroscopic network. Nearly all viroids present cosediment with this chromatin network (Schumacher *et al.*, 1983a). It cannot, however, be concluded that viroids are associated with the chromatin, because viroids may be caught in the meshes of the network in a completely unspecific manner.

B. Nucleoli

The components of the nucleus may be separated if the nuclei are homogenized by sonication. According to reports on studies with animal cells, during the sonication the chromatin is fractured to smaller pieces whereas the nucleoli are left intact (Pederson, 1974). The details of the

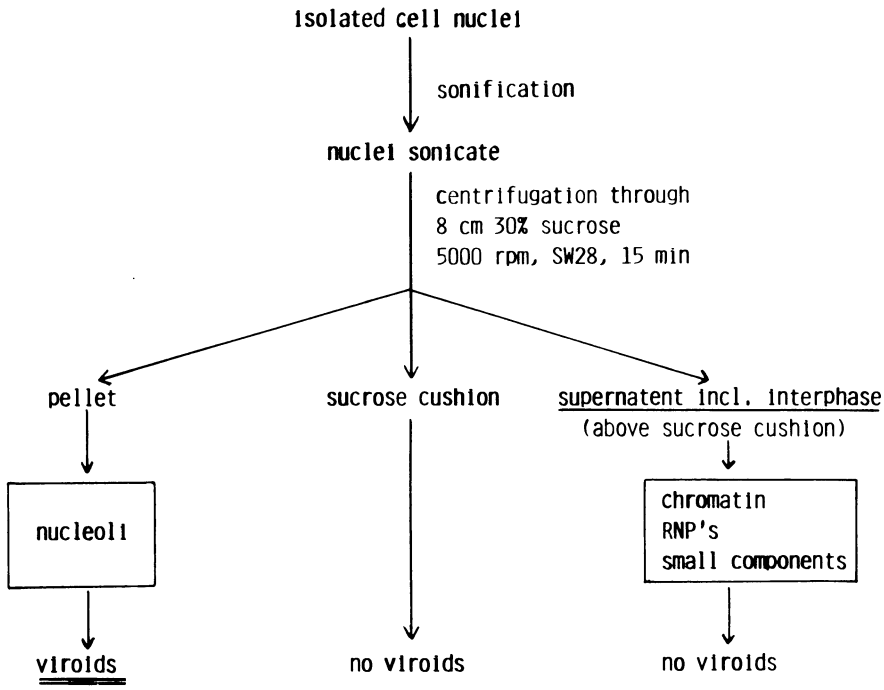

FIGURE 3. Fractionation of nuclear components from PSTV-infected leaf tissue. Viroids were found in the nucleolus-containing pellet.

fractionation may be seen from the scheme in Fig. 3. The pellet of the final sucrose gradient centrifugation contains highly purified nucleoli, as analyzed by several techniques (Pederson, 1974; Schumacher *et al.*, 1983a). The gel electrophoresis in Fig. 4 shows that the vast majority of viroids were found with the nucleoli, whereas only traces could be detected in the supernatant, which contains the fractured chromatin, ribonucleoprotein particles, and low-molecular-weight nuclear components. The cushion of the gradient was free of viroid. When the copy number of viroids was determined in the pellet fraction, again several thousand viroid molecules per nucleolus were observed as previously found for intact cells and nuclei. This demonstrated that no significant amount of viroid was lost during the fractionation and assay procedure and that the bulk of viroid RNA is associated with the nucleoli (Schumacher *et al.*, 1983a).

C. Nucleolar Nucleosomes

The nucleolus is the site of ribosomal RNA synthesis and processing and of ribosome assembly. Not only are the ribosomal RNAs, proteins, and small nuclear RNAs, e.g., U3 RNA, organized in the nucleolus but

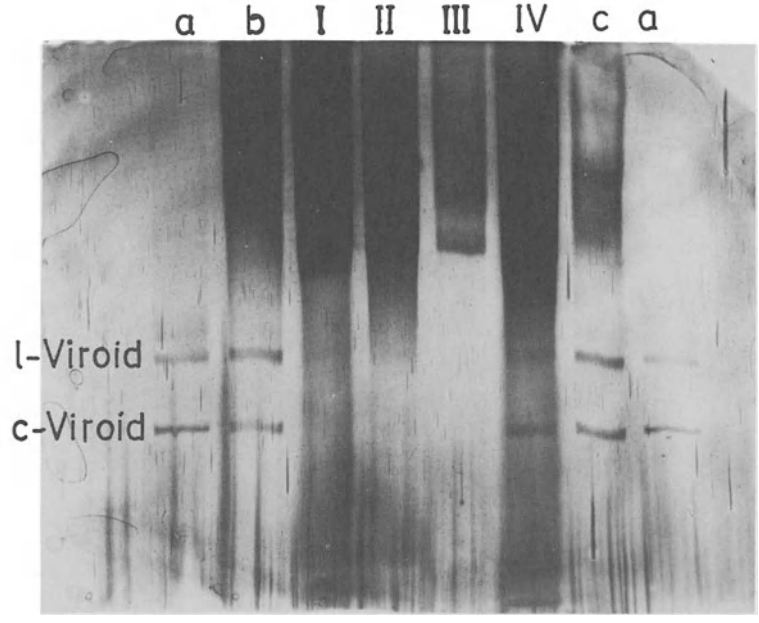

FIGURE 4. Analysis of the distribution of viroid RNA in subnuclear fractions. As in Fig. 2, the second direction of a bidirectional gel electrophoresis is shown. Lane a: 16 ng purified linear and circular forms of viroid; lanes b and c: nucleic acids extracted from nuclei; lanes I–IV refer to fractionation scheme in Fig. 3: subnuclear top fraction (I), upper two-thirds of the sucrose cushion (II), lower third of the sucrose cushion (III), nucleolar pellet fraction (IV). From the comparison of the viroid bands in lane IV and the very faint bands in lanes I and II, it is evident that >95% of the viroid RNA is associated with the nucleolar pellet fraction. From Schumacher *et al.* (1983a) with permission.

also the part of the chromatin that contains the ribosomal DNA. The DNA part forms together with histones a nucleosomal structure (Leweke and Hemleben, 1982). It was argued that viroids may be associated with the nucleosomes of the nucleolus because of their known affinity to histones (see next section). The nucleosomal part may be split out from the nucleolus by DNase I treatment and sonication and may be separated from the high-molecular-weight RNA-containing fraction by centrifugation. The procedure is listed in Fig. 5. The supernatant contains the chromatin degraded to smaller pieces, which are about the size of nucleosomes. Viroids were detected in this chromatin-containing fraction. As a control experiment, the same procedure was applied to the chromatin fraction of the nucleoplasm after removing the nucleoli. No viroids were detected in this preparation, as had to be expected because of the location of viroids in nucleoli.

The viroid-containing complexes from the nucleosomal fraction of the nucleoli were further characterized by their sedimentation behavior in a sucrose gradient centrifugation. Figure 6 shows the gel electrophoretic

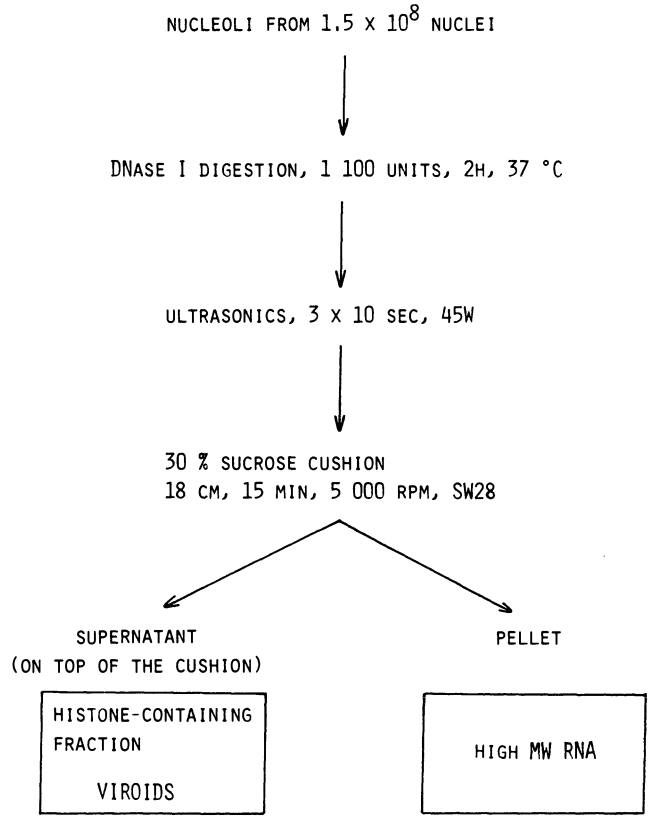

FIGURE 5. Fractionation scheme of the nucleolar components from PSTV-infected leaf tissue. Viroids were found in the chromatin-containing supernatant.

analysis of the nine top fractions. The three fractions (3, 4, 5) containing the viroid band correspond to $s_{20,w}$ values of 7.5 S, 12 S, and 15 S. Consequently, viroids are present in the chromatin-containing fraction from the nucleolus, not in an uncomplexed state, but associated with the nucleosomes. The average s value of 12 S indicates a complex with monomeric or possibly dimeric nucleosomes. At present, the most precise location of viroids is that part of the nucleoli in which histones and ribosomal DNA form nucleosomal structures.

Sites of viroids and corresponding copy numbers are listed in Table I.

IV. NATURE OF VIROID COMPLEXES

In situ associations of viroids may be formed via viroid–nucleic acid or viroid–protein interactions. Other interactions as with membranes or polysaccharides do not have to be taken into account according to our present knowledge.

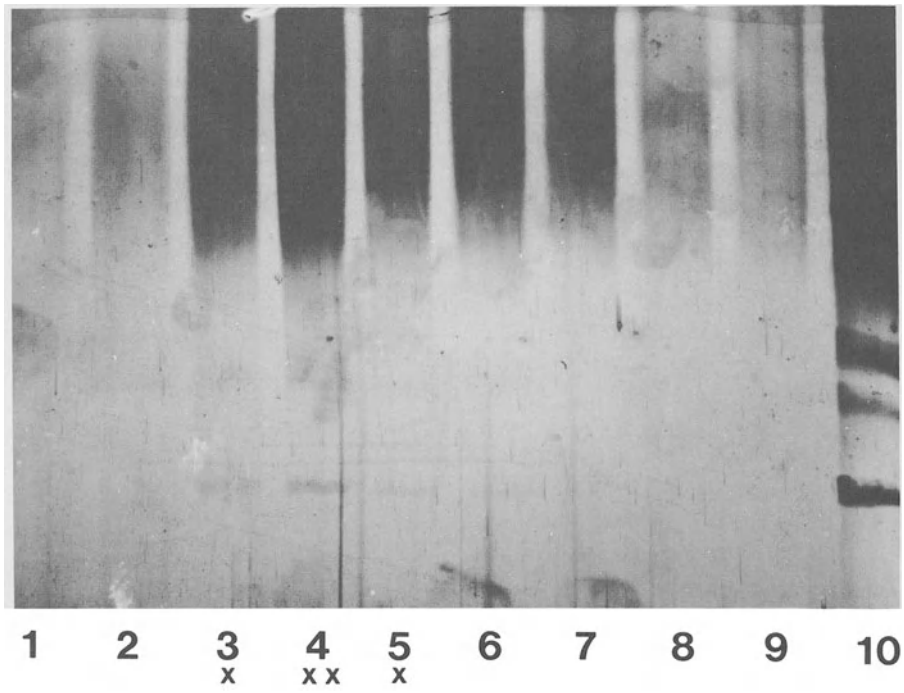

FIGURE 6. Viroid detection in a sucrose gradient of the nucleosomal fraction from nucleoli. The nine slowest fractions (starting from the top) were tested for the presence of viroids by bidirectional gel electrophoresis. Lanes 1–9: fractions 1–9; lane 10: PSTV-containing crude extract as reference. The viroid bands in lanes 3–5 are weak but clearly visible; the crosses indicate their relative intensities in the original gel. From Wolff *et al.* (1985) with permission.

A. Ionic Strength Dependence

It is known from the literature that increasing ionic strength dissociates most protein–nucleic acid complexes (Zieve and Penman, 1981; Record *et al.*, 1978), but always raises the strength of nucleic acid–nucleic acid complexes (Record *et al.*, 1978). To differentiate between both possibilities, the viroid-containing nucleoli have been incubated in a series of buffers with increasing ionic strength and tested for the release of viroids (Schumacher *et al.*, 1983a; Schumacher, 1984). Nucleoli and re-

TABLE I. Distribution of Viroids (PSTV) in Leaf Cells from Tomato Plants

Intact cells	10–10,000 viroids (depending upon the progress of the infection)
Nuclei	10–10,000 viroids
Chloroplasts	1–2 viroids (?)
Lysed nuclei	All viroids cosediment with the chromosomal network
Nucleoli	90% of all viroids from the nucleus

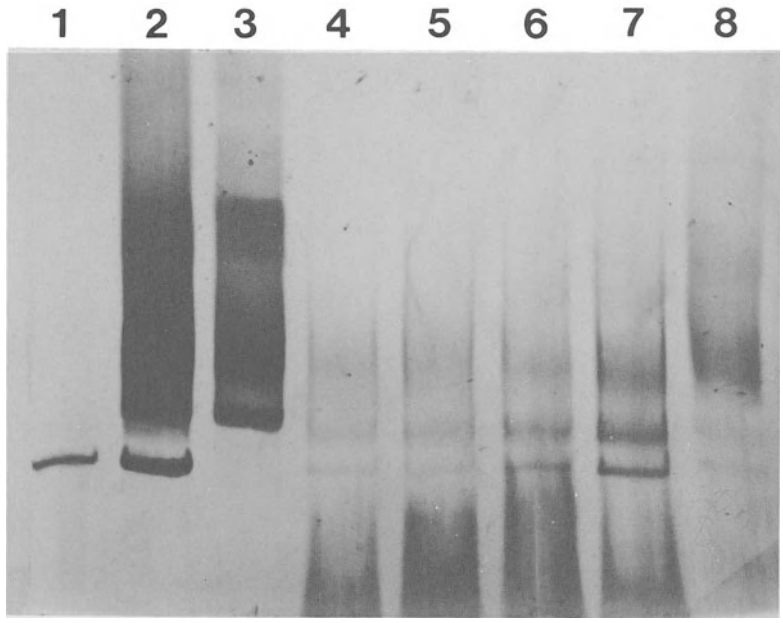

FIGURE 7. Ionic strength dependence of the release of viroids from nucleoli. Nucleoli from PSTV-infected leaf tissue were incubated with 50 mM (8), 100 mM (4), 200 mM (5), 300 mM (6), and 400 mM (7) NaCl and centrifuged at 45,000 rpm for 1 hr. The supernatant was tested for the content of viroids by bidirectional gel electrophoresis. For comparison, lane 1 shows 30 ng purified PSTV, lane 2 crude RNA extract from PSTV-infected tissue, and lane 3 crude RNA extract from noninfected tissue. From Schumacher (1984).

leased viroids were separated by a centrifugation step. As seen from the gel analysis in Fig. 7, a substantial increase in the release of viroids occurs between 300 and 400 mM ionic strength. This result clearly favors the presence of viroid–protein complexes. Indeed, with specific viroid–protein complexes (see next section), the same ionic strength dependence was observed.

B. Viroid-Binding Proteins

A search for viroid-binding proteins has been carried out by an *in vitro* reconstitution experiment (Wolff *et al.*, 1985). The proteins from a nuclear extract were separated on an SDS-polyacrylamide gel, renatured, blotted to nitrocellulose filters, and tested for viroid binding. A particular technique to visualize the nonradioactive viroids—bound to a protein—was developed. It is described in Fig. 8. The viroids were incubated with the proteins fixed on the filter; afterward they were cross-linked covalently with the corresponding proteins and hybridized against a radio-

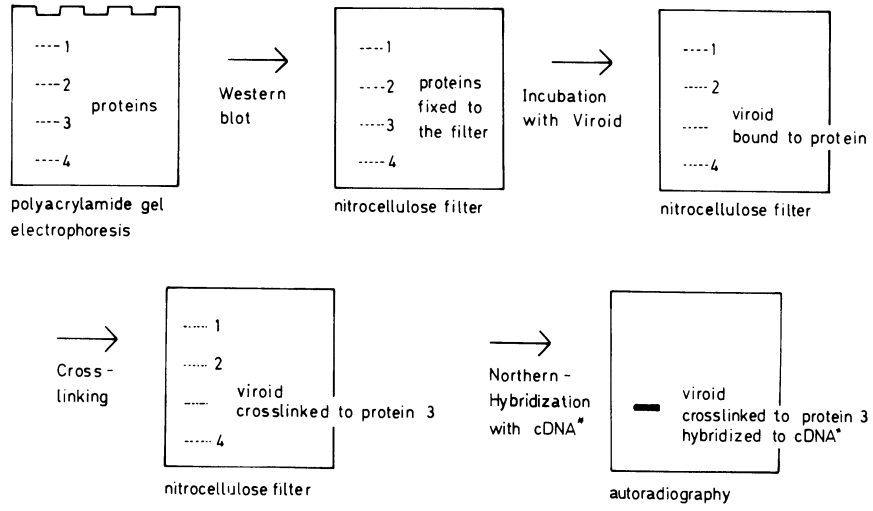

FIGURE 8. Procedure to detect *in vitro* reconstituted PSTV-protein complexes. Step 1: separation of the proteins by SDS gel electrophoresis; step 2: transfer of the proteins to nitrocellulose filters (Western blot) after renaturation; step 3: incubation with viroid RNA to establish binding; step 4: fixation of the complexes by cross-linking with glutaraldehyde; step 5: detection of the viroid RNA by molecular hybridization using ^{32}P-end-labeled cDNA (Northern hybridization). From Wolff *et al.* (1985) with permission.

active cDNA probe specific for the viroid under investigation. In an autoradiograph the positions of the proteins that have bound viroids are visible. The control experiments are described in the original literature. In Fig. 9 such an autoradiograph is shown. It reveals the well-know pattern of four histones, and after longer exposure a 41,000-dalton protein and a very weak band of a 31,000-dalton protein appear.

At first glance, the viroid–histone complexes may be regarded as unspecific associations,which reflect merely the basic character of the histones. Viroids are complexed, however, also *in vivo* with the histone-containing fraction of the nucleolus as outlined above. Under this aspect both types of experiments fit very well together. The binding of viroids to histones is also in accordance with the idea of the DNA similarity of viroids. Such similarity was argued first on the basis of the structural and thermodynamic properties of viroids (Riesner *et al.*, 1979).

The complex of viroids with the 41,000-dalton protein has been established so far only in *in vitro* experiments. Therefore, a physiological significance of the complex cannot be inferred at present. It is, however, remarkable and most probably not accidental that among the many nuclear proteins besides the histones, only the 41,000-dalton protein shows a high affinity to viroids. The ionic strength dependence of viroid–protein complexes, studied in *in situ* nucleolar associations or in reconstitution complexes, is very similar (Wolff *et al.*, 1985).

Proteins that consist of several subunits and require the intact qua-

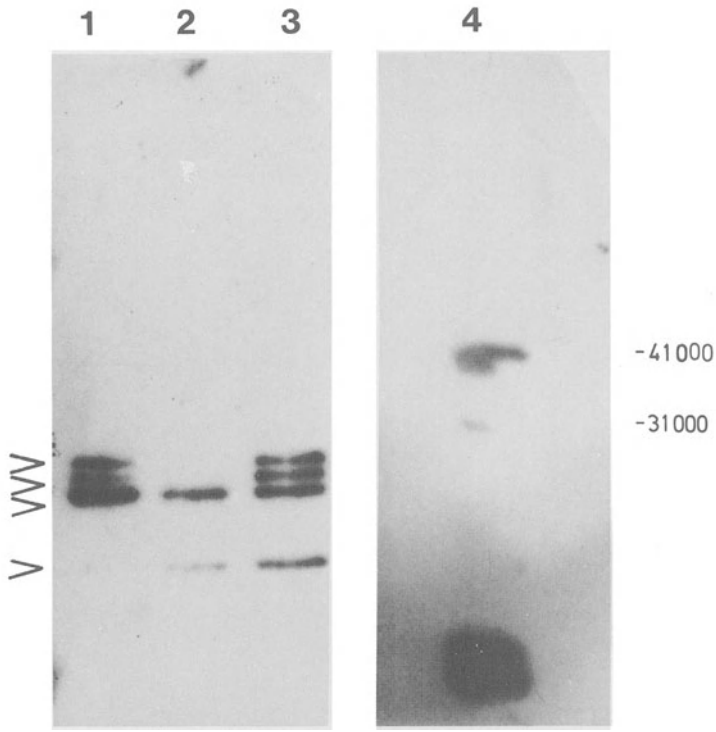

FIGURE 9. *In vitro* viroid–protein complexes. An autoradiograph obtained by the procedure of Fig. 8 is shown. Lane 1: proteins from nuclei; lane 2: proteins from chromatin; lane 3: proteins from nucleoli; lane 4: proteins from nuclei after long exposure in the autoradiograph. The gel runs in lanes 1–3 and in lane 4 are of different length. The arrows point to the main histone bands; the other viroid-binding proteins are indicated by the molecular weight values. Data from Wolff *et al.* (1985).

ternary structure for viroid binding could not be found in reconstitution studies because they dissociate during SDS gel electrophoresis and cannot be renatured. It was found by incorporation studies that viroids are accepted as a template by DNA-dependent RNA polymerase II (see Chapter 5, this volume). Complexes of viroids with the polymerase II from wheat germ have been studied by other physical techniques, and it was shown that viroids bind stronger than all other RNAs tested to polymerase II (Goodman *et al.*, 1984). It supports the concept that viroids resemble some DNA-like features.

It should be mentioned that viroids were also tested for their interaction with the disease-related protein P14. The expression of this protein is highly stimulated by viroid infection (Camacho Henriquez and Sänger, 1982). Despite an intensive search, a direct interaction between P14 and viroids could not be detected (P. Wörgötter, A. Camacho Henriquez, and D. Riesner, unpublished)

V. RECONSTITUTION OF VIROID–HOST INTERACTIONS

Since the interaction of viroids with isolated cellular proteins could be reconstituted *in vitro*, it was investigated whether the interactions with larger components of the cells and even with complete organelles may also be restored by adding viroids to the corresponding components of the noninfected cell.

A. Nuclei

Viroids were incubated with nuclei isolated from noninfected tissue (Schumacher, 1984). After varying time intervals, the nuclei were separated from the incubation mixture by centrifugation, washed thoroughly, and tested for their viroid content by gel electrophoresis. The process of viroid uptake is shown in Fig. 10. Viroids enter the nucleus with a characteristic time of 10 to 20 min. The final concentration was about 5000 viroids per nucleus. This number is close to the *in vivo* copy number of 10,000 in highly infected tissue. In concentration terms, it means that from a concentration of 10^{-9} M in the incubation mixture, the viroids enter the nucleus up to an intranuclear concentration of 10^{-7} M. They are more or less sucked into the nucleus.

Viroids that enter the nucleus could also be followed by physical methods (Kapahnke, 1984). The dye homoethidium-dimer binds strongly to primary and secondary binding sites of viroids whereby the dye in the primary sites shows an enhanced and the dye in the secondary sites a quenched fluorescence. If the viroids with dyes in both sites enter the nucleus, the dye molecules bound in the secondary sites are transferred to primary sites of the chromosomal DNA. Consequently, the entrance of viroids may be recorded by an increase in the fluorescence of the dye. Nearly the same time constant as in the experiment described before was observed. Furthermore, the process is not specific to viroids, because pieces of double-stranded DNA of similar size enter the nucleus comparably fast. In order to exclude the possibility that the diffusion of free dye molecules, i.e., dissociated from the nucleic acid, into the nucleus is recorded, the transfer of a dye from a very large DNA, which cannot enter the nucleus, to the chromosomal DNA was recorded; in this control experiment a much slower increase in fluorescence was observed.

B. Nucleoli

We also tried to reconstitute viroids into their *in vivo* site of association, i.e., in the nucleolus (Wolff, unpublished study). Similarly to the experiments described above, viroids were incubated with nuclei, but

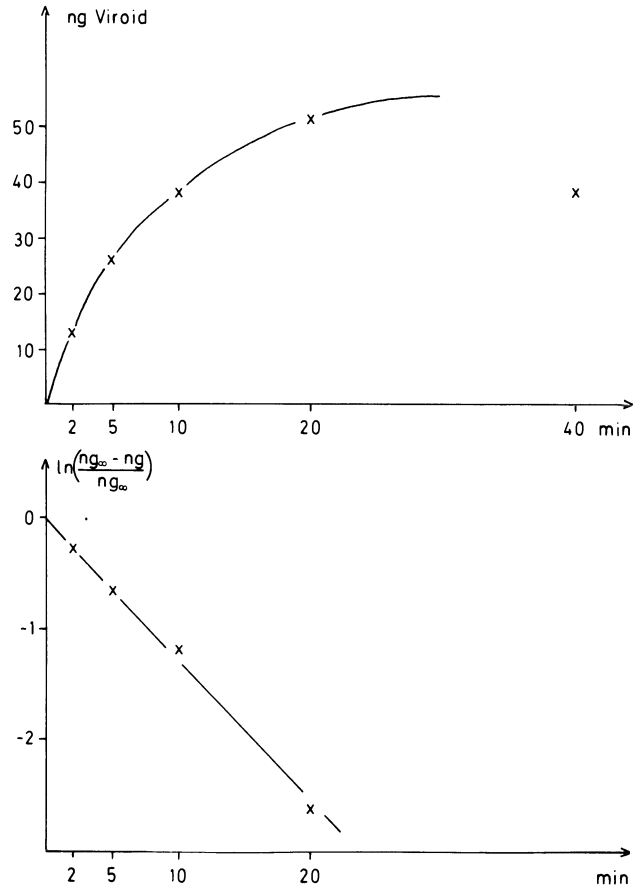

FIGURE 10. Time-dependent uptake of viroids into nuclei from noninfected leaf tissue. 300 ng PSTV (10^{-9} M) was incubated with 6×10^7 nuclei in 1 ml buffer at 4°C. The nuclei were centrifuged, resuspended for washing, centrifuged again, and tested for their viroid content by bidirectional gel electrophoresis. Data from Schumacher (1984).

afterwards the nucleoli were prepared and tested for the presence of viroids. The experiments have been repeated several times, but viroids could never be found in the nucleoli. Therefore, one has to conclude that viroids, although they enter the nucleus, do not proceed into the nucleolus. This result will be discussed further in relation to results from replication studies (see section VII).

VI. LOCATION OF VIROID INTERMEDIATES

If the analysis of viroids is carried out by bidirectional gel electrophoresis, the circular form of viroids is always detected. Because viroids are circular only in their mature form, all results about location of viroids

in nucleoli and subnucleolar fractions refer to mature viroids and do not imply *a priori* an identical location of replicative intermediates of viroids. In order to localize intermediate forms of viroids, the analysis has to be carried out with radioactive hybridization probes. Mühlbach and Sänger and their co-workers (Spiesmacher *et al.*, 1983, 1985) have carried out these studies with nuclei isolated from PSTV-infected potato cells. Their hybridization probes were specific for the (+) strand or for the (−) strand of PSTV. Several species of longer-than-unit-length (−) PSTV as well as oligomeric forms of (+) PSTV were detected in the nuclei. According to current models of replication (see Chapter 5, this volume), these forms are replicative intermediates. The results demonstrate that besides the mature form of viroids, the intermediates of replication are also present in the nucleus. So far, the studies on replicative intermediates could not differentiate between different subnuclear locations.

VII. LOCATION AND FUNCTION

From the fact that viroids are located at specific sites, it may be expected that these sites are relevant for viroid function. This subject will be discussed briefly under the aspects of replication, pathogenesis, and origin of viroids.

The discussion of viroid location with respect to their replication suffers from the present situation, in that the replicative intermediate forms of viroids have not yet been attributed to subnuclear sites. According to current models, the invading viroid is transcribed into multimeric (−) strands by DNA-dependent RNA polymerase II. This enzyme is located in the nucleoplasm. It agrees with the finding that viroids, when incubated with nuclei, do enter the nucleoplasm but not the nucleolus. The viroids found in the nucleolus have to be regarded as products of intranuclear replication. According to a recent report, the (−) strand is transcribed into the oligomeric (+) strand by DNA-dependent RNA polymerase I (Spiesmacher *et al.*, 1985). It is known that this enzyme normally transcribes ribosomal RNA from the ribosomal DNA genes inside the nucleolus. Thus, the studies on the replication and on the location of viroids fit very well together in that they show independently that the sites of viroid (+)-strand synthesis and of storage of the mature viroids are identical. One should assume that splitting and ligation of oligomeric viroids to mature viroid circles occur also in the nucleolus. Furthermore, one has to assume that the transport of viroid information from the nucleoplasm to the nucleolus occurs at the stage of the oligomeric (−) strands.

Because the bulk part of viroids is stored in the nucleolus, one is tempted to assume that the nucleolus is the site of the primary pathogenic action. It is, however, not known whether any of the proteins that were described in this chapter as interacting with viroids are involved in path-

ogenesis. Origin and pathogenesis of viroids have been discussed in the literature as related topics, because viroids may have originated as "escaped introns" and may presently be pathogenic because they interfere with the normal splicing process (Diener, 1981; Gross *et al.*, 1982; Riesner *et al.*, 1983). This hypothesis of pathogenic action was based on the sequence homology between the conserved region of viroids and the 5′-terminal sequence of snRNA U1, which is according to current models assumed to be involved in the splicing process. Because of the location of viroids in nucleoli but of snRNA U1 (together with its involvement in splicing) in ribonucleoprotein particles, this model of the pathogenic action of viroids cannot be supported further. From more recent systematic searches for homologies, the possibility of viroids having originated from rDNA genes was derived (Diener and Hadidi, 1985). Sequence homologies between viroids and snRNA U3, which is actually located in the nucleolus, have also been found (Schumacher *et al.*, 1983a; Kiss *et al.*, 1983). Involvement of RNA U3 in the splicing of precursor ribosomal RNA was inferred only from theoretical studies (Crouch *et al.*, 1983; Bachelerie *et al.*, 1983); experimental evidence is not available. One has to summarize that some indications point to a connection of viroid origin and pathogenesis and the splicing and processing of ribosomal RNA. Although this would fit nicely with the location of viroids in the nucleolus, it has to be regarded as a rough working hypothesis for future studies.

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CHAPTER 5

VIROID FUNCTION
Viroid Replication

HEINZ L. SÄNGER

I. INTRODUCTION

In contrast to the rapid progress that has been made in establishing the molecular structure and properties of viroids, the elucidation of their intriguing mechanism of replication and pathogenesis has been rather slow and frustrating. This is mainly due to system-inherent experimental limitations in higher plants, which are less well suited to study the dynamic aspects of protein and nucleic acid metabolism at the cellular level than bacterial and animal cell systems. However, a shift of emphasis toward investigating the cell biological and functional aspects of viroids, in particular their mode of replication, has recently become apparent.

All these studies have been greatly facilitated and promoted by the development and use of appropriate plant cell systems and by the application of various new techniques and sensitive molecular probes for analysis. Thus, denaturation of the nucleic acids with glyoxal and dimethyl sulfoxide allowed their gel electrophoretic separation in a truly denatured condition. In combination with the subsequent "Northern blotting" of the electrophoretically separated nucleic acids to hybridization membranes (Thomas, 1980), in particular by electrotransfer, this method became a powerful analytical tool for the characterization of viroid-specific nucleic acids. Molecular hybridization became more specific and reliable when the previously used ^{125}I-labeled viroid probes were replaced by viroid-complementary single-stranded cDNA as produced by reverse transcription of viroid RNA *in vitro* (Palukaitis and Symons, 1978; Rohde and Sänger, 1981; Rohde *et al.*, 1981a,b) and by chemically synthesized strand-specific DNA primer molecules (Spiesmacher *et al.*, 1983).

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The most important improvements were achieved after recombinant DNA technology had been introduced to viroid research. With the successful molecular cloning of PSTV cDNA (Owens and Cress, 1980; Cress and Owens, 1981; van Wezenbeek *et al.*, 1982), viroid-specific double-stranded cDNA became available. This allowed its subcloning in bacteriophage M13 from which the strand-specific single-stranded viroid cDNAs could be produced, which are required for the detection and reliable discrimination of viroid RNA forms of (+) and (−) polarity (Owens and Diener, 1982; Bruening *et al.*, 1982; Cress *et al.*, 1983; Tabler and Sänger, 1984; Hutchins *et al.*, 1985). More recently, viroid (+) and (−) RNA synthesized *in vitro* in microgram quantities and with extremely high specific radioactivity with the aid of the SP6-RNA polymerase transcription system and cloned viroid DNA as template (Tabler and Sänger, 1985) have become available for molecular hybridization. This will further improve the sensitivity and reliability of the detection of the intermediate forms of viroid replication, because RNA–RNA hybrid molecules are much more stable than RNA–DNA hybrids.

With the original report on the unique nature of PSTV, it had become evident that the viroid, when introduced into susceptible host cells, is replicated autonomously without the requirement of a helper virus (Diener, 1971b). From this basic fact, two main questions arose: How are these small RNAs inducing the synthesis of their RNA progeny molecules in the susceptible host cell and how are they inciting the development of disease in certain host plants, although they are well replicated in others without causing any apparent symptoms?

In view of the extensive knowledge of the molecular properties of PSTV and of the relative ease with which it can be transmitted, propagated, bioassayed, and detected, it is not surprising that most of the investigations on viroid replication have been carried out with this prototype viroid. The combined application of all the previously mentioned new techniques has allowed new experimental approaches by which certain parts of the mechanism of viroid RNA transcription and processing could be unraveled. As a consequence, an overall scheme for viroid replication has now emerged that most probably will only need minor modifications and corrections in light of future investigations.

II. THE QUESTION OF VIROID TRANSLATION

Unlike certain viruses, viroids cannot carry their own replicase with them, because they are "naked" RNA molecules, i.e., they are not encapsidated in a protein coat and envelope. Furthermore, it is obvious that the potential genetic information of the viroid molecule with its 240–380 nucleotides is insufficient to code for a complete viroid-specific replicase, even if overlapping reading frames and three rounds of translation are assumed. But it is, nevertheless, conceivable that the viroid RNA mol-

ecule, arbitrarily called viroid (+) RNA, or its complementary strand, the viroid (−) RNA, could be translated *in vivo* into small polypeptides that might be capable of converting a host enzyme into a viroid-specific replicase. If so, this would resemble a strategy similar to the one used by the RNA bacteriophage Qβ, in which case a phage-coded 65,000-molecular-weight protein is combined with three host proteins (ribosomal protein S1 and the protein synthesis elongation factors EF-Tu and EF-Ts) to create the Qβ-specific replicase with its four subunits (Kamen, 1975). Also, overlapping reading frames could be used to code for entirely different proteins, as realized by the DNA bacteriophage $\phi \times 174$ (Sanger *et al.*, 1977). Attempts were made, therefore, to demonstrate mRNA functions for viroid RNA; however, when viroid RNA was used in cell-free *in vitro* protein-synthesizing systems (Davies *et al.*, 1974; Hall *et al.*, 1974) or injected into *Xenopus* oocytes (Semancik *et al.*, 1977), neither viroid-specific polypeptides nor any interference of the viroid RNA with the translation of genuine mRNA could be detected.

Another approach to investigate possible viroid translation consisted in the analysis of the leaf proteins of various viroid-infected and healthy host plants. These studies also revealed no viroid-specified novel polypeptides; but pronounced differences in the accumulation of certain host-encoded proteins could be observed (see Chapter 1, this volume). The contention that viroids are not translated gained further support from the sequence analysis of PSTV, which showed that neither the PSTV (+) RNA nor the PSTV (−) RNA contains any AUG initiation codons (Gross *et al.*, 1978; Matthews, 1978) and that the GUG codons that are actually present in the PSTV sequence are not capable of initiating translation in eukaryotic cells (Sherman *et al.*, 1980). Thus, speculations on possible GUG-initiated PSTV translation products (Matthews, 1978) also lost their justification. Presently known nucleotide sequences of about a dozen additional viroids and viroid strains have further strengthened the concept that their (+) or (−) RNAs are not translated.

AUG initiation codons are also not present in the sequences of the various PSTV (Gross *et al.*, 1981; Schnölzer *et al.*, 1985) or CEV (Visvader *et al.*, 1982; Gross *et al.*, 1982) isolates established more recently. But the sequence of CSV contains one AUG codon (Haseloff and Symons, 1981; Gross *et al.*, 1982) and ASBV contains three AUG codons (Symons, 1981). The potential polypeptide products that could be translated from the corresponding sequences range in length from 24 to 63 amino acid residues. It has been suggested that viroid (−) RNA might act as mRNA (Matthews, 1978). However, neither PSTV (−) RNA nor CSV (−) RNA, as constructed from the corresponding (+) RNA sequence, contains any AUG initiation codons. The (−) RNA of CEV (Visvader *et al.*, 1982) and ASBV (Symons, 1981), however, contain one AUG codon each, which would result in polypeptides with 42 and 5 amino acid residues, respectively. Because of the drastic differences in the open reading frames in different viroids, a whole series of different mechanisms of replication

would have to be invoked if translation were involved. Finally, if one considers that despite pronounced differences in their sequences, these different viroids exhibit a surprisingly close similarity in their overall secondary structure, this latter concept becomes rather unlikely. Consequently, there is general agreement at present that in contrast to the viruses, the RNA genomes of the viroids are not translated into viroid-specific polypeptides. But translation of a small peptide that could act, for example, as a viroid-specific recognition factor cannot be completely ruled out, as yet. Under these premises, viroid-specific polypeptides are most probably not involved in viroid replication and pathogenesis. Consequently, the replication and processing of viroids must proceed entirely with the aid of the machinery that is preexisting or becomes activated in the viroid-infected host cell.

III. POTENTIALLY POSSIBLE PATHWAYS OF VIROID REPLICATION

Theoretically, various pathways are conceivable by which viroid RNA could become replicated in the host cell with the aid of preexisting nucleic acid-synthesizing host enzymes. Because healthy plant cells are known to contain two classes of RNA-synthesizing enzymes, namely the DNA-dependent RNA polymerases I, II, and III (see Duda, 1976; Wollgiehn, 1982) and the RNA-dependent RNA polymerase (see Fraenkel-Conrat, 1979), viroids could be replicated either via the viroid RNA–viroid DNA–viroid RNA route or via the viroid (+) RNA–viroid (−) RNA–viroid (+) RNA pathway.

A. Presumed DNA-Directed Viroid Replication

The finding that actinomycin D inhibits PSTV replication in leaf strips (Diener and Smith, 1975) and in purified cell nuclei (Takahashi and Diener, 1975) suggested that viroids might be replicated from viroid-specific DNA templates. Since no reverse transcriptase activity has been described from higher plants, it was assumed that the viroid-specific DNA complement could already be present in the host cell as an integral part of the host genome. It was anticipated that it existed in a repressed state in healthy plants and that the newly infecting viroid RNA might act as a regulatory molecule and trigger derepression of the presumed viroid-complementary DNA. Upon its activation it would serve as template for one of the DNA-dependent host RNA polymerases, from which the viroid progeny RNA would then become synthesized. Conclusive evidence for this pathway would be the presence of viroid-specific DNA in viroid-infected tissue.

Efforts to identify these viroid-specific host DNA sequences by molecular hybridization using ^{125}I-labeled viroid (+) RNA resulted in claims that such DNA is present not only in viroid-infected (Semancik and Geelen, 1975; Hadidi *et al.*, 1976) but also in uninfected plants (Hadidi *et al.*, 1976). Both of these claims were later shown to be in error. In more rigorous solution-, filter-, and Southern-hybridization experiments with highly purified viroid RNA (Zaitlin *et al.*, 1980; Branch and Dickson, 1980) or with cloned viroid cDNA (Hadidi *et al.*, 1981), it was found that the supposed viroid–host DNA hybrids detected earlier had actually been complexes between genomic host DNA and certain ^{125}I-labeled cellular ribosomal RNA contaminants that were present and had therefore also become labeled in the viroid probes. The sensitivity of the appropriate molecular hybridization analysis was demonstrated to be adequate for the detection of less than one copy of viroid-specific DNA per haploid genome (Branch and Dickson, 1980). These experiments thus ruled out the presence of even a single continuous viroid copy in host DNA, but did not exclude the possibility that PSTV sequences are randomly located on host chromosomes or that the host DNA contains only a portion of the PSTV RNA genome. In fact, such short viroid-complementary sequences could still serve as recognition sites and be involved in viroid replication and/or pathogenesis. But there is no experimental evidence that such DNA sequences could act as templates for the synthesis of viroid progeny molecules.

The absence of any viroid-specific DNA in the host genome is further strengthened by the observation that the primary structure of viroids is faithfully maintained irrespective of the host plants in which they are replicated (Dickson *et al.*, 1978; Niblett *et al.*, 1978; Owens *et al.*, 1978). Additional support comes finally from the existence of different isolates of one and the same viroid characterized by differences in virulence and chain length which are also maintained upon replication in one and the same host plant (Gross *et al.*, 1981; Schnölzer *et al.*, 1985; Visvader and Symons, 1985). Thus, all the data available at present strongly suggest that viroids are replicated via the RNA–RNA pathway with viroid (+) and (−) RNA serving as template.

B. RNA-Directed Viroid Replication

With the accumulation of evidence that no viroid- or host-specific DNA is involved in viroid replication, it has become increasingly obvious that viroids are replicated via the RNA–RNA pathway. Therefore, studies were directed toward detecting the intermediate forms that one could expect to find in this type of replication mechanism. The search was particularly focused on viroid-specific (−) RNA that could serve as template for the synthesis of infectious viroid (+) RNA progeny, the viroid

proper. To facilitate subsequent discussion of the various aspects of viroid replication, its various products found to date will first be discussed individually.

IV. INTERMEDIATES AND PRODUCTS OF VIROID REPLICATION

A. Monomeric Circular Viroid (+) RNA, the "Mature" Viroid Proper

About 2 years before the unequivocal and positive identification of RNA as the infectious moiety of viroids, inactivation experiments of PSTV (Diener, 1970) and CEV (Semancik and Weathers, 1970) with various nucleases showed that viroid infectivity was partially resistant to phosphodiesterase. Therefore, it was assumed that the infectious entity might be either an exonuclease-resistant, possibly circular, structure or that its termini are masked in such a way that this enzyme cannot attack the terminal nucleotide. Since alkaline phosphatase did not render the RNA exonuclease-sensitive, "masking" could not be due to phosphorylation of the terminal nucleotides (Diener, 1970). Visualization of a native viroid by electron microscopy was first achieved in 1973 by Sogo *et al.* who found, however, more or less base-paired double-stranded short strands in their PSTV preparations.

Evidence that these double-stranded structures were actually collapsed and highly base-paired single-stranded circular RNA molecules was finally provided by the electron microscopic analysis of viroid preparations that had been denatured under conditions known to fully dissociate intramolecular base pairing (Sänger *et al.*, 1976). Since single-stranded circular RNA molecules had not been found previously, it was mandatory to unambiguously demonstrate that the circularity of this RNA was not due to cohesive ends that might not have become separated during the preparation of the samples for electron microscopy. Therefore, an additional biochemical analysis was performed with the corresponding end-labeling-techniques to demonstrate the existence of free 3′- and 5′-termini. The failure of these end-labeling experiments substantiated the electron microscopic finding in a reliable biochemical way. All these preparations contained predominantly circular molecules and only a low percentage of linear forms was present, which were considered to represent mainly nicked circles. The evidence provided by these combined studies led to the conclusion that viroids are covalently closed single-stranded circular RNA molecules existing in their native state as highly base-paired rodlike structures. As will be discussed in the following section, some controversies regarding the actual relationship between the

circular and linear viroid arose when McClements and Kaesberg (1977) found that in their preparations about 70% of the molecules were linear forms. But today, due to the results from many investigations on about a dozen different viroids, it is generally accepted that the accumulating mature end products of the replication process of all presently known viroids are circular RNA molecules.

B. Oligomeric Linear Viroid (−) RNA

The existence of viroid (−) RNA was first reported by Grill and Semancik (1978) who detected it by hybridization with ^{125}I-labeled CEV in nucleic acid preparations from CEV-infected *Gynura aurantiaca* plants, but not from healthy ones. The thermal denaturation properties of the complexes in which the (−) RNA occurred were consistent with those of RNA–RNA duplexes and not with properties of RNA–DNA hybrid molecules. The (−) RNA was later also found in plants infected by PSTV (Owens and Cress, 1980; Branch *et al.*, 1981; Rohde and Sänger, 1981) and ASBV (Bruening *et al.*, 1982).

Detailed characterization of the PSTV (−) RNA strands revealed that they are heterogeneous in size, forming either four (Branch *et al.*, 1981; Spiesmacher *et al.*, 1983) or six (Mühlbach *et al.*, 1983) discrete bands in polyacrylamide gels, thus representing multimers of the PSTV unit-length RNA with 359 nucleotides. They seem to be present in complexes containing extensive double-stranded regions, but after treatment with RNases under conditions favoring digestion of single-stranded regions, the high-molecular-weight, unit-length (−) strands appear (Branch *et al.*, 1981). Fingerprint analysis of hybridized ^{125}I-labeled PSTV following recovery from the hybrids demonstrated that all regions of the PSTV molecules are represented in the (−) RNA strands (Zelcer *et al.*, 1982). Further characterization of the double-stranded structures indicated that monomeric circular and linear (+) PSTV strands are complexed with multimeric (−) RNA strands and that synthesis of the double-stranded complexes increases simultaneously with synthesis of the single-stranded PSTV (+) RNA (Owens and Diener, 1982).

With the identification in infected tissue of viroid-complementary RNA sequences (Grill and Semancik, 1978) and the demonstration of full-length copies (Owens and Cress, 1980) and even oligomeric forms of such viroid (−) RNA molecules (Rohde and Sänger, 1981; Branch *et al.*, 1981), it became evident that viroids are replicated by RNA-directed RNA synthesis. These findings and the detection of oligomeric (+) RNA molecules in viroid-infected cells (Bruening *et al.*, 1982; Owens and Diener, 1982; Spiesmacher *et al.*, 1983; Branch and Robertson, 1984) furthermore suggested a "rolling circle"-type mechanism for viroid replication. To account for all the different forms of viroid RNA detected, a final processing

of the oligomeric viroid (+) RNAs is required in which specific cleavage generates the monomeric linear molecules that are then ligated to yield the monomeric circular viroid RNA progeny. Thus, viroid processing is reminiscent of the two characteristic steps in splicing of pre-mRNAs, which also involves excision of an RNA molecule (the intron) and subsequent ligation of an RNA (the exon). Although the corresponding host enzymes could be utilized in these key steps in the viroid replication cycle, it is also conceivable that viroid processing might involve a non-enzymatic mechanism as in the case of the self-splicing group I introns.

C. Oligomeric Linear Viroid (+) RNA

The existence of oligomeric viroid (−) RNA strands led to the assumption that the subsequent synthesis of the viroid progeny might also involve oligomeric (+) RNA forms from which the monomeric circular and linear viroid (+) RNA molecules are generated by cleavage and circularization. Molecular hybridization and size analysis revealed that, in addition to accumulated monomeric PSTV proper, dimeric and trimeric PSTV (+) strands are present in isolated nuclei and in total RNA preparations from PSTV-infected potato cells (Spiesmacher *et al.*, 1983) (see Fig. 1) and in total RNA from PSTV-infected tomato leaves (Branch *et al.*, 1981; Branch and Robertson, 1984). Up to five repeats of the viroid monomer were reported for ASBV (Bruening *et al.*, 1982) and in the case of CCCV dimeric forms were found to accumulate to high levels in co-

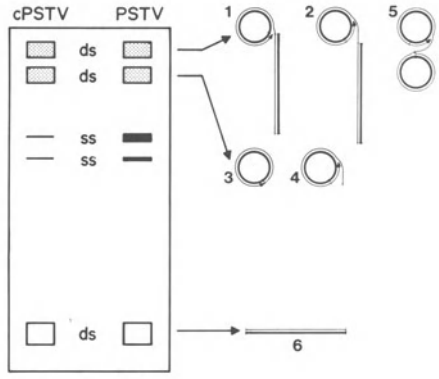

FIGURE 1. Structural elements arising during the process of PSTV replication according to Owens and Diener (1982). They are based on the major viroid-related RNA species characterized and on tentative structures proposed for double-stranded (ds) PSTV RNA. The mobility of each of the five PSTV-related zones on the schematically represented Northern blots from 5% polyacrylamide gels on DBM paper shown on the left has been calculated relative to that of the monomeric circular PSTV proper. There are three zones containing ds PSTV RNA and two containing ss PSTV (+) or (−) RNA. The left lane shows the autoradiographic pattern after probing for PSTV (−) RNAs, the right lane the one after probing for PSTV (+) RNAs. Shading indicates relative amounts present in a total RNA preparation. Circular PSTV (+) RNA was the most abundant species, while the PSTV • $cPSTV_L$ duplex consisting of linear PSTV (+) RNA • linear PSTV (−) RNA is shown as an empty box to indicate that it was not detected unless RNA samples had been digested with RNase. Thick lines, monomeric PSTV (+) RNA strands in the ds PSTV RNAs [consisting of the circular PSTV (+) RNA template and the linear and circular PSTV (+) RNA progeny]; thin lines, linear PSTV (−) RNA strands of various lengths.

conut palms (Haseloff *et al.*, 1982), especially in late stages of infection (Mohamed *et al.*, 1982).

D. Viroid (+) · (−) RNA Hybrid Molecules

In the replication of small RNA plant viruses, at least two types of virus-related RNA species seem to be involved, the double-stranded replicative form (RF) and the partially double-stranded replicative intermediate (for review, see Siegel and Hariharasubramanian, 1974). Although the enzymes catalyzing viroid RNA and plant virus RNA replication are evidently different, one might nevertheless expect that viroid replication involves analogous double-stranded complexes of viroid and viroid-complementary RNA. Such intermediate double-stranded viroid RNA complexes have indeed been detected in the case of PSTV.

In a detailed study, Owens and Diener (1982) applied CF11-cellulose chromatography to fractionate the total RNA from PSTV-infected tomato leaves into single- and double-stranded RNA. These RNA species were then characterized by a combination of RNase treatment, electrophoretic separation on polyacrylamide gels under native and denaturing conditions, and Northern blot analysis under stringent conditions. The hybridization probes specific for the detection of PSTV (+) or (−) RNA were produced by labeling the 5′-termini of a *Bam*HI-cleaved PSTV cDNA-containing plasmid clone with [γ-^{32}P]-ATP and polynucleotide kinase, digestion of the [^{32}P]-DNA with *Hae*III, and electrophoretic purification of the two PSTV-specific fragments. The 300- and 59-base-pair fragments provided specific probes for nucleotides 147–87 of PSTV (+) RNA and 88–146 of PSTV (−) RNA, respectively.

Characterization of the intermediates of PSTV replication showed that in accordance with their previous finding (Owens and Cress, 1980) and with replication studies on plant virus RNA, most PSTV (−) RNA is isolated in an RNase-resistant duplex also containing PSTV (+) RNA. However, the presence of multimeric PSTV (−) RNA strands that are complexed with both the circular and the linear PSTV (+) RNA strand is rather unusual. The synchronous synthesis of single-stranded PSTV was found to be accompanied by a simultaneous marked increase in double-stranded PSTV RNA, which strongly suggests that the *in vivo* precursors of these double-stranded PSTV RNAs are involved in PSTV replication. The association of multimeric linear PSTV (−) RNA strands with monomeric circular PSTV (+) RNA progeny molecules can be most readily explained if one assumes that PSTV (−) RNA is synthesized on this circular RNA as template and that this synthesis continues past the origin of replication, leading to the synthesis of linear dimeric and higher multimeric PSTV (−) RNA molecules. Since no multimeric PSTV (+) RNA molecules could be detected, it was further postulated that the PSTV (+) RNA progeny that is synthesized from the linear multimeric PSTV

(−) RNA template during the second RNA–RNA transcription step is cleaved to monomers and circularized while still complexed with its template. Apart from the viroid-specific circularity and its particular operational requirements, the viroid replication scheme proposed from these studies resembles, in some respects, the rolling circle mechanism previously advanced to explain the replication of certain viral RNAs (Brown and Martin, 1965).

V. STUDIES ON VIROID REPLICATION *IN VIVO*

The earlier studies on viroid replication *in vivo* were carried out with total plants, sprouts, detached leaves, and leaf disks, as these were the only experimental systems available for this purpose at that time. To follow viroid replication, the accumulation of extractable viroid RNA was generally determined spectrophotometrically in cylindrical 5% polyacrylamide gels on which the total cellular RNA had been separated. Also, radioactive labeling experiments were carried out but most of them must be considered as "long-term" incorporation studies that only allowed a limited insight into the actual mode of viroid replication. Moreover, all these systems exhibit severe limitations in the uptake and distribution of the radioactive precursors and different inhibitors of nucleic acid and protein biosynthesis, which greatly interfered with the performance of reliable pulse-labeling experiments. Finally, adequate probes and procedures were not available that would have allowed one to decide whether only a small number of cells in the infected plant tissue exhibit a high rate of viroid replication or whether viroid RNA is synthesized at a low rate in practically all cells of the tissue at the same time. This question is still unresolved. Because of these limitations, many of these earlier studies on viroid replication resulted in contradictory results and conclusions that, in retrospect, can be related to the inadequacies of the experimental systems available at that time. Consequently, the discrepancies in and between these studies should not be overemphasized. Despite all their problems, these studies nevertheless paved the way for the subsequent work in that they led to increasing experimental experience and the development of more appropriate systems and more critical attitudes toward them.

Since molecular hybridization has become the most powerful tool for the study of viroid replication and its various products, a note of caution should be added here. The unusual secondary structure of viroids must be considered in all molecular hybridization analyses. Their high degree of intramolecular self-complementarity has been shown to result in the hybridization of ^{125}I-labeled PSTV (+) RNA with unlabeled monomeric circular and linear PSTV (+) RNA under high salt/low temperature conditions (Rohde and Sänger, 1981). Comparable homopolar hybridization can be expected for viroid (−) RNA. This potentially possible

aberrant behavior of the probes may lead to erroneous results and interpretations. Therefore, a combination of well-defined homogeneous hybridization probes and stringent hybridization conditions is required to obtain reliable data on the actual nature of the molecules searched for.

A. Replication in Intact Plants

To optimize viroid yield, the first studies were focused on the dependence of viroid replication in intact plants on environmental conditions. In the initial experiments aimed at the characterization of the infectious entity, no UV-absorbing component could be found to coincide with the distribution of infectivity in polyacrylamide gels in the case of both PSTV (Diener, 1971b; Diener and Smith, 1971) and CEV (Sänger, 1972). But when concentrated preparations were used for electrophoretic analysis, a specific UV-absorbing peak resembling viroid RNA could be demonstrated for PSTV (Diener, 1972) and for CEV (Semancik and Weathers, 1972; Semancik *et al.*, 1973). A systematic study on the influence of temperature on the synthesis of PSTV RNA in Rutgers tomatoes (Sänger and Ramm, 1975) showed that growth temperature has a pronounced effect on viroid replication and that raising the temperature from 18–20°C to 30–32°C may cause a more than 300-fold increase in viroid yield. Also, the period of incubation, i.e., the mean time required for the first appearance of disease symptoms, is reduced from several weeks at temperatures around 20°C to about 1 week at temperatures above 30°C.

Furthermore, the intensity of the light under which the infected host plants are grown has a pronounced effect on viroid replication, as reflected in the period of incubation. It was found for CEV-infected *G. aurantiaca* that the mean incubation period at 1000-, 15,000-, and 36,000-lx daylight equivalents was 60, 42, and 30 days, respectively (Sänger and Ramm, 1975).

These incorporation studies and the corresponding autoradiographs clearly substantiated the inordinate temperature-dependence of PSTV and CEV replication which had been derived from the spectrophotometric scanning of cylindrical gels. The quantification of these data showed that, based on the incorporation of ^{32}P, the relative concentration of PSTV RNA at 18–20°C is less than 0.01% of the transfer RNA and that it increases at 30–32°C to about 3%. Provided the infected plants are growing at an optimal temperature and provided that they are harvested 3–6 weeks after inoculation, the PSTV RNA may constitute up to 0.3% of the total RNA extracted.

When these estimations were carried out with CEV-infected *Gynura*, it appeared that the relative concentration of CEV RNA at 30–32°C was only 0.8–1.3% of the tRNA, when all symptom-bearing leaves were harvested and used for extraction 2 months after inoculation.

Similar experiments with entire tomato plants and ^{32}P incorporation

for 1–5 days were carried out by Hadidi and Diener (1977) in a study of the *de novo* synthesis of PSTV. They found that PSTV synthesis constituted 0.4% of the low-molecular-weight RNA and 0.08% of the total RNA extracted from tomato leaves. Moreover, they observed that PSTV infection appreciably increased the synthesis of a so-called "9 S RNA"—a cellular RNA species now known as 7 S RNA that is present in all organisms from bacteria to man.

In a subsequent study with the same experimental system of intact plants and ^{32}P incorporation, Hadidi and Diener (1978) investigated the kinetic relationship between the monomeric single-stranded circular and linear form of PSTV that had been found in electron micrographs of denatured PSTV (McClements, 1975; Sänger *et al.*, 1976; McClements and Kaesberg, 1977). They tried to clarify whether linear PSTV molecules are formed *in vivo* as a normal part of viroid replication or whether they arise *in vitro* by breakage of the circular PSTV molecules during viroid extraction and purification.

They found that circular and linear PSTV molecules were the first and second detectable products, respectively, of PSTV replication regardless of the stage of the infection. The observation that linear PSTV was predominant after long periods of incorporation, whereas circular PSTV was prevalent after short periods of incorporation, suggested that linear PSTV arises from circular PSTV by nicking *in vivo* and that its formation is not an artifact of isolation and purification as suggested by Sänger *et al.* (1976).

It should be mentioned in this context that today there is general agreement that the linear forms generally found in all viroid preparations may arise in both ways. A minor part of them represent so-called "natural" linears that were generated *in vivo* from multimeric precursors by nucleolytic cleavage and that have not yet been circularized. The larger fraction of linears are evidently nicked circles arising during isolation and purification.

Temperature-shift experiments, which are widely used in studies on the replication of animal viruses in cultured cells, have only been applied in viroid replication studies by Owens and Diener (1982). Differential temperature treatment of PSTV-infected tomato seedlings had indicated that this method could be used as an experimental system in which PSTV replication is at least partially synchronized. This system was therefore used to estimate by electrophoretic analysis the relative amounts of PSTV (+) and (−) RNA at various times after the temperature increase. When viroid replication was blocked by the low temperature, the upper part of the tomato seedlings contained only traces of PSTV and small amounts of double-stranded PSTV RNA. Twenty-four hours after the shift to the permissive temperature (31°C), the concentration of PSTV had increased approximately 100-fold, which was accompanied by a marked increase in the amount of double-stranded PSTV RNA. The dsPSTV/PSTV ratio was maintained over the next 74 hr, a period during which the PSTV

concentration remained constant. The concentration of PSTV in the unsynchronized plants was about 1000-fold greater than that in the synchronized plants before the temperature shift and about 10-fold greater than that in the synchronized plants 24 hr after the temperature shift.

The application of such temperature-shift experiments to one of the newly developed viroid-synthesizing plant cell systems would be a very promising approach for studying viroid replication—especially when temperature-sensitive viroid mutants could be selected and used in these studies.

B. Replication in Plant Protoplasts and Cultured Cells

For several reasons, plant protoplasts are particularly suitable for studying viroid replication. Unlike entire plants, the cells of a protoplast preparation can be inoculated in a synchronous way and the uptake of radioactive precursors which are required for an appropriate analysis of the replication process is rapid and poses fewer problems. Moreover, the environmental and growth conditions for protoplasts can be easily controlled and standardized. Consequently, the biochemical events of viroid biosynthesis are more or less synchronized in such experimental systems—which greatly facilitates appropriate investigations at the cellular level.

The first such studies were carried out with protoplasts isolated from tomato leaf tissue (Mühlbach and Sänger, 1977). Their metabolic capacity was substantiated by their ability to regenerate new cell walls within 3 days after their isolation, to divide within a week, and to produce complete tobacco mosaic virus (TMV) particles after infection with TMV RNA *in vitro*. Viroid replication could be followed in these protoplasts by incorporation of [^{3}H]uridine after their *in vitro* inoculation, so that this experimental system also allowed studies with inhibitors of nucleic acid transcription (see below).

Further progress in the study of viroid replication was achieved when permanent plant cell cultures became available which were capable of continuous viroid replication. In callus cultures from a PSTV-infected wild-type potato (*Solanum demissum* L.) and tomato (*Lycopersicon peruvianum* L. Mill.) and in cell suspension cultures derived from potato (*Solanum tuberosum*), PSTV replication was found to persist for several years through many subcultures. The continuous *de novo* viroid synthesis was substantiated by the incorporation of [^{3}H]uridine and of [^{32}P]orthophosphate into PSTV RNA and its subsequent analysis by gel electrophoresis and autoradiography (Mühlbach and Sänger, 1981). Similar observations of sustained long-term PSTV replication were reported for suspension cultures prepared from callus tissue of tomato (Zelcer *et al.*, 1981) and wild-type potato (Mühlbach *et al.*, 1983). Under optimal conditions of culture and rapid growth of these cells, the maximum rates

of ^{32}P-incorporation into PSTV RNA varied between 0.4% of that into soluble RNA (Zelcer *et al.*, 1981), 2–3% of that into tRNA (Mühlbach and Sänger, 1981), and 10% of that incorporated into the 2 M LiCl-soluble cellular RNA (Mühlbach *et al.*, 1983).

The *S. demissum* cell suspension culture with its comparatively high rate of viroid replication allowed the direct demonstration, without complicated sample processing, of the presence of intermediate forms of replication. Electrophoretic and molecular hybridization analysis revealed that linear, oligomeric, single-stranded PSTV (−) RNA molecules from two to six times the chain length of the PSTV (+) RNA monomer with its 359 nucleotides (Mühlbach *et al.*, 1983) are synthesized in these cells.

The rapidly growing PSTV-infected *S. demissum* cell suspension culture has also been used for the production of PSTV by large-scale fermentation in volumes of up to 800 liters. During their culture, the physiological state of these cells was monitored by *in vivo* ^{31}P NMR spectroscopy. The scale-up from 10-liter cell inoculum grown in shake flasks to the harvest of the 800-liter suspension culture took 38 days and provided 112 kg of biomass. Northern blots revealed the presence of about 700 μg of PSTV per kg cultured cells, so that the 112 kg of biomass harvested from the 800-liter fermentor would represent about 80 mg of PSTV. Thus, such cell cultures provide not only the basis for investigating the dynamic aspects of viroid replication, but also the appropriate system for the purification and characterization of the transient products of the replication cycle.

The continuous viroid replication in permanent plant cell cultures contrasts strongly with the results obtained with conventional plant viruses, which are rather quickly excluded from rapidly dividing cells and can, thus far, only be propagated in large quantities in intact plants.

C. Replication in Isolated Plant Cell Nuclei

Bioassays of subcellular fractions from leaf tissue of PSTV-infected tomato (Diener, 1971a) revealed quite early that viroid infectivity is predominantly located in the nuclear fraction and primarily associated with the chromatin.

As discussed in detail in Chapter 4, these early findings could be substantiated by a direct quantitative analysis, which showed that 200–10,000 copies of the PSTV (+) RNA progeny molecules are present inside the nucleus, where they are closely associated with the nucleolus (Schumacher *et al.*, 1983).

Incorporation studies with radioactive precursors of RNA biosynthesis and nuclei isolated from PSTV-infected tomato leaves suggested that PSTV replicated in the nucleus (Takahashi and Diener, 1975). However, this approach did not allow detection of discrete bands of radiola-

beled RNA species representing the postulated intermediates of viroid replication.

With the development of permanent suspension cultures of healthy and continuously PSTV-synthesizing wild potato (*S. demissum*) cells (Mühlbach and Sänger, 1981; Mühlbach *et al.*, 1983), the adaptation to these cell systems of an improved method for the isolation of nuclei from tobacco cell suspensions and the availability of "tailor-made" strand-specific DNA probes for molecular hybridization (Spiesmacher *et al.*, 1983), the presumed presence of the replicative intermediate forms of PSTV within the nucleus could be reinvestigated. Unlabeled total nucleic acids were isolated from purified nuclei, fully denatured by glyoxylation, separated electrophoretically on polyacrylamide gels, electroblotted to transfer membranes, and probed with synthetic ^{32}P-labeled DNA primers for the presence of PSTV-specific (+) and (−) RNA molecules (Spiesmacher *et al.*, 1983). With this technique, different linear oligomeric forms of PSTV RNA replication could be detected in nuclei in addition to the monomeric circular and linear PSTV (+) RNA progeny, which normally accumulates to quantities that can be visualized by staining. In particular, linear PSTV (+) RNA oligomers about two- and threefold the length of the monomer were found. The PSTV (−) RNA-specific probe detected PSTV (−) RNA species in the range from monomeric up to tetrameric forms in isolated nuclei and up to sixfold unit-length molecules in intact cells (Mühlbach *et al.*, 1983). All these products proved to be DNase I resistant and RNase A sensitive, which clearly proved their RNA nature.

To demonstrate that PSTV and its various oligomeric forms are truly synthesized *de novo* within the nucleus, it is necessary to incubate nuclei with radioactive precursors of RNA synthesis and to show that the viroid-specific products are radioactively labeled and hence newly synthesized during this period of incubation. However, when the nuclear RNAs synthesized in the presence of [^{32}P]-UTP were separated on gels and analyzed on Northern blots, no distinct viroid-specific bands were revealed. A heterodisperse distribution of large amounts of endogenous nuclear transcription products resulted in a "smear" on the autoradiographs, under which the newly synthesized PSTV was totally buried. Therefore, an alternative technique, so-called "transcription-hybridization analysis," was applied. In this approach (Spiesmacher *et al.*, 1985), the radioactively labeled nuclear transcription products are hybridized at 75°C to purified, membrane-bound, unlabeled, single-stranded PSTV DNA of (+) and (−) polarity obtained by molecular cloning in bacteriophage M13 (Tabler and Sänger, 1984). By using this highly specific and sensitive analysis, it could be clearly demonstrated that nuclei isolated from PSTV-infected potato cell suspension cultures are capable of synthesizing PSTV (+) RNA quite efficiently. In contrast, the amount of newly synthesized PSTV (−) RNA appeared to be much lower than that of PSTV (+) RNA. With the quan-

tities employed, PSTV (−) RNA was only detectable by its hybridization to a membrane-bound tetrameric PSTV DNA, but not with the corresponding mono-, di-, and trimeric forms.

From these data it can be concluded that the two steps of "RNA–RNA transcription" involved in viroid replication must proceed within the cell nucleus. Considering the additional experiments with transcription inhibitors (see next section) and the already mentioned accumulation of the PSTV (+) RNA progeny in the nucleolus, further experiments are now required to demonstrate that the synthesis of PSTV (+) RNA also takes place in the nucleoli. However, nucleoli are functionally transient and structurally rather unstable subnuclear structures, and their isolation from plant cells in a transcriptionally highly active form poses considerable problems so that progress in this direction may be slow.

VI. INHIBITION STUDIES ON VIROID REPLICATION

From the analysis of the effects on viroid replication of inhibitors of different metabolic pathways or enzymes, direct or indirect conclusions can be drawn about the involvement of certain enzyme systems of the infected host cell in viroid biosynthesis. Unfortunately, most inhibitors are only of limited specificity and may also cause unwanted side effects. Moreover, their uptake and intracellular distribution usually pose serious problems. Consequently, evaluation of the results of any inhibition studies requires a detailed consideration of the experimental conditions under which they were carried out.

Inhibitors of RNA transcription can be used to distinguish between the different host RNA polymerases involved in viroid replication. The first studies along this line were carried out by Diener and Smith (1975) who used an *in vivo* system in which viroid replication in leaf strips from healthy and PSTV-infected plants treated with water or actinomycin D was monitored by incorporation of radioactively labeled uracil into the newly synthesized RNA. They found that viroid replication was almost completely inhibited by this drug and obtained similar results with an *in vitro* RNA-synthesizing system in which purified cell nuclei from healthy or PSTV-infected tomato leaves were used as enzyme source (Takahashi and Diener, 1975). Mitomycin C, however, a specific inhibitor of DNA polymerase, did not inhibit viroid replication. These findings and the fact that actinomycin D is known to inhibit cellular DNA-directed RNA synthesis in plant cells but does not seriously interfere with the replication of several plant viral RNAs strengthened the contention that PSTV is transcribed from a DNA template. It was assumed that the transcription proceeded with the aid of the preexisting cellular DNA-dependent RNA polymerase system, which is responsible for normal RNA transcription. It was also postulated that the PSTV-specific DNA template could be part of the normal genome of all plant species that are susceptible

to PSTV. Under these premises, the introduction of PSTV into such cells would lead to derepression of the PSTV-specific DNA segment, production of PSTV, and, in certain hosts, to disease. Alternatively, a complementary DNA could be produced in susceptible hosts after inoculation with PSTV. But it was also pointed out that the progeny viroids could be transcribed from RNA templates and that this process could be dependent on the continued synthesis of a short-lived host RNA that might serve as primer for RNA-directed viroid replication (Diener and Smith, 1975; Diener, 1979). Such a scheme would be analogous to that operating in influenza virus RNA replication, in which globin mRNA has been identified as primer for the transcription of viral RNA and could explain the actinomycin D sensitivity of viroid replication even though it is RNA directed.

The sensitivity of viroid replication to actinomycin D has been confirmed in a study of CPFV synthesis in protoplasts from tomato leaves (Mühlbach and Sänger, 1979). In addition to this antibiotic, the effect of mushroom toxin α-amanitin on viroid replication was investigated, because this drug allows the unambiguous discrimination between the different host DNA-dependent RNA polymerases possibly involved in the replication process. In eukaryotic cells, α-amanitin is known to inhibit selectively the DNA-dependent RNA polymerases II and III at low and high concentrations, respectively, whereas RNA polymerase I is not inhibited at all (Lindell *et al.*, 1970; Zylber and Penman, 1971; Weinmann and Roeder, 1974; Wieland and Faulstich, 1978). The corresponding experiments showed that, at 50 μg/ml culture medium, α-amanitin inhibits viroid replication to about 75%, whereas the biosynthesis of the prominent cellular RNA species tRNA, 5 S RNA, 7 S RNA, and rRNA is not appreciably affected. Experiments with tomato protoplasts inoculated with TMV RNA showed that the replication and accumulation of TMV was not affected by α-amanitin under these conditions. These controls showed that the marked inhibition of viroid replication by α-amanitin is unlikely to be due to a secondary effect of this drug on cell metabolism in general.

In view of the concentration-dependent specificity of the α-amanitin inhibition, interpretation of such *in vivo* experiments depends on knowing the intracellular concentration of this inhibitor, which was therefore determined by using tritiated toxin. It was found that under the conditions where viroid replication is specifically inhibited, the intracellular concentration of the inhibitor was in the range of 10^{-8} M, i.e., at a concentration that specifically inhibits RNA polymerase II. Since inhibition of RNA polymerase III requires a 1000-fold higher concentration (Weinmann and Roeder, 1974) and since RNA polymerase I is not affected at all by α-amanitin, it was postulated that the DNA-dependent RNA polymerase II of the host cell is involved in viroid replication (Mühlbach and Sänger, 1979). But it was emphasized explicitly in this report that the observed α-amanitin inhibition cannot be considered as definite proof for the direct replication of PSTV RNA by RNA polymerase II. Several mechanisms

can be conceived by which this enzyme system could be indirectly involved as a result of its central role in the synthesis of mRNAs in the host cell. Even the remote possibility existed that viroid replication could proceed through a yet unknown pathway that is α-amanitin-sensitive.

Results that contradicted these inhibition data have been reported by Grill and Semancik (1980) on the basis of infiltration of CEV-infected *G. aurantiaca* leaf tissue or of PSTV-infected potato tuber sprouts with varying concentrations of actinomycin D. The authors concluded that the antibiotic had no specific inhibitory effect on viroid replication and that the results reported earlier were due to a general toxic effect of actinomycin D on cell metabolism. But, as has been discussed in detail (Diener, 1981), the evidence presented must be regarded with caution, because both the execution of the experiments and the results obtained from them led to a series of unresolved and unexplained contradictions that render such a conclusion somewhat equivocal.

In two later studies with so-called "nucleus-rich preparations" from *G. aurantiaca* (Flores and Semancik, 1982; Semancik and Harper, 1984), α-amanitin sensitivity was also established for the synthesis of CEV—thus confirming the experimental evidence for the postulated involvement of DNA-dependent RNA polymerase II of the host cell in viroid replication. From the detection of trace levels of α-amanitin-resistant CEV synthesis that is inhibited by low $(NH_4)_2SO_4$ concentrations (25 mM), a possible involvement of DNA-dependent RNA polymerase I and/or III-like activity in viroid replication was inferred. Because no molecular hybridization techniques were applied in this work, the authors were unable to discriminate between the newly synthesized CEV (+) and (−) RNA. Thus, they interpreted their data in terms of a general lack of specificity of the three nuclear DNA-dependent RNA polymerases with viroid synthesis and took the precaution to propose all choices in advance—namely that all three RNA polymerases are involved in CEV replication (Semancik and Harper, 1984).

The most recent inhibition studies of Spiesmacher *et al.* (1985) took advantage of the rapid progress that has been achieved in experimental and analytical systems. They used highly purified nuclei obtained by two steps of Percoll centrifugation. The nuclei were isolated from a cell suspension culture established about 4 years previously from PSTV-infected potato cells (Mühlbach and Sänger, 1981), which have been capable of continuously producing PSTV ever since. The transcriptional activity of the nuclei was determined by [^{3}H]-UMP incorporation and found to be optimal at a combination of 5 mM $MgCl_2$ with 40 mM $(NH_4)_2SO_4$. The incorporation of UMP by PSTV-infected nuclei was usually about twice that in healthy nuclei and fully dependent on the presence of all four nucleotides, indicating that the labeling of the products resulted from genuine transcription and not from labeling due to terminal transferase activity. RNA synthesis was linear for the first 30 min and then slowed down but continued for an additional 90 min. It was inhibited up to nearly

90% by 10^{-6} M α-amanitin, implying that this proportion of the total activity could be attributed to the activity of the highly α-amanitin-sensitive DNA-dependent RNA polymerase II. Actinomycin D at a concentration of 10 μg/ml inhibited RNA synthesis by approximately 60% and preincubation of nuclei with 10 μg/ml RNase-free DNase I for 30 min at 29°C reduced the UMP incorporation to 0.1%.

In order to determine the size of the newly synthesized RNA, transcription experiments were performed in the presence of [^{32}P]-UTP and the products were then glyoxylated and gel separated under fully denaturing conditions.

With the highly specific and sensitive technique of "transcription-hybridization analysis" on Northern blots and dot blots, it could be unequivocally demonstrated that PSTV (+) and (−) RNA molecules are synthesized in isolated nuclei.

With the experimentally well-established preconditions, the differential sensitivity of RNA polymerases I, II, and III to α-amanitin and actinomycin D was expected to yield unequivocal evidence on the possible involvement of these host enzymes in PSTV replication. Therefore, the effects of these two drugs on the synthesis of PSTV (+) and (−) RNA were analyzed. α-Amanitin was added to the incubation mixture to give a final concentration of 10^{-6} M, and actinomycin D to 10 μg/ml. α-Amanitin inhibited the synthesis of both PSTV (+) and (−) RNA more efficiently than actinomycin D. Densitometric analysis of the original autoradiographs revealed that α-amanitin inhibited the synthesis of PSTV (+) and (−) RNA by about 80%, which again confirmed that the DNA-dependent RNA polymerase II is involved in PSTV replication. On the other hand, actinomycin D inhibited PSTV (+) RNA synthesis by 65%, but PSTV (−) RNA synthesis only by 10%, which indicates that the latter is largely resistant to this drug and that two RNA polymerases differing in their sensitivity to actinomycin D must be involved in PSTV replication. On the basis of the reported sensitivity of RNA polymerase I-mediated rRNA synthesis to actinomycin D, it was concluded that it is the nucleolar DNA-dependent RNA polymerase I that synthesizes PSTV (+) RNA. It could not be decided from these inhibition experiments which of the lesser-sensitive RNA polymerases II or III mediates the synthesis of PSTV (−) RNA. However, the specific inhibition of PSTV synthesis by an α-amanitin concentration (10^{-6} M) that is known to inhibit RNA polymerase II but not III, strongly suggests that DNA-dependent RNA polymerase II is involved in the synthesis of PSTV (−) RNA and not polymerase III.

VII. REPLICATION MODELS

With increasing experimental evidence for the existence of specific oligomeric products as putative intermediates arising during the process

of viroid replication, it became possible to conceive by analogy mechanisms by which these products could be generated. As a consequence, a series of replication schemes and models have been advanced. They reflect the great interest and intellectual challenge emanating from the still largely enigmatic process of viroid replication. Naturally, all of these models are in some way or other hypothetical and probably none of them will finally turn out to be completely correct in all of its elements.

Historically, the concepts for viroid replication have been developed from products found and mechanisms proposed for the replication of RNA viruses. In all these cases, at least two types of virus-related RNA species, the double-stranded replicative form (RF) and the partially double-stranded replicative intermediate (RI), are involved in the replication cycle (Brown and Martin, 1965; Siegel and Hariharasubramanian, 1974). Of these models, the one introduced by Brown and Martin (1965) was of particular relevance, because it invoked a hypothetical circular (−) RNA molecule as template from which larger-than-unit-length (+) RNA strands are transcribed and then cut down to the monomeric (+) RNA progeny. Interestingly, the most essential element of this model, the single-stranded circular RNA, remained hypothetical for about 10 years until these features were unequivocally established for PSTV RNA by a combination of electron microscopic, biochemical, and biophysical analyses (Sänger *et al.*, 1976). Nevertheless, the Brown and Martin model became the progenitor of the "rolling circle" model, because it could be well applied to the replication of the single-stranded circular DNA of bacteriophage $\phi \times 174$ (Gilbert and Dressler, 1968). When molecular hybridization studies revealed that multimeric viroid (−) RNA molecules (Branch *et al.*, 1981; Rohde and Sänger, 1981; Bruening *et al.*, 1982; Owens and Diener, 1982), as well as multimeric viroid (+) RNA molecules (Bruening *et al.*, 1982; Branch and Robertson, 1984) are present in the total RNA extracted from infected plants, it became evident that multimeric viroid molecules might be synthesized by a "rolling circle"-like mechanism.

A. The Model of Owens and Diener

The tentative elements of a viroid replication model have been depicted by Owens and Diener (1982). They are derived from the analysis of PSTV-specific RNA species in LiCl-fractionated total RNAs from infected tomato plants after differential nuclease digestion, gel electrophoretic separation, and DNA–RNA hybridization on Northern blots. The authors largely interpreted the presumed nature of the elements by invoking the known RF and RI forms arising during the replication of viral RNA genomes. Because of the limited data available at that time, Owens and Diener (1982) refrained from developing a detailed model of the replication process. The essential elements (Fig. 1) are the slowly migrating multimeric ds PSTV RNAs, one strand of which is considered to be a

multimeric PSTV (−) RNA (elements 1, 2, and 5). These duplexes are presumably formed by synthesis of PSTV (−) RNA on the infecting circular PSTV (+) RNA as template (element 3), with the synthesis continuing past the origin of replication (element 4), thus leading to the synthesis of linear dimers and higher multimers of PSTV (−) RNA (elements 1 and 2). The model further posits that monomeric linear PSTV (+) RNA is synthesized from the multimeric linear PSTV (−) RNA template and circularized while still complexed to this template (element 5). Multimeric PSTV (+) RNA was not detected in these studies but its synthesis on a multimeric linear PSTV (−) RNA template was anticipated. To accommodate the synthesis of small amounts of monomeric single-stranded circular and linear PSTV (−) RNA, the more slowly migrating double-stranded PSTV (+) RNA zones were assumed to contain dimeric and higher multimeric PSTV (−) RNA molecules (elements 1, 2, and 5). The more rapidly migrating ds PSTV (+) RNA zone would consist of duplexes of circular PSTV (+) RNA monomers with ss PSTV (−) RNA tails of various lengths (elements 3 and 4). The model also accounts for the formation of more rapidly migrating linear PSTV (+)·(−) RNA duplexes (element 6) during digestion of the most slowly migrating ds PSTV (+) RNA zone with RNase.

B. The Models of Branch and Robertson

The first detailed description of a viroid replication scheme involving a rolling circle-like mechanism was provided by Branch *et al.* (1981) on the basis of their evidence for the existence of multimeric PSTV (−) RNA. Additional data obtained from Northern blot analysis with ^{125}I-labeled PSTV and CEV RNA of the products of PSTV and CEV replication after their differentiation by CF-11 cellulose chromatography and nuclease treatment led to the development of two alternative hypothetical models for viroid replication (Branch and Robertson, 1984) that involve either one or two rolling circle mechanisms (Figs. 2 and 3). The replication cycle with only one rolling circle mechanism (Fig. 2) starts with the introduction of the infecting circular viroid (+) RNA into the cell and the initiation of viroid (−) RNA synthesis (step 1). The circular viroid (+) RNA serves as template on which multimeric linear viroid (−) RNA molecules are synthesized in the rolling circle fashion (step 2). The multimeric linear viroid (−) RNA, with each viroid repeat delineated by putative short dividers (marked ~~), then serves as template for the production of multimeric viroid (+) RNA strands (step 3), which must be cleaved to give monomeric, i.e., unit-length, molecules with characteristic end-groups (as in step 4) and which are finally circularized (step 5) to yield the circular monomeric viroid (+) RNA progeny.

The author favors this scheme with a single rolling circle mechanism because it relies exclusively on viroid-specific nucleic acid species that

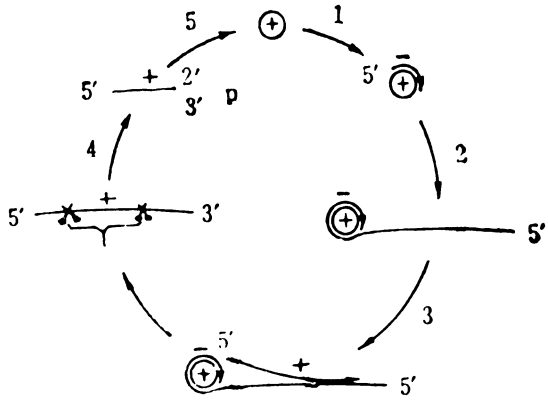

FIGURE 2. Viroid replication model according to Branch and Robertson (1984) involving a single rolling circle mechanism. The infecting circular viroid (+) RNA is copied into a multimeric (−) RNA strand (steps 1 and 2). After being synthesized on its multimeric (−)-strand template (step 3), the multimeric (+) RNA is shown as an isolated species indicating that rearrangements may be required to facilitate processing. It is also assumed that cleavage and ligation might take place while the (+) strand remains within the replication complex. A 2′,3′-phosphate moiety (arising after cleavage—step 4) is indicated by an arrowhead.

can readily be detected in PSTV-infected plants and incorporates all the viroid-related RNAs that have been well characterized.

The second model involving two rolling circle mechanisms is shown in Fig. 3. It provides an alternative way to generate multimeric viroid (+) RNA and still accounts for the presence of multimeric viroid (−) RNAs. In this scheme the infecting circular viroid (+) RNA serves as template for the first rolling circle synthesis of multimeric viroid (−) RNA strands (steps 1 and 2). These give rise to monomeric linear viroid (−) RNA intermediates (steps 2 and 3; shown in brackets to represent uncertainty in the reaction mechanism) that are then circularized (step 3b) and copied in a second rolling circle mechanism to yield multimeric viroid (+) RNA

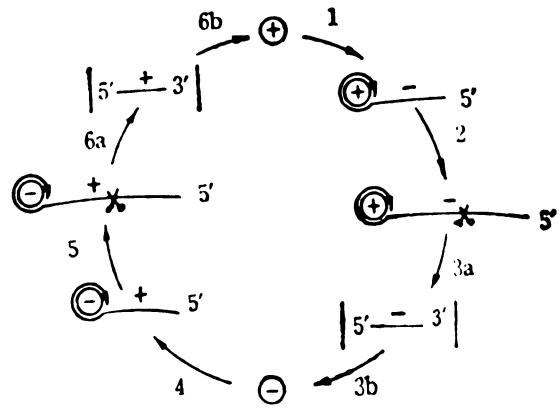

FIGURE 3. Viroid replication model according to Branch and Robertson (1984) involving two rolling circle mechanisms. This replication cycle is a variant of the one outlined in Fig. 2. It illustrates how multimeric viroid (−) RNAs could be generated by copying of a circular (+) RNA template in the first rolling circle fashion (step 1), then processing (step 2) and conversion into a circular (−) RNA (step 3a,b). A second rolling circle mechanism would generate multimeric viroid (+) RNA which is cleaved to monomers and finally circularized to give the viroid (+) RNA progeny. The liner monomeric (−) and (+) strands (see steps 3a and 6a) are shown in brackets to indicate that these may be unstable reaction intermediates.

molecules (steps 4 and 5), which are finally cleaved to monomers (step 6a) and circularized (step 6b) to yield the mature viroid proper.

Choosing between the pathways shown in Figs. 2 and 3 depends on the experimental demonstration of complexes containing circular (−) strands and multimeric (+) strands. Additional experiments are also needed to explain the observation that both (+) and (−) RNA strands accumulate preferentially as RNAs with sizes that are multiples of unit length, perhaps due to pauses in synthesis or specific cleavage, or a combination of the two processes. Since their nature and involvement in viroid replication and processing had not yet been clearly identified, the corresponding host RNA polymerases, RNases, and RNA ligases are not explicitly incorporated in the two replication schemes.

C. The Model of Ishikawa *et al.*

The viroid replication model proposed by Ishikawa *et al.* (1984) involves a single rolling circle mechanism and is based on their characterization of viroid-related RNA molecules in HSV-infected cucumber leaves. In addition, the ideas resulting from infectivity assays with monomeric and tandemly connected, dimeric and tetrameric HSV (+) and (−) RNA molecules synthesized *in vitro* with a bacterial RNA polymerase/promoter system (see Section IX) were incorporated into the scheme. Because the profiles of HSV-related RNAs in the total nucleic acids from HSV-infected cucumber leaves and their characteristics are essentially the same as in the PSTV–tomato system (Branch *et al.*, 1981; Branch and Robertson, 1984), HSV seems to replicate in the same way as PSTV.

The model of Ishikawa *et al.* (1984) is shown in Fig. 4. An RNA polymerase (RPase) recognizes a specific site on the circular RNA and initiates the synthesis of viroid (−) RNA (step 1). The transcription proceeds beyond the initiation site and thus generates multimeric viroid (−) RNA molecules that are associated with the monomeric circular (+) RNA template (step 2). The subsequent synthesis of the viroid (+) RNA is assumed to start (step 3a) at a specific site in this double-stranded (+)·(−) RNA structure (indicated by a broken circle) and to proceed along the multimeric (−) RNA template on which single-stranded multimeric viroid (+) RNA molecules are transcribed (step 3b). The multimeric linear viroid (+) RNAs are then subjected to endonucleolytic cleavage at specific sites (step 4) and linear monomeric viroid (+) RNA molecules are generated that have 2′,3′ cyclic phosphates at their 3′-termini. These linear monomers are finally converted to the mature monomeric circular viroid (+) RNA (step 5) for which process an enzyme with properties similar to those of the wheat germ RNA ligase is invoked.

An essential and indispensible element of this model is a double-stranded region on the replicating complex (indicated by a broken circle) that consists of the monomeric circular viroid (+) RNA template and the

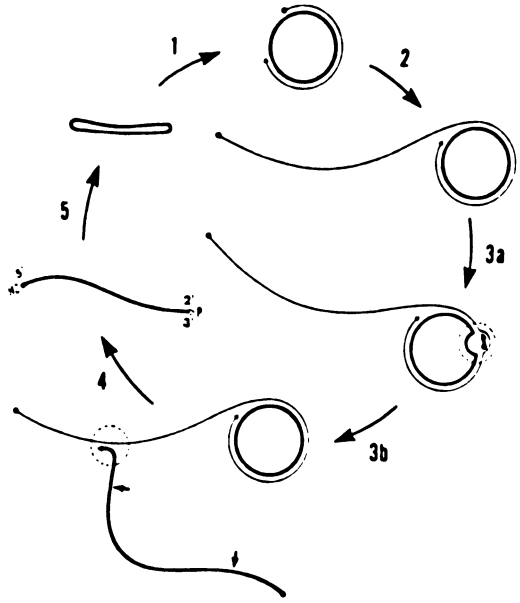

FIGURE 4. Viroid replication model involving one rolling circle mechanism as proposed by Ishikawa *et al.* (1984). Thick and thin lines indicate HSV (+) and (−) RNAs, respectively. Arrowheads and closed circles mark the 3′- and 5′-termini of RNA molecules, respectively. The short arrows point to the cleavage sites for a hypothetical endonuclease. Broken circles in the structures following steps 3a and 3b represent the hypothetical RNA polymerase for (+)-strand synthesis. The details of this replication scheme are described in the text.

multimeric (−) RNA transcript. A double-stranded domain is considered to be responsible for the recognition by the RNA polymerase that synthesizes the multimeric viroid (+) RNA. Indirect evidence for this assumption came from the findings that the tetrameric HSV (−) RNA molecules were noninfectious and that they became infectious after they had been incubated together with noninfectious monomeric HSV (+) RNA (see Section IX.A).

Except for the assumption that the specific initiation of the synthesis of multimeric viroid (+) RNA requires a double-stranded structure and that transcription steps 1–3 are carried out by different RNA polymerases, the model coincides with the one of Branch and Robertson (1984) that also involves a single rolling circle mechanism (Fig. 2).

D. The Model of Hutchins *et al.*

A replication model for viroids and virusoids involving two rolling circle mechanisms has been developed by Hutchins *et al.* (1985) on the basis of their comparisons of multimeric (+) and (−) RNA forms of three viroids (ASBV, CEV, and CCCV) and three virusoids, i.e., viroidlike RNAs (RNA 2) of lucerne transient streak virus (LTSV), velvet tobacco mottle virus (VTMoV), and *Solanum nodiflorum* mottle virus (SNMV). Both types of agents are structurally very similar but, in contrast to viroids, virusoids require the linear viral RNA (RNA 1) of the corresponding plant virus with a chain length of about 4,500 nucleotides for their replication and

encapsidation (see Chapter 8, this volume). Therefore, virusoids can be considered to be satellite RNAs of these viruses.

According to this new version (Fig. 5), the infecting monomeric circular (+) RNA is copied by a host RNA polymerase to produce an oligomeric (−) RNA strand (step A) that is processed by a nonenzymatic mechanism (step B) to the linear (−) RNA monomers and a mixture of linear (−) RNA oligomers. The latter are considered to arise either by inefficient processing of the oligomeric (−) RNA strands or by head-to-tail ligation of the linear (−) RNA monomers (step C_2) by the same enzyme that circularizes the linear (−) RNA monomers and oligomers to covalently closed circles (step C_1 and C_3, respectively). The monomeric circular (−) RNA then serves as template on which a host RNA polymerase synthesizes in a second rolling circle fashion oligomeric (+) RNA (step D), which is nonenzymatically processed (step E) to the linear (+) RNA monomer and a mixture of linear (+) RNA oligomers. As in the case of the linear (−) RNA oligomers, the linear (+) RNA oligomers arise either by inefficient processing of the multimeric (+) RNA strands or by head-to-tail ligation of the (+) RNA monomers (step F_2). The circular (+) RNA monomers and oligomers are finally produced by circularization of the appropriate precursor molecules (step F_1 and F_3, respectively). Overall, this model is similar to the second model proposed by Branch and Robertson (1984) as shown in Fig. 3.

The model of Hutchins *et al.* (1985) accounts for most of the (+) and (−) RNA species found in extracts of viroid- and virusoid-infected plants. The variation in pattern of RNA species observed with the different viroids and virusoids studied is assumed to be due to the variable accumulation of these RNA species at different points of the replication cycle.

The monomerization of the multimeric transcripts in steps B and E is believed to proceed by two different mechanisms. In certain cases, such as in ASBV, it is evidently nonenzymatic "self-cleavage" that operates on its multimeric (+) and (−) RNA forms. In other cases, specific endonucleases might be required for the processing of the (+) and (−) RNA multimers.

Regarding the intramolecular circularization and the intermolecular head-to-tail ligation of monomeric and oligomeric RNA forms of both polarities, Hutchins *et al.* (1985) invoke a host enzyme with properties similar to those of the ATP-dependent RNA ligase from wheat germ. As will be discussed in Section X.B, this enzyme is, in fact, capable of circularizing at least a fraction of the linear viroid (+) RNA molecules isolated from plants infected with PSTV (Kikuchi *et al.*, 1982; Branch *et al.*, 1982).

The second rolling circle mechanism by which multimeric (+) RNA strands are generated is assumed to proceed on a circular monomeric (−) RNA as template. Since circular dimeric (+) and (−) RNAs have also been detected in ASBV-infected avocado leaves, it is conceivable that

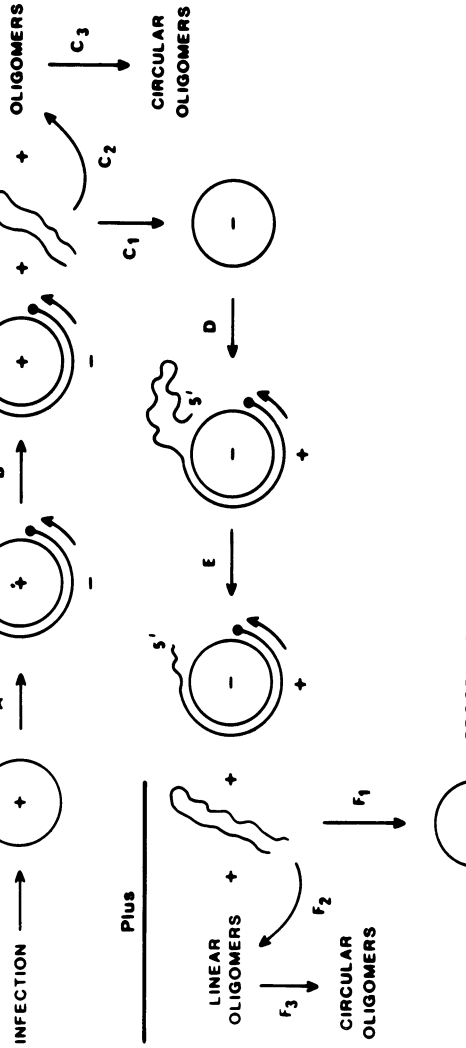

FIGURE 5. Model for the replication of viroids and virusoids according to Hutchins *et al.* (1985) involving two rolling circle mechanisms. This model is very similar in its elements to the second model proposed by Branch and Robertson (1984) and shown in Fig. 3. The small filled circle represents the RNA polymerase and the associated arrow the direction of RNA synthesis. The details of the replication cycle are described in the text.

dimeric circular molecules could also act as templates in the rolling circle mechanism. Monomeric (−) RNA species were also present in extracts of plants infected with CEV, SNMV RNA 2, and LTSV RNA 2, but it has not yet been determined whether they are circular and/or linear forms. In CCCV- and VTMoV RNA 2-infected plants, the presence of monomeric (−) RNA has yet to be unambiguously demonstrated.

VIII. *IN VITRO* TRANSCRIPTION OF VIROID RNA WITH PURIFIED POLYMERASES

A. Transcription with DNA-Dependent Plant RNA Polymerases

The assumption that the DNA-dependent RNA polymerase II of the host cell might be involved in viroid replication was tested in *in vitro* experiments (Rackwitz *et al.*, 1981). For this purpose, the enzyme had been purified from tomato tissue and wheat germ. With either enzyme, ssDNA templates were found to be transcribed less efficiently than dsDNA templates and transcription of ssDNA templates was considerably improved in the presence of oligo-RNA primers.

Synthetic and natural RNAs also served as templates for transcription, thus confirming previous results with the homologous enzyme from pea and cauliflower (Sasaki *et al.*, 1974a,b, 1976). TMV RNA and soluble RNA (5 S RNA and tRNA) from yeast were relatively poor templates, whereas the synthetic template/primer poly(rA)/oligo(rU) was transcribed with high efficiency. Most surprisingly, however, of all natural RNA templates tested, viroid RNA was transcribed with the highest efficiency by DNA-dependent RNA polymerase II from tomato or wheat germ. Although overall transcription was about the same when circular or linear PSTV RNA molecules were used as templates, the pattern of product formation varied slightly among different template preparations. When the transcription products were analyzed under denaturing conditions in which circular and linear viroid molecules are clearly separated, it became evident that the largest transcripts from both polymerases had the same migrating properties and hence the same size as the linear viroid molecule. Several control experiments demonstrated that this RNA is a genuine transcript and not due to terminal labeling of linear viroid template molecules. In addition to the full-length copies, several smaller viroid transcription products were found. Further experiments are needed to decide whether the latter originate by site-specific nicking or by run-off transcription on randomly nicked template molecules.

The highly specific interaction between viroid RNA and polymerase II was further substantiated by the inhibition of transcription at 10^{-8} M α-amanitin and by binding studies between ^{125}I-labeled PSTV RNA and

tomato polymerase II using the cellulose filter technique and by Northern blot analysis. Viroid RNA was found to form a binary complex with polymerase II, to compete with DNA for the template binding site on the enzyme, and to strongly inhibit DNA-directed RNA synthesis.

Recent investigations have shown that polymerase II initiates specifically at nucleotides 168, 300, and 49 of PSTV, CSV, and CEV, respectively. It was found, in addition, that polymerase III also is capable of specific initiation and that it transcribes viroid RNA into complete copies whereas, so far, polymerase I has produced only smaller transcription products at very low yield (unpublished work in the author's laboratory).

B. Transcription with RNA-Dependent Plant RNA Polymerases

Higher plants are characterized by the presence of an RNA-dependent RNA polymerase which, therefore, is another candidate for viroid replication. Such enzyme activities have been detected in several healthy plant species (Duda *et al.*, 1973; Duda, 1976; Ikegami and Fraenkel-Conrat, 1978, 1979b; Romaine and Zaitlin, 1978; Lazar *et al.*, 1979; Takanami and Fraenkel-Conrat, 1982), including tomato (Boege and Sänger, 1980), a well-known host plant for many viroids. An RNA-dependent RNA polymerase activity is increased about two- to sixfold after infection by various RNA plant viruses (Fraenkel-Conrat, 1979) and especially cucumber mosaic virus has been reported to elicit particularly high enzyme activity in cucumber cotyledons (Gilliland and Symons, 1968; May *et al.*, 1969; May and Symons, 1971). It thus appears that upon virus infection these enzymes are taken over for the replication of the viral RNA, a conjecture that is still controversial. In analogy with the well-characterized RNA replicase from *Escherichia coli* infected with RNA bacteriophage Qβ (Kamen, 1975), the RNA-dependent RNA polymerase in virus-infected plants has also been designated "RNA replicase." Although the catalytical and biochemical properties of the RNA replicase seem to be very similar to those of the RNA-dependent RNA polymerase preexisting in the uninfected plant cell, the actual relationship between these two enzyme activities is still unresolved. Therefore, the enzyme from healthy plants should be called RNA-dependent RNA polymerase. Its biological role in plant cell metabolism is still unknown. It has been suggested that it may serve to synthesize dsRNAs, possibly with regulatory functions (Ikegami and Fraenkel-Conrat, 1979b).

Regarding viroid replication, it was found that RNA-dependent RNA polymerase from virus-free healthy tomato leaf tissue accepts PSTV RNA as template and produces full-length PSTV copies *in vitro* as judged from their electrophoretic mobility on denaturing 5% polyacrylamide gels and from molecular hybridization on Northern blots under stringent conditions (Boege *et al.*, 1982). Viroid transcription requires the presence of Mn^{2+} and/or Mg^{2+} ions and is not inhibited by 10^{-5} M α-amanitin. The

percentage of viroid template copying under optimal conditions was found to vary around 0.1%. Of special interest is the appearance of high-molecular-weight products that barely enter the 2.5% stacking gel or the 5% resolving gel. With other RNA templates, these enzymes were found to produce extremely heterodisperse transcription products of low molecular weight, irrespective of the nature and size of the template or the source from which they were isolated (Duda, 1976; Romaine and Zaitlin, 1978; Chifflot *et al.*, 1980). Therefore, the full-length viroid copy is the first well-defined and homogeneous product reported to be synthesized *in vitro* by an RNA-dependent RNA polymerase from healthy plants. A comparable synthesis of distinct transcription products has so far only been reported for the RNA-dependent RNA replicase from barley leaves infected with brome mosaic virus (Hardy *et al.*, 1979).

Regarding the relation between the structure of the RNA templates and their transcription efficiency, it was previously found that RNA-dependent RNA polymerase transcribes ssRNA more efficiently than dsRNA (Ikegami and Fraenkel-Conrat, 1978, 1979a,b; Fraenkel-Conrat, 1979; Boege and Sänger, 1980). In view of these results, the observed viroid transcription into full-length copies is not too surprising, because viroid RNA displays features of both ds- and ssRNAs. The elucidation of the details of the transcription process and of the nature of the transcripts that are larger than unit length requires further biochemical analysis.

IX. VIROID REPLICATION AS INITIATED BY CLONED VIROID DNA

Although unexpected findings have been a common experience in viroid research, it was, nevertheless, a surprise when Cress *et al.* (1983) reported that despite the RNA–RNA pathway of normal viroid replication, ds PSTV-specific DNA as produced by molecular cloning is infectious. They showed that dimeric forms are capable of initiating a normal PSTV RNA replication cycle when mechanically inoculated into tomato seedlings by leaf rubbing in the presence of carborundum as abrasive, as is commonly done with the PSTV proper. Interestingly, the monomeric forms exhibited at best only very low activity in initiating viroid replication. The progeny viroid synthesized in the inoculated plants proved to be normal PSTV RNA with a sequence complementary to that of the cloned PSTV DNA used for inoculation. Similar results were obtained when certain cloned monomeric and oligomeric ds cDNAs of HSV were mechanically inoculated onto cucumber plants (Meshi *et al.*, 1984). These data could be confirmed and extended in three aspects of general interest by Tabler and Sänger (1984). It was found that (1) not only ds PSTV DNAs but also all bacteriophage M13-cloned dimeric and multimeric ss PSTV DNAs can initiate PSTV RNA replication, irrespective of whether they represent the PSTV (+) or (−) DNA strand; (2) that certain cloned mon-

omeric ss, as well as ds, PSTV DNAs exhibit the same initiation activity; and (3) that "noninfectious" subgenomic *AvaI* fragments of the cloned PSTV DNA consisting in their (+) strand of 167 and 192 nucleotides, respectively, are capable of initiating normal PSTV replication when inoculated as a mixture. Again, it could be shown by molecular hybridization that the inoculated PSTV-specific cloned DNAs are not replicated as such in the tomato plants but that predominantly circular and some linear PSTV RNA molecules of unit length accumulate as in the case of a normal viroid infection.

From all these data the question arises as to how these DNAs initiate the viroid RNA replication cycle, which normally does not involve DNA. The subsequent accumulation of only viroid RNA progeny proves that the DNA must have served as template for PSTV RNA synthesis. It is still unknown which of the three nuclear DNA-dependent RNA polymerases of the host plant accepts and transcribes the cloned viroid DNA. Evidently, multimeric viroid RNA transcripts are formed that are then processed to the monomeric circular viroid proper.

A. Infectivity of Cloned Double-Stranded Viroid DNA

The ability of cloned ds viroid DNA to initiate the synthesis of ss viroid RNA progeny resembles that of the cloned dsDNA copies of the linear RNA genomes of bacteriophage Qβ (Taniguchi *et al.*, 1978) and poliovirus (Racaniello and Baltimore, 1981) to initiate synthesis of the respective viral RNAs. But the intact recombinant DNAs of these two viruses were highly infectious when they contained a monomeric cDNA insert. Primary transcription of these cloned DNAs by the DNA-dependent RNA polymerases of the host could initiate within either plasmid sequences or the adjacent homopolymeric "tails," but the full-length PSTV cDNA clones do not contain such homopolymeric "tails." Neither Qβ phage production nor PSTV replication depends upon orientation of the cDNA insert, which indicates that no promoter is active in the plasmid vector. The low infectivity of the vector-inserted viroid units is greatly increased after their excision from the vector DNA (Table I). This increase in infectivity can be explained by assuming that, prior to their transcription, the excised monomeric viroid DNA templates are recombined *in vitro* to multimeric templates from which (as in the case of normal viroid replication) multimeric viroid RNA transcripts are generated, which then undergo cleavage and ligation to circular PSTV monomers. Under these premises, it is quite plausible that dimeric and higher multimeric forms of head-to-tail-connected, ds viroid DNA units are highly infectious, because only these DNAs allow the transcription into sufficient quantities of (+) or (−) viroid RNA molecules which represent the entire PSTV genome and multimers thereof from which the normal replication cycle can start.

TABLE I. Autoexcision, Self-Cleavage, and Self-Cyclization of RNAs

System	Type of RNA processed	Requirements for the process	Termini of RNA Products 5′	Termini of RNA Products 3′	Subsequent self-cyclization
Autoexcision					
Tetrahymena	Pre-rPNA IVS	G or GTP	OH-	2′,3′ P	Yes
Neurospora	Cytochrome *b* mRNA IVS	G or GTP	P-	3′-OH	Yes
Yeast	Mitochondrial RNA IVS	G or GTP	P-	3′-OH	Yes
Self-cleavage					
Phage T_4	Precursor RNA p2Spl	Mg^{2+}	OH-	P	No
PSTV	Dimeric (+) RNA	Mg^{2+}	?	?	No
ASBV	Dimeric (+) and (−) RNA	Mg^{2+}	OH-	2′,3′ P	No
RNase P					
E. coli	Precursors of tRNA	M1RNA	P-	3′-OH	No

The assumption of *in vivo* recombination of the inoculated viroid cDNA gains support from the finding that coinoculation of two noninfectious subgenomic PSTV DNA fragments leads to a viroid infection. When inoculated together, these fragments are presumably ligated *in vitro* to a complete and hence infectious DNA complement of the viroid RNA genome. The existence of appropriate termini predisposes these fragments to become ligated in the correct fashion. Such *in vivo* DNA ligation has already been proposed to explain how cloned noninfectious DNA fragments of the cauliflower mosaic virus (CaMV) DNA genome regain infectivity when inoculated simultaneously (Lebeurier *et al.*, 1982).

Details of how PSTV DNA is transcribed into (+) or (−) PSTV RNA have yet to be delineated. In the case of the viroid DNA still inserted in the vector DNA, transcription could start inside the prokaryotic vector sequences and then proceed through the viroid DNA.

B. Infectivity of Cloned Single-Stranded Viroid DNA

Cloned ss PSTV DNAs of (+) and of (−) polarity are capable of inducing PSTV RNA replication and disease symptoms in tomato plants. This is the first example where a recombinant ssDNA of a pathogen is infectious. This finding is rather surprising since ssDNA, which is normally not present in eukaryotic cells (except in certain small viruses whose DNA genome is circular), is nevertheless able to give rise to a replicable pathogenic RNA genome. The replication of the ssDNA vi-

ruses, which include the gemini viruses, depends on the replication functions of the host which convert the ss viral DNA to a ds molecule, which then serves as a template for transcription. One could assume that the infectious circular ss M13 DNA containing one to four units of PSTV DNA is made ds after it has entered the tomato leaf cells. However, since this ssDNA is also accepted by the DNA-dependent RNA polymerases I, II, and III *in vitro* (unpublished results from the author's laboratory), the possibility exists that the cloned ss PSTV DNA is directly transcribed into PSTV RNA. It cannot be distinguished at present which of these two modes of transcription is actually responsible for the infectivity of the M13-cloned ss PSTV DNAs of (+) or (−) polarity.

The interpretation of the infectivity of the M13-cloned ss PSTV DNA is complicated by the finding that the ss monomeric *Bam*HI unit of (+) polarity is highly infectious, whereas the corresponding (−) form is not. One would expect, therefore, that the cloned ds *Bam*HI monomer would be infectious, even if still inserted in its vector, because it contains the potentially infectious *Bam*HI (+) DNA strand. However, the ds monomeric *Bam*HI PSTV DNA unit cloned into pBR322 or into M13mp11 in (−) orientation had a very low specific infectivity. This is in accordance with the situation in all conventional viruses with a circular DNA genome. On the other hand, the dsDNA of the M13mp11 construct carrying a (+) *Bam*HI unit of PSTV DNA had a significantly higher infectivity index. The obvious discrepancy between the results of the infectivity assays obtained with M13-inserted *Bam*HI clones depending on the polarity of ssDNA and on the orientation of insertion of the dsDNA led to the analysis of the sequences adjacent to the junction between the M13 vector DNA and PSTV DNA. In the mp11 strain of bacteriophage M13, the *Sma*I site is positioned at the right side of the *Bam*HI site. Coincidently, the sequence of 11 nucleotides (GGATCCCCGGG), which creates the two adjacent *Bam*HI and *Sma*I restriction sites in the polylinker of the mp11 strain of bacteriophage M13, is also present in the PSTV sequence. Consequently, a *Bam*HI monomer that is inserted into the *Bam*HI site of M13mp11 in (+) orientation results in a clone that possesses this segment of 11 nucleotides twice. Therefore, this recombinant clone consists of the complete 359 nucleotides of the monomeric PSTV sequence plus 11 PSTV-specific nucleotides that originate from the vector. The (−) orientation of insertion of the *Bam*HI unit, or the insertion in both possible orientations into the *Bam*HI site of plasmid pBR322, however, will only lead to a clone consisting of 359 plus 6 PSTV-specific nucleotides originating from the vector. This difference of five nucleotides seems to be essential for the infectivity of the cloned PSTV DNA. Interestingly, the region in question is part of the central region that is strictly conserved in all viroids sequenced so far, with the exception of ASBV. In fact, because of this strict conservation, it has been postulated that this central region is essential for viroid replication. Site-specific mutations will hopefully help to determine the importance of individual nucleotides in this region for the replication and subsequent processing of the PSTV RNA molecule.

C. Viroid-Related RNA Transcripts in *E. coli*

During the cloning of viroid cDNA, the interesting question arose as to whether or not infectious viroid-related RNAs are produced in the *E. coli* strains that harbor the various recombinant plasmids. In one study, Cress *et al.* (1983) inserted the cloned PSTV cDNA sequences downstream from either the tet or lac UV5 promoter in the plasmids pBR322 and pGL101H, respectively. Theoretically, such a construction was expected to permit the transcription of the PSTV cDNA sequences in *E. coli* under the control of these efficient promoters. For analysis they extracted the RNA from exponentially growing *E. coli* cultures and determined the presence of any PSTV RNA transcripts in *E. coli* and also their polarity by Northern blot analysis of the RNA after its electrophoretic fractionation under fully denaturing conditions using strand-specific probes. These studies revealed that each of the four *E. coli* clones tested contained PSTV-related RNAs that were heterogeneous in size but of only one polarity. Susceptibility to pancreatic RNase digestion proved that the hybridizing material was RNA. Apparent discrete PSTV-related RNAs between 359 and 718 nucleotides in length were visible in all four RNA preparations analyzed and the transcripts from the dimeric cDNA were noticeably longer than those from the monomeric cDNA. RNA transcripts having (+) polarity like PSTV proper were more abundant than PSTV (−) RNA transcripts. *E. coli* harboring the pGL101H plasmids contained a higher concentration of PSTV-related RNA than the pBR322 recombinants. The total RNAs extracted from *E. coli* were assayed by mechanical inoculation onto tomato seedlings, resulting in subsequent appearance of the characteristic disease symptoms, and by dot-spot hybridization. Infectivity from the total *E. coli* RNA was observed for a recombinant dimeric clone that produces PSTV (+) RNA transcripts but not with the corresponding one producing PSTV (−) RNA transcripts. A low level of infectivity was also obtained from the PSTV (+) RNA transcribed from a monomeric cDNA clone, suggesting that RNAs containing nonduplicated PSTV sequences can also be replicated in the host cell. The (−) RNA produced in *E. coli* from the corresponding monomeric clone did not produce an infection.

The presence of high levels of infectious PSTV RNA transcripts in *E. coli* cells was also observed when they harbored a pBR322 plasmid containing four head-to-tail-connected *Bam*HI units of PSTV DNA in (+) orientation (Tabler and Sänger, 1984). In this case, the insertion was such that the PSTV (+) DNA strand is connected with the strand of the plasmid DNA that is numbered from the 5′ to the 3′ terminus by convention and denoted the (+) strand. The infectious RNA transcripts produced in *E. coli* were therefore PSTV (+) RNA molecules. In both studies the RNA nature of the infectious entities was established by their RNase sensitivity and DNase resistance.

All these data show that the RNA-synthesizing machinery of *E. coli* is in principle capable of synthesizing *in vivo* infectious viroid RNA

molecules, provided the appropriate viroid-specific and potentially infectious cDNA is introduced into this prokaryote by means of recombinant DNA techniques. It is not surprising, however, that the transcripts obtained are predominantly heterogeneous in size because the RNA processing system active in *E. coli* cells is certainly different from the one operating in the viroid host plants and generating the viroid proper. Further examination of the size and structure of infectious viroid RNAs synthesized in *E. coli* cells upon introduction of appropriate recombinant plasmid DNAs may, nevertheless, help to clarify the precise structural requirements for the infectivity and replicability of viroid RNA and DNA molecules.

X. *IN VITRO* SYNTHESIS OF VIROID RNA WITH RNA TRANSCRIPTION SYSTEMS

In its early days, viroid research was greatly hampered by the very limited amounts of highly purified viroid RNA available. With the development of various efficient purification procedures, rapid progress was made in the elucidation of the structure of viroids and their biochemical and biophysical properties *in vitro*. At present, this problem of scarcity of material has come up again but now with the short-lived primary transcripts of the viroid replication process and the delineation of the molecular mechanism of their synthesis. Unlike the viroid proper, these oligomeric intermediate forms of (+) and (−) RNA are usually present in the infected plant tissue only at such extremely low levels that their isolation and purification in the amounts required for biochemical studies will be very cumbersome if not impossible. Unlike the viroid proper, these intermediates cannot be visualized by one of the highly sensitive silver staining procedures and they are only detectable on Northern blots with the aid of molecular hybridization. For this purpose, (+)- and (−)-strand-specific, ss viroid cDNAs obtained by molecular cloning in bacteriophage M13 have been used as probes. This technique allows estimation of the size of the intermediate RNA forms of viroid replication and determination of their sensitivity against nucleases and transcription inhibitors. On the basis of these data, various models for the mechanism of replication and processing of viroid RNA could be developed. Future work will have to verify these models by delineating the different steps of these processes and by analyzing the corresponding molecules biochemically.

One way to approach these problems experimentally involves the *in vitro* synthesis of biochemical amounts of RNA molecules analogous to the short-lived replicative intermediates and to use them as substrates for investigating the various transcription and processing activities in *in vivo* and *in vitro* experiments. Moreover, such *in vitro*-synthesized ssRNAs are excellent probes for blot and *in situ* hybridization, because they are at least about ten times more sensitive than nick-translated DNA probes

with the same number of counts and because the RNA–RNA hybrids are of greater stability. In view of the very promising perspectives for future investigations on viroid replication, the synthesis of viroid RNA with the aid of *in vitro* RNA transcription systems and their application will be discussed in detail.

A. Viroid Synthesis with Bacterial RNA Polymerase/Promoter Systems

Ohno *et al.* (1983) first used a bacterial RNA polymerase/promoter system to synthesize viroid RNA *in vitro*. The availability of various cloned and infectious multimeric viroid DNAs prompted them to insert their tandemly repeated dimeric and tetrameric HSV cDNA sequences into plasmid pGL101, which is a derivative of plasmid pBR322 that carries a 100-base-pair lac UV5 promoter. The recombinant cDNA was inserted downstream from the promoter which is specific for the RNA polymerase of *E. coli*. After amplification to sufficient quantities and after appropriate linearization, this recombinant DNA was used as template in an *in vitro* transcription system. Under the control of the promoter present on the template, purified *E. coli* RNA polymerase synthesized the corresponding PSTV RNAs in the test tube in a so-called "run-off" transcription process. Based on the orientation of insertion, the transcripts synthesized *in vitro* with *E. coli* RNA polymerase were expected to contain dimeric and tetrameric HSV (+) or (−) RNA, respectively. Except for the extra 36 bases at their 5′-terminus, which are derived from the lac promoter region, and the 138 bases at the 3′-terminus originating from the plasmid pGL101, these *in vitro*-produced multimeric viroid RNAs are equivalent to the presumed intermediates of viroid replication detectable *in vivo*.

Bioassays on cucumber plants revealed that all dimeric and tetrameric HSV (+) RNAs were highly infectious, whereas the infectivity of the ones of (−) polarity was low. However, in a subsequent study, the same authors reported that the HSV (−) RNAs were noninfectious (Ishikawa *et al.*, 1984).

It should be mentioned that Fig. 3 of Ohno *et al.* (1983) clearly shows that the run-off transcripts obtained in the pGL101 system are neither pure nor uniform. This inhomogeneity in the products of transcription is most probably caused by genuine and cryptic promoters present on the plasmid DNA and by the ability of *E. coli* RNA polymerase to initiate transcription not only at all these signals, but also at the termini of the linearized DNA template.

B. Viroid Synthesis with Bacteriophage RNA Polymerase/Promoter Systems

Most of the problems encountered in the previous *in vitro* transcription systems do not seem to exist in two newly developed systems, the

SP6 (Butler and Chamberlin, 1982; Melton *et al.*, 1984) and the T7 (Davanloo *et al.*, 1984) systems which employ purified bacteriophage-encoded RNA polymerase and their associated promoters. Of these the SP6 system is most widely used at present, because an SP6 promoter sequence has been cloned into convenient general-purpose plasmid cloning vectors and SP6 RNA is commercially available from several suppliers.

1. *In Vitro* Synthesis of Viroid RNA with the SP6 RNA Polymerase

The SP6 transcription system (Melton *et al.*, 1984) has been applied for the *in vitro* synthesis of a series of oligomeric linear (+) and (−) PSTV RNAs, both on a preparative scale yielding amounts of up to several hundred micrograms and for producing highly radiolabeled hybridization probes (Tabler and Sänger, 1985). In this detailed investigation, it could be shown that the highly structured PSTV RNA can easily be synthesized by the SP6 RNA polymerase in virtually any size and quantity in a homogeneous and pure form. A transcription efficiency of up to 600 copies per DNA template and several hundred micrograms of (+) and (−) PSTV RNA could be produced routinely on a preparative scale. This efficiency exceeds that of the previously reported SP6 transcription of the DNA of the human β-globin pre-mRNA (Melton *et al.*, 1984) where only 10–20 copies were obtained per template molecule. The comparatively high yields obtained can be related to the higher enzyme concentration and the prolonged time of incubation used for transcription. Since the original plasmid vector pSP62 had been utilized in this study, the PSTV (+) RNAs produced contain 47 and about 15 vector-derived bases at their 5′- and 3′-terminus, respectively, whereas in the PSTV (−) RNAs these foreign flanking sequences consist of 61 and 29 bases, respectively.

2. Infectivity of the SP6-Produced Viroid RNA

Infectivity studies of these *in vitro*-produced PSTV RNAs showed (Tabler and Sänger, 1985) that the PSTV (+) RNA oligomers have the same specific infectivity as the natural PSTV proper, of which as little as 1 ng is sufficient to infect a tomato plant, i.e., to initiate viroid replication. In contrast, the monomeric SP6 PSTV (+) RNA and the corresponding dsDNA template, both with their flanking vector sequences, were noninfectious, which is most probably due to the inability of these composite forms to be properly cleaved and circularized into a correct PSTV RNA monomer. Most surprisingly, however, the corresponding oligomeric PSTV (−) RNAs were at least 10^4-fold less infectious. Infections could only be obtained when at least 10 μg of oligomeric forms was inoculated per plant. The (−) RNA monomers proved to be noninfectious. However, when these multimeric (−) RNAs were partially protected prior to inoculation by mixing or hybridizing them with noninfectious (+) RNA fragments or by "capping" their 5′-termini, an increase in the number of infections was observed. These findings contrast with the previous

observation that all cloned oligomeric ds and ss PSTV DNAs including also those of (−) polarity are highly infectious (Tabler and Sänger, 1984).

XI. THE PROCESSING OF VIROID RNAs

As already discussed, different forms of viroid RNA are detectable in infected plant tissue. The oligomeric forms of (+) or (−) polarity must originate from the infecting circular or linear viroid molecule. Inoculation with *in vitro*-synthesized oligomeric (+) RNAs leads on the other hand to the accumulation of the ordinary monomeric viroid RNA (Ohno *et al.*, 1983; Robertson *et al.*, 1985; Tabler and Sänger, 1985; Visvader *et al.*, 1985; Owens *et al.*, 1986). Thus, it is evident that the various existing forms of viroid RNA are convertible into each other *in vivo*. Of particular interest for the mechanism of viroid replication are the processing reactions, namely the cleavage and ligation processes between the oligomeric forms and the corresponding linear and circular monomers.

A. Interconversion of Monomeric Circular and Linear Viroid Molecules *in Vitro*

Ten years ago, PSTV was discovered to be the first example of a natural circular RNA (Sänger *et al.*, 1976). In addition to the circular form isolated from infected plant tissue, all preparations of PSTV also contained linear monomeric RNA. The relative amounts of circular and linear form depended on the isolation procedures applied. Owens *et al.* (1977) provided evidence that both circular and linear PSTV molecules are infectious. The purified circular RNA of PSTV, CEV, and CSV can be nicked artificially and thus be converted into linear forms in a Mg^{2+}-catalyzed reaction (Sänger *et al.*, 1979). As a consequence, the infectivity rate of these linear PSTV molecules generated by Mg^{2+}-nicking was reduced about 10^3 times compared to the circular form from which they had originated. In addition to these randomly nicked linear forms, a so-called "natural" linear form exists. This form is isolated in Mg^{2+}-free buffers containing EDTA; it is practically as infectious as the circular form—as shown for CSV (Palukaitis and Symons, 1980). The 5′-terminus of a preparation of "natural" linear PSTV RNA was determined by end labeling and subsequent sequence analysis by Kikuchi *et al.* (1982). They could show that natural linear PSTV RNA has nicks between C_{181} and A_{182} or C_{348} and A_{349}. Based on the high specific infectivity of the natural linear viroid RNAs, one can speculate that they represent processing intermediates and that their defined 5′- and 3′-termini allow the *in vivo* ligation to the circular form, in contrast to the randomly Mg^{2+}-linearized PSTV RNAs. This assumption is justified because preparations of natural linear PSTV could be ligated to circular PSTV when incubated in extracts of wheat germ or

Chlamydomonas, which contain ligase activity (Branch *et al.*, 1982; Kikuchi *et al.*, 1982).

Hashimoto *et al.* (1985) compared the infectivity rates of differently *in vitro*-linearized PSTV RNA circles in a detailed study. They found evidence that the nature of the resulting termini rather than their map position is important for the biological activity of the linear forms of PSTV. They obtained a similar infectivity rate, as compared to PSTV circles, when the linear molecules had a 2′,3′ cyclophosphate at the 3′-terminus, irrespective of where the nick was located. Linear molecules with 3′-termini having a 3′-phosphate were significantly less infectious and those without a 3′-phosphate were noninfectious. Interestingly, the type of RNA ligase detected in wheat germ by Konarska *et al.* (1981), joins two ends of an RNA substrate when it possesses a 2′,3′ cyclic phosphate at its 3′-terminus and a 5′-phosphate terminus. Assuming a similar enzyme activity is present in other plants, such as tomato, it would become evident that only linear molecules with a 2′,3′ cyclophosphate, i.e., those produced by RNase U_2, are highly infectious, because they can act as substrate for the RNA ligase. These viroid RNA species could be ligated to the infectious circle, provided their 5′-end had been phosphorylated *in vivo*. At the site of circularization, a 2′-phosphomonoester, 3′,5′ phosphodiester linkage should be formed. In the case of the structurally related virusoids, such an extra 2′ phosphate was indeed detected, but not in the case of viroids. Its absence could, however, be due to phosphatase activities. It remains to be clarified as to whether the circularization of monomeric linear viroid RNA follows this mechanism.

Summarizing, it has now been shown that monomeric circular viroid RNA can be linearized and that some linear forms, including the so-called natural linears, can in principle be circularized *in vitro*. Because the *in vitro* linearization of viroid circles is a more or less artificial catabolic reaction, one is forced to assume that *in vivo* the circular monomeric forms of viroid RNA are made out of linears and not the other way around. The linear monomers, which act as precursors for the viroid circles, must be precisely released from the oligomeric (+) RNAs. It is unclear as to whether the process of cleavage and subsequent ligation proceeds at a defined position of the viroid molecule or whether the processing can occur randomly. The strict conservation in size for some viroids, i.e., PSTV, indicates that a precise processing reaction is necessary.

B. Cleavage of Oligomeric Viroids into Monomers

The oligomeric forms of viroid RNA, which are considered to be replicative intermediates, have thus far not been isolated, because they are present only in low concentrations in the infected tissue. Molecular cloning of viroid cDNA and the application of bacteriophage-encoded RNA polymerases like SP6 or T7 has made possible the synthesis of oligomeric viroid RNAs *in vitro*. The processing of these transcripts,

which should resemble the natural oligomeric RNAs, can now be studied *in vitro*.

Robertson *et al.* (1985) have reported that dimeric PSTV (+) RNAs are cleaved to monomers with an efficiency of 1–5% when incubated under conditions previously described for the autocatalytic self-cleavage of the rRNA of the protozoan *Tetrahymena*. Visvader *et al.* (1985), however, mentioned that they could not observe self-monomerization when they incubated a dimeric (+) transcript of CEV under these conditions. Several controls have to be done in order to test whether the *in vitro* monomerization of PSTV (+) RNA dimers or other viroids follows the same mechanism as elucidated for the self-splicing of *Tetrahymena* or whether host proteins are required, not only for the circularization of (+) RNAs, but also for the monomerization.

An open question is whether there also exist processing reactions on the level of (−) RNAs. As Branch and Robertson (1984) have pointed out, theoretically a monomeric circular (−) RNA species could be formed that would serve as template for the synthesis of the oligomeric (+) RNAs. The presence of such monomeric (−) RNA in infected tissue would indicate that (−) RNA processing exists. Its detection is, however, hampered by the excess of the corresponding monomeric (+) RNA, which obviously migrates to the same position in different gel systems. Due to the intramolecular self-complementarity of all viroid RNAs, strand-specific Northern hybridization is extremely difficult, especially if one compound dominates. The excess monomeric (+) form bound on the nitrocellulose filter may be able to hybridize to some extent to the (+) probe, simulating a (−) signal. On the other hand, the bound (+) form may be able to hybridize to the bound (−) form as well and thus prevent hybridization of the minus probe. Recently, the detection of a signal comigrating with the monomeric circular ASBV was obtained with a probe specific for (−) polarity (Hutchins *et al.*, 1985). Although further analyses and controls are required to prove unequivocally the existence of such a circular viroid RNA of (−) polarity, the processing, i.e., the specific cleavage and circularization, could, at least in the case of ASBV, occur as a possible pathway of viroid replication.

C. Perspectives

In the last couple of years, enormous progress has been made in understanding the various mechanisms of RNA processing. This was greatly stimulated firstly by the introduction of bacteriophage-encoded RNA polymerases, which allow the *in vitro* synthesis of any RNA, provided its cDNA has been cloned. Secondly, it has become possible to prepare enzymatically active extracts of nuclei which allow the study of processing reactions other than the autocatalyzed ones *in vitro*. The combination of these two techniques has led to a deep insight into the mechanism of excision of introns from pre-mRNA as lariats, the 3′ end pro-

cessing, and also the splicing of transfer and ribosomal RNAs. Further investigation will show whether it will be possible to prepare similar nuclear extracts from plants, which exhibit processing activity for viroids. This would allow the direct analysis of the specific cleavage and circularization of *in vitro*-synthesized viroid RNA oligomers. It will be of special interest whether only nuclear extracts of viroid-infected plants will be able to process viroid RNA or whether the enzymes required are preexisting in the healthy host plant.

XII. STRUCTURAL REQUIREMENTS FOR THE REPLICABILITY OF VIROIDS

One of the most interesting problems in viroid research is the question: What makes viroid RNAs autonomously replicable although they do not code for proteins?

This process is far from being understood. In order to address this question, one has to consider the natural structural flexibility of viroid RNAs which still allow replication.

A characteristic structural element of all viroids is their circularity and high degree of self-complementarity, leading to the typical rod-shaped RNA molecule. Sequence homologies among viroid species, however, vary significantly, thus illustrating the fact that very different RNA sequences are in principle replicable, sometimes even on the same host (PSTV, CEV, and CSV, for example, can infect tomato). On the other hand, the sequences of individual viroid species are fairly well conserved. This is surprising insofar as viroids replicate via the RNA–RNA pathway only. Consequently, they are synthesized by one of the RNA polymerases, which are known to exhibit an error rate on the order of 10^{-3}. If there were no selection toward replicability, the viroid sequence should quickly become diverse.

The sequence duplication and also the dimerization of CCCV (see Chapter 2, this volume) illustrate that in addition to the point mutations discussed before, major sequence rearrangements of viroids are possible without interfering with replicability or infectivity. Keese and Symons (1985) have pointed out that a similar partial sequence duplication could possibly have also occurred at the left half of the PSTV sequence (in its customary presentation) during evolution. This sort of "chromosomal" mutation could have also played a role in the rearrangement of functional domains among different viroid species. For each individual domain, various degrees of homology can be seen among different viroid species, indicating that an exchange of sequences could have indeed occurred during mixed infections (Keese and Symons, 1985).

The existence of infectious cDNAs and infectious RNA transcripts thereof will now allow deliberate alteration of the viroid sequence by *in vitro* mutagenesis. Thus, the role of the individual nucleotides for replicability can be studied in detail. So far, however, all attempts to mutate

viroids by manipulating their infectious cDNA have failed. Ishikawa *et al.* (1985) introduced mutations into the HSV sequence by "end polishing" of different restriction sites of their cloned cDNA. All these constructs have been noninfectious. The results of Owens *et al.* (1986) show that even the substitution of a single base, namely a C → U transition at position 284 of PSTV, renders the cDNA noninfectious. Only transformation of *Agrobacterium tumefaciens* by a Ti plasmid containing this mutated viroid cDNA downstream from the efficient 35 S promoter of cauliflower mosaic virus and inoculation of tomato plants with the bacteria induced infection. The viroid RNA progeny, however, consisted of reverted RNA. This illustrates dramatically that the biological properties of the viroid are very sensitive to even slight alterations of its primary structure. This finding is rather surprising because the newly introduced U_{284} should still be able to base-pair to some extent with the G_{76}. Furthermore, such a U residue is found in CEV, CSV, and TASV just outside the lower portion of the central conserved region and is therefore the first nucleotide in which PSTV differs from the other viroids mentioned before.

In addition to the introduction of point mutants into PSTV, the *in vitro* recombination of two different viroids has been tested (Owens *et al.*, 1986). For this purpose the cDNAs of PSTV and TASV have been connected via the central conserved region which they have in common. When testing these tandem chimeric viroid constructs, an infection with either PSTV or TASV was observed. The "monomeric" chimeric constructs, however, were noninfectious.

Considering these mutagenesis experiments, we have to be aware that the viroid structures that we observe in nature are highly adapted toward replicability and that they are not accidentally formed. Minimal changes lead to abolition of replication and therefore also to loss of infectivity, although the viroids do not code for proteins. On the other hand, we have seen that the viroids are able to mutate to some extent naturally on the level of individual nucleotides and by rearrangement of major parts of the genome. Further experiments will have to be done to better understand which parts of the viroid molecules are tolerant to nucleotide changes and which are unalterable.

XIII. STRUCTURAL REQUIREMENTS FOR THE PROCESSING OF VIROID RNA

As we have seen, the processing of viroid RNAs is a central step of their replication. The capacity of the oligomeric viroid (+) RNA of being specifically cleaved to monomers and subsequently ligated to circles must be predetermined by the viroid sequence, as are the signals for mRNA splicing or 3′ end processing. The processing of viroids has so far been studied mainly in an indirect approach, namely by infectivity assays of cloned cDNA constructs of different viroids or RNA transcripts thereof.

Before analyzing the structural requirements for processing of viroid RNA, we have to briefly summarize the results of these infectivity assays.

The various infectivity tests of wild-type sequences have shown firstly that the (+) RNA forms are infectious and not the (−) form (Ishikawa *et al.*, 1984; Tabler and Sänger, 1985). Therefore, the infection observed after inoculation with different forms of cloned viroid cDNA (Cress *et al.*, 1983; Meshi *et al.*, 1984; Tabler and Sänger, 1984) can be related to *in vivo* transcription of these DNAs into (+) RNA since the cDNA does not replicate. Presumably the infectious ssDNAs of (+) or (−) polarity are completed to duplex DNA before transcription.

Secondly, bioassay has revealed that DNA constructs or RNAs transcribed thereof are only infectious if they are dimers or contain at least parts of the viroid genome duplicated, or if the inoculated constructs are ligatable *in vivo* to such longer-than-unit-length products (see Diener, 1986). For example, all dimeric viroid DNA constructed are highly infectious (Cress *et al.*, 1983; Meshi *et al.*, 1984; Tabler and Sänger, 1984), whereas monomeric constructs are at best marginally infectious when flanked by vector sequences. If the monomeric DNA is, however, exactly excised by restriction enzymes, the rate of infectivity is greatly increased (Meshi *et al.*, 1984; Tabler and Sänger, 1984). The protruding ends of the restriction fragments can be religated *in vivo* to form oligomeric or circular RNAs allowing the transcription of longer-than-unit-length (+) forms. The occurrence of such *in vivo* ligation could be demonstrated by testing for infectivity separate subgenomic DNA fragments which together represent the complete PSTV genome. Coinoculation of these two fragments resulted in infection of tomato plants (Tabler and Sänger, 1984).

In addition to such DNA constructs, which allow *in vivo* ligation to circles or oligomers, at least partially duplicated DNA or RNA constructs are infectious and therefore biologically active. This can only be interpreted by assuming that in all tests performed, a (+) RNA circle has to be formed which then initiates normal viroid replication. Hence, indirectly, the bioassay tests whether a given (+) RNA will be properly processed, i.e., whether a monomeric viroid RNA is specifically cleaved out and then circularized. As a further consequence, one has to assume that all constructs that are marginally infectious or noninfectious are either processed with low efficiency or not processed at all. In principle, it does not matter whether a DNA construct is tested or whether the (+) RNA is bioassayed directly. Because the DNA to be analyzed requires an additional *in vivo* transcription step, its specific infectivity can be expected to be lower. This is in line with the experimental data which show that the infectivity levels of SP6 (+) RNAs of PSTV are about two orders of magnitude higher than those of equal amounts of the corresponding DNAs (Tabler and Sänger, 1984, 1985).

The finding that head-to-tail-connected dimers are highly infectious in contrast to monomers is not restricted to viroids. Similar observations have been made for DNA viruses with circular genomes, such as SV40, cauliflower mosaic virus (Lebeurier *et al.*, 1982), or cassava latent virus.

Assuming that circularization proceeds at a defined position, it is, as in the case of viroids, unlikely that a linear monomer of the viral genomes constructed by molecular cloning has the correct "beginning" which would allow circularization. Furthermore, the vector sequences adjacent to the monomer would also inhibit correct circularization.

Theoretically, cleavage and correct subsequent circularization of viroid (+) RNA can only be expected when the linear precursor RNA possesses the processing site twice and when the two copies are located one genome length apart from each other. In order to delineate the position of this "circularization site," one has to consider constructs that are smaller than a genuine head-to-tail-connected dimer, but still able to induce infectivity because of the duplication of the relevant processing domain.

There are several indications from different viroids that processing occurs within the central conserved region. Since this stretch of about 20 nucleotides is highly conserved among the different viroid species (with the exception of ASBV), it has already been speculated that this part of the viroid genome has an essential function.

Meshi *et al.* (1985) have deleted a dimeric cDNA clone of HSV from both ends. Clones longer than one HSV genome are highly infectious only when they possess a duplicated region of about 60 nucleotides. This region, which can presumably be even smaller, contains the upper portion of the central conserved region, indicating that this domain is indeed involved in the processing of viroid RNA.

As discussed previously, a full-length cDNA clone consisting of the entire 359 nucleotides of PSTV plus 11 vector-derived, but PSTV-specific nucleotides (numbers 87–97) had a significantly higher level of infectivity than the corresponding clone with a duplication of only 6 nucleotides (numbers 87–92) (Tabler and Sänger, 1984). The duplication of 5 nucleotides (numbers 93–97) (CCGGG), positioned within the central conserved region, seemed to be essential for infectivity. Visvader and Symons (1985) have mutated their infectious cDNA clone of CEV at the G_{97} position in the middle of these 5 nucleotides into an A or U residue and shown that the excised mutated cDNA itself was not infectious. When, however, the mutation was located at the left half of the duplication, with the right half remaining unmutated, the construct was infectious, leading to wild type CEV RNA progeny. Therefore, the authors concluded that cleavage occurs at one of the three G nucleotides 97–99 of CEV, corresponding to nucleotides 95–97 in PSTV. This suggested position of the processing site would be in line with the model proposed by Diener (1986), in which he shows that all partially duplicated viroids can assume a very stable base-paired structure involving the central conserved region and secondary hairpin I. This structure is assumed to be essential for precise cleavage and subsequent ligation into circular molecules.

Our unpublished studies suggest that other positions than that proposed by Visvader and Symons can act efficiently as processing sites. Further analysis has to be done in order to find out whether there exists

a single processing site or more than one and where this site (these sites) is (are) located.

It will also be necessary to study the processing reactions directly rather than by the indirect approaches discussed so far. A first attempt was made by Robertson *et al.* (1985) who reported that PSTV RNA dimers can monomerize when incubated in a neutral tris-HCl buffer containing ammonium and magnesium ions. The site of cleavage was determined to be between positions 250 and 270. This position does not correlate with the cleavage site as indicated by the infectivity data. Thus, it remains to be determined how far these *in vitro* observations are relevant *in vivo*. An alternative approach that relates more closely to the *in vivo* situation will be to directly study the processing of oligomeric viroid RNAs by incubating them in a nuclear extract of their host plant.

XIV. CONCLUSIONS

1. Viroids are most probably not translated into a polypeptide, and all components required to replicate the infecting viroid RNA molecule must preexist in the host cell into which it has been introduced.
2. All viroids studied so far are replicated by direct RNA–RNA transcription without a viroid- or host-specific DNA being involved.
3. As the end product of viroid replication, monomeric circular viroid (+) RNA molecules, i.e., the "mature" infectious viroid proper, accumulate predominantly.
4. This mature viroid is usually accompanied by a small and variable fraction of infectious monomeric linear forms which have either not yet become circularized or/and which could have been generated from the mature viroid circles by nucleolytic cleavage.
5. Multimeric viroid (+) and (−) RNA strands and complexes containing both forms are detectable by molecular hybridization in viroid-infected cells, suggesting that the synthesis of at least one if not of both strands proceeds in a "rolling circle"-like mechanism.
6. Inhibition studies on isolated nuclei have revealed that the first step in viroid replication, i.e., transcription of the infecting monomeric viroid (+) RNA into multimeric (−) RNA copies, is performed by the nucleoplasmic DNA-dependent RNA polymerase II, whereas the second step, transcription of this multimeric (−) RNA into (+) RNA multimers, is performed by the nuclear DNA-dependent RNA polymerase I.
7. The monomeric circular viroid (+) RNA progeny molecules are generated from their multimeric precursors in a process reminiscent of RNA splicing, i.e., by specific cleavage and ligation which

may combine self-splicing-like capabilities inherent in the replicating RNA itself with the action of preexisting host enzymes.

8. The virusoids and certain viral satellite RNAs, two other classes of small pathogenic RNA molecules, form structures in infected cells which suggest that their mechanism of replication is very similar in many respects to that of the viroids.

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CHAPTER 6

VIROID FUNCTION
Molecular Biology of Viroid–Host Interactions

ROBERT A. OWENS AND ROSEMARIE W. HAMMOND

I. VIROID PATHOGENESIS—A STRUCTURE/FUNCTION PARADIGM

Viroids provide a unique opportunity to study the relationship between the structure of an RNA molecule and its various biological functions. These unusual pathogens are sufficiently small that independent experimental and theoretical studies have produced identical models for their secondary structure (see Chapter 3, this volume). The recent demonstration that viroid cDNAs cloned by recombinant DNA techniques and their RNA transcripts are infectious (e.g., Cress *et al.*, 1983; Ohno *et al.*, 1983a; Tabler and Sänger, 1984) allows the use of site-specific mutagenesis techniques to probe the structure/function relationships suggested by comparative sequence analysis.

Current models for viroid pathogenesis provide an instructive paradigm for viroid structure/function relationships, but design of experiments to test certain of their predictions can be complicated by the many uncertainties/inconsistencies that remain unexplained. Our discussion of site-specific mutagenesis begins by comparing such models, but we emphasize alternative approaches and strategies rather than specific (but as yet unpublished) experimental results. Specific *in vivo* and *in vitro* assays for individual viroid functions are urgently needed to complement currently used bioassay techniques for characterizing altered viroid cDNAs.

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A. Current Models for Viroid Pathogenesis

Useful models of viroid pathogenesis must accommodate the observation that viroids replicate without detectable symptoms in many of their hosts (Diener, 1982). Because PSTV accumulates in nuclei of infected cells and appears to lack mRNA activity *in vitro* and *in vivo*, Diener (1971, 1977) suggested that PSTV may function as an "abnormal regulatory RNA" that interacts directly with its host cell. The concept of viroids as abnormal regulatory RNAs has been widely accepted (see Zaitlin, 1979) and provides the basis for the models of viroid pathogenesis discussed below.

Early models (e.g., Diener, 1977) were closely related to contemporary models for eukaryotic gene regulation (Britten and Davidson, 1969; Davidson and Britten, 1979). Since that time, portions of the PSTV and CEV sequences that control symptom expression have been identified by comparative analysis of viroid primary and secondary structures. Current models for viroid pathogenesis attempt to explain how independent field isolates of PSTV or CEV with almost identical sequences can differ greatly in their pathogenicity while viroids exhibiting much greater sequence divergence induce very similar symptoms.

Comparative sequence analysis of PSTV (Schnölzer *et al.*, 1985) and CEV sequence variants (Visvader and Symons, 1985) has correlated differences in symptom expression with sequence changes in the "pathogenesis" and "variable" regions of the native structure (see Fig. 1). All PSTV isolates examined thus far contain 359 nucleotides, and sequence differences among the various isolates are comparatively minor. Only changes in a "virulence modulating" region on the left side of PSTV were correlated with pathogenicity in tomato (see Fig. 1). CEV variants could also be grouped according to the symptoms produced in tomato, but members of the two resulting groups contain a variable number of nucleotides (370–375) and at least 26 sequence differences. Unable to correlate CEV pathogenicity with changes in a single region, Visvader and Symons (1985) suggested that viroid pathogenicity may be influenced by sites within either or both the "pathogenesis" and "variable" regions.

Current models for viroid pathogenesis are based on the assumption that a viroid's structure *in vitro* can be used to predict its behavior *in vivo*. Flores (1984) has suggested that viroid–host interactions may be controlled by static conformational features of the viroid, i.e., the number and type of bulged nucleotides within the region diagrammed in Fig. 2. Noting that nucleotide changes within this region of PSTV alter its structural stability, Schnölzer *et al.* (1985) have proposed that the relative ability of this "virulence modulating" region to undergo premelting transitions during thermal denaturation *in vitro* parallels its ability to interact with unspecified host components *in vivo*.

Although PSTV symptom severity can be correlated with either the number and size of bulge loops in the "virulence modulating" region or

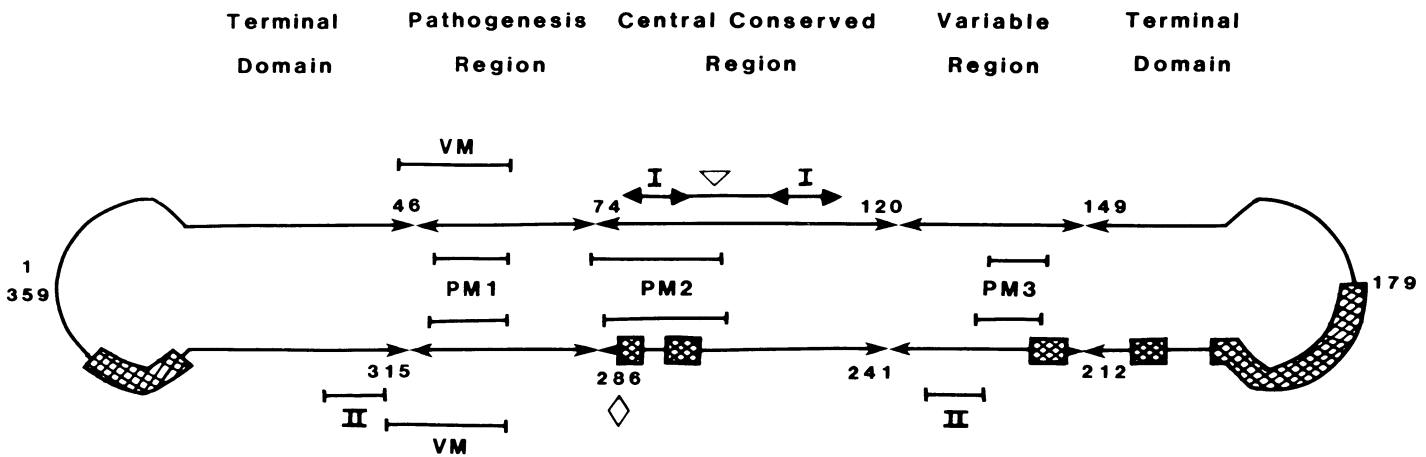

FIGURE 1. Relative locations of PSTV nucleotides important for its structure *in vitro* and its function *in vivo*. Boundaries of the five structural and functional domains proposed by Keese and Symons (1985) are indicated by solid arrowheads within the native structure of PSTV; nearby numbers give exact nucleotide positions. Nucleotides comprising premelting regions (PM) 1–3 and the "virulence modulating" (VM) region (Schnölzer *et al.*, 1985) as well as secondary hairpins I and II (Chapter 3, this volume) are shown by bars inside or outside the native structure. Nucleotides forming the stem of hairpin I are found at the boundaries of a palindrome believed to be involved in processing of multimeric replicative intermediates (Diener, 1986). The shaded boxes indicate sequences homologous to the "box" sequences of group I introns (Chapter 7, this volume). Unique restriction sites in PSTV cDNA are depicted by the open triangle (*Bam*HI) and diamond (*Hin*dIII).

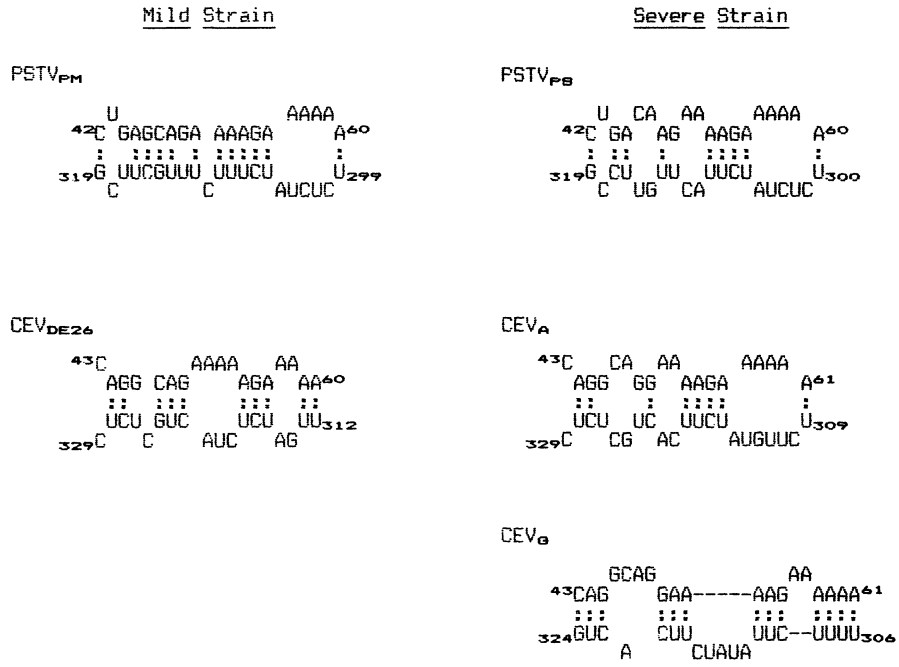

FIGURE 2. Alternative conformations for the "virulence modulating" regions of PSTV and CEV. Homologous portions of published secondary structures for various isolates of PSTV and CEV have been redrawn from the original references (PSTV$_{pm}$, Schnölzer *et al.* (1985); PSTV$_{ps}$, Sänger (1982); CEV$_{DE26}$ and CEV$_{A}$, Visvader and Symons (1985); CEV$_{G}$, Gross *et al.* (1982)). PSTV$_{PM}$, PSTV$_{PS}$, and CEV$_{A}$ are structures that were considered by Flores (1984) in the formulation of his model for pathogenicity. Classification into mild and severe strains, as reported by Schnölzer *et al.* (1985) and Visvader and Symons (1985), is based upon symptom expression in tomato.

its thermal stability *in vitro*, the general applicability of such an approach is unclear. Visvader and Symons (1985) have stated that pathogenicity-related sequence changes in CEV cannot be correlated with the calculated stabilities of a "virulence modulating" or "pathogenesis related" region corresponding to that of PSTV. Furthermore, there are significant differences among published secondary structures for the corresponding portions of CEV isolates that produce similar symptoms (Fig. 2). Both CEV$_{A}$ and CEV$_{G}$ are believed to induce severe symptoms in tomato (Visvader and Symons, 1985), but the ability of differences in static conformation or thermal stability to explain observed differences in CEV symptom expression depends upon the secondary structures considered.

Such apparent disagreement is not surprising, even in view of the 71% sequence homology between the pathogenicity domains of PSTV and CEV (Keese and Symons, 1985), because essentially nothing is known about viroid structure *in vivo*. Comparison of the secondary and tertiary structures of members of the PSTV viroid group by established techniques (Domdey *et al.*, 1978; Branch *et al.*, 1985) may help refine the models discussed above, but a complete understanding of pathogenicity will require knowledge of viroid structure *in vivo*.

B. Possible Mechanisms for Disease Induction

Although comparative sequence analysis suggests that a single region within PSTV controls symptom expression in tomato, work to characterize the viroid–host interactions involved in viroid pathogenesis is just beginning. Two potential mechanisms for this interaction have been proposed—interference with host mRNA splicing mediated by viroid sequences homologous to small nuclear RNA U1 and inhibition of mRNA synthesis via competition for DNA-dependent RNA polymerase II. In their present forms, only one of these mechanisms may involve nucleotides from the "virulence modulating" region of PSTV.

1. Interference with mRNA Splicing

Excision of introns from eukaryotic nuclear mRNAs requires the participation of U1 RNA, one of a family of uridine-rich small nuclear RNAs; complementarity between the 5′-end of U1 RNA and the ends of the intron may ensure the correct excision of the intron sequence and accurate joining of the exon sequences (Lerner *et al.*, 1980; Rogers and Wall, 1980; Krainer and Maniatis, 1985). Two characteristics of viroids, their circularity and localization within the nucleus, suggested that they may have originated from circularized introns that escaped degradation and gained the ability to be replicated by cellular enzymes (Roberts, 1978; Crick, 1979; Diener, 1979).

At least three investigators (Diener, 1981; Dickson, 1981; Gross *et al.*, 1982) have independently noted the presence of regions homologous to U1 RNA in PSTV and related viroids. One model for the interaction of viroid RNA with host mRNA splicing (Dickson, 1981) is based upon the presence of sequence elements in PSTV and CSV that are identical to those near the 5′-end of U1 RNA. Although these sequences (PSTV nucleotides 307–311 and 113–118) are not contiguous, they are located within two of the three regions whose sequence varies among PSTV isolates. The severity of symptoms induced in chrysanthemum by PSTV, CSV, and CEV could be correlated with increased complementarity to intron–exon junctions.

Figure 3 illustrates a second and longer region of homology with U1 RNA independently identified by Diener (1981) and Gross *et al.* (1982). Because these nucleotides (positions 257–279) occur within the lower portion of the central conserved region (see Fig. 1), all members of the PSTV viroid group contain an almost identical sequence. Although these nucleotides occur within a stable, double-stranded portion of the native structure, formation of secondary hairpin I (Chapter 3, this volume) would allow this region to form a palindromic structure in which the nucleotides homologous to U1 RNA remain largely single-stranded and able to interact with host mRNAs *in vivo* (Gross *et al.*, 1982). HSV also contains a similar region of homology with the 5′-end of U1 RNA, but not within its central region (Ohno *et al.*, 1983b). Thus, viroids (or the complemen-

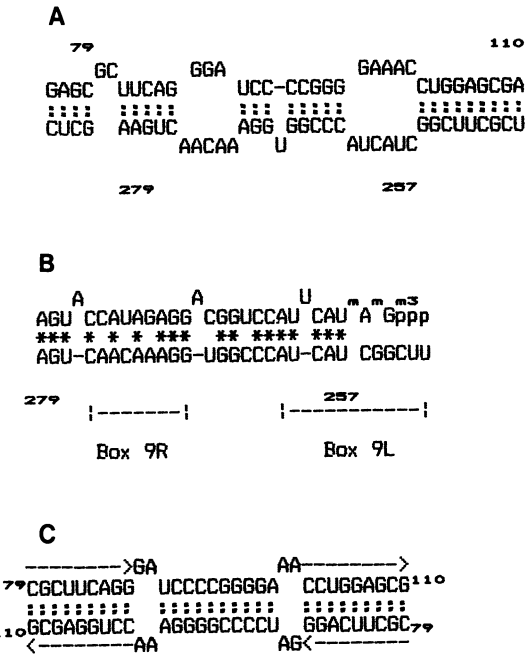

FIGURE 3. Potential structural interactions involving nucleotides within the central conserved region of PSTV. The native structure of the central conserved region (A) has been redrawn from Sänger (1982). Sequence homologies between the lower portion of the central conserved region and either U1 RNA or group I intron "boxes" 9L and 9R (B) have been redrawn from Diener (1981) and Dinter-Gottlieb (Chapter 7). The probable involvement of a highly stable, base-paired structure (C) involving the upper portion of the central conserved region in the processing of viroid replicative intermediates has been discussed by Diener (1986).

tary RNAs involved in their replication) could inhibit host mRNA splicing by binding to the same sequences in host mRNA precursors that are recognized by U1 RNA (Dickson, 1981; Diener, 1981; Gross *et al.*, 1982).

Computer-assisted sequence analysis has detected statistically significant homologies with other eukaryotic snRNAs—homologies between PSTV and the nucleolar U3B snRNA as well as between ASBV and U5 snRNA have been described (Kiss and Solymosy, 1982; Kiss *et al.*, 1983). PSTV/U3B homologies are particularly interesting because they are colinear and involve 81% of the U3B molecule and six regularly arranged regions in the left side of PSTV. Unlike U1 RNA, U3B RNA and PSTV are both found within the same subcellular compartment.

More recent evidence indicating that excision of introns from nuclear pre-mRNAs produces "lariat" RNAs containing an unusual nuclease-resistant branch site (Padgett *et al.*, 1984) has dampened earlier speculations about the origin of viroids from normal cellular mRNAs. Viroids appear not to contain similar 2′–5′ phosphodiester linkages. Nevertheless, the sequence homologies with snRNAs discussed above continue to provide a plausible mechanism for viroid pathogenesis.

2. Competition for RNA Polymerase

Two observations, the specific inhibition of viroid replication by α-amanitin (Mühlbach and Sänger, 1979; Spiesmacher *et al.*, 1985) and the ability of plant DNA-dependent RNA polymerase II to use purified PSTV as a template for *in vitro* synthesis of full-length PSTV (−)-strand RNA

(Rackwitz *et al.*, 1981), have led Sänger and colleagues to suggest that viroid replication may compete with cellular mRNA synthesis for available RNA polymerase II. RNA polymerase I also seems to be involved in PSTV replication because low concentrations of actinomycin D preferentially inhibit the synthesis of PSTV (+) strands in isolated nuclei (Spiesmacher *et al.*, 1985; Chapter 5, this volume). Palukaitis and Zaitlin (1983) have pointed out that the right terminal loop or PSTV exhibits considerable sequence homology with a promoter sequence for mouse RNA polymerase I.

Although recent studies of the *in vitro* interaction of cellular proteins with PSTV have shown that purified wheat germ RNA polymerase II specifically recognizes a single site on the native structure of PSTV (Goodman *et al.*, 1984; Wolff *et al.*, 1985), the location of this sequence could not be unambiguously determined. Essentially nothing is known about the *in vitro* interaction of RNA polymerase I with PSTV-related RNAs except that polymerase I appears unable to specifically initiate (−)-strand RNA synthesis *in vitro* (Sänger, 1984).

At the present time, competition for cellular RNA polymerases or their associated transcription factors remains a plausible mechanism for viroid pathogenesis, but *in vitro* experimentation would be more convincing if it included comparisons of the relative activities of mild and severe viroid strains. Although their concentrations in infected tissue appear similar, mild and severe strains of PSTV produce dramatically different biological effects. Riesner *et al.* (1983) have suggested that the localization of RNA polymerase II and PSTV within different nuclear compartments (chromatin and the nucleolus, respectively) may limit their ability to interact *in vivo*, but RNA polymerase I is located in the same compartment as PSTV.

C. Possible Relationships between Viroid Structure and Function

Figure 1 shows the relative locations of certain sequences within PSTV that may be involved in pathogenesis or replication-related events. Such sequences can be identified by comparative sequence analysis of naturally occurring viroid isolates as long as the phenotype (e.g., symptom expression) is not lethal. Other techniques are required to identify sequences involved in essential functions such as the initiation/termination of RNA replication or conversion of viroid multimers to mature circular progeny. Site-specific mutagenesis of infectious cDNAs in proving very useful in such studies (see Sections II.A and II.B).

Although independent viroid isolates may differ by only a single nucleotide exchange (Schnölzer *et al.*, 1985), most isolates contain multiple nucleotide changes. The importance of compensating nucleotide changes in maintaining both the native structure and secondary inter-

actions such as hairpin I has been recognized (Randles *et al.*, 1982; Sänger, 1982). RNA–protein interactions often require a certain secondary structure rather than a specific nucleotide sequence (Gralla *et al.*, 1974; Peattie *et al.*, 1981), and the same is true for the RNA enzyme function involved in the RNA splicing of group I introns (Weiss-Brummer *et al.*, 1983; Waring *et al.*, 1985). The possible role of self-splicing in viroid replication and/or pathogenesis is described in Chapter 7.

Several of the secondary interactions proposed in the literature appear to be mutually exclusive, e.g., the sequence homologies between the lower portion of the central conserved region of PSTV and the 5′-end of U1 RNA or group I intron "boxes" 9L and 9R (see Fig. 3). Site-specific mutagenesis of infectious viroid cDNAs allows evaluation of the functional importance of such secondary interactions as hairpins I and II or the group I intron "boxes" *vis-à-vis* the familiar viroid native structure. That viroids may assume several very different conformations during various portions of their replication/pathogenesis cycle is becoming increasingly clear.

II. SITE-SPECIFIC MUTAGENESIS OF INFECTIOUS cDNAs

Certain of the mutagenesis strategies to be described below can be considered extensions of the comparative sequence analysis approach that has been successfully applied at the RNA level (i.e., generation of viroid pseudorevertants in which the introduction of a secondary mutation restores a potential structural interaction disrupted by the primary mutation). Others involve creation of novel chimeric viroid RNAs that have no naturally occurring equivalents. In light of the experimental complexity inherent in site-specific mutagenesis and bioassay of viroid cDNAs, knowledge of the factors controlling their infectivity is essential.

A. Minimal Requirements for cDNA Infectivity

Cress *et al.* (1983) first demonstrated the infectivity of cloned double-stranded viroid cDNAs. Intact pBR322 recombinants containing tandem dimers of PSTV cDNA were highly infectious, and the nucleotide sequence of the resulting PSTV progeny was in complete agreement with that of the cloned PSTV cDNA. Infectious (+)-strand RNA transcripts (RNA molecules containing the sequence of PSTV) could also be isolated from *E. coli* cells harboring certain recombinant plasmids. Subsequent systematic comparisons of the infectivity of (+)- and (−)-strand HSV and PSTV RNAs transcribed *in vitro* strongly suggest that only (+)-strand transcripts are infectious (Ishikawa *et al.*, 1984; Tabler and Sänger, 1985).

Although these observations have been extended by several groups, it is not yet known in detail how viroid cDNAs initiate the infection

process. Viroid-related RNAs transcribed from cDNA templates could either enter a "rolling circle" mechanism for viroid replication directly or first undergo cleavage and ligation to form a circular template for replication. Present evidence strongly favors the second alternative and is summarized below.

Cress *et al.* (1983) also provided the first evidence that tandem multimers of viroid cDNA are not required for infectivity, i.e., PSTV (+)-strand RNAs transcribed *in vivo* from a PSTV cDNA template that contained only a four-nucleotide sequence duplication (positions 145–148) appeared to be weakly infectious. Tabler and Sänger (1984) reported similar results with single- or double-stranded PSTV cDNAs containing five-nucleotide sequence duplications (positions 220–224). Not all full-length viroid cDNAs are infectious, however. CEV cDNAs whose termini are derived from that viroid's right terminal loop were not infectious (Visvader and Symons, 1985), while Shikata and colleagues have shown that HSV cDNAs whose termini are derived from that viroid's left terminal loop were also not infectious (Ishikawa *et al.*, 1984).

All members of the PSTV viroid group contain the sequence GGAUCC within the upper portion of the central conserved region (see Fig. 1), and the resulting unique *Bam*HI recognition site in their cDNAs has been widely used for molecular cloning (van Wezenbeek *et al.*, 1982; Cress *et al.*, 1983; Kiefer *et al.*, 1983; Visvader and Symons, 1983; Tabler and Sänger, 1984). When a full-length PSTV cDNA insert with *Bam*HI termini is transferred from pBR322 to certain of the pUC plasmid or M13mp phage vectors, the fortuitous identity of their overlapping *Bam*HI–*Sma*I sites causes the extent of sequence duplication generated by the molecular cloning to increase from 6 (GGAUCC) to 11 nucleotides (GGAUCCCCGGG).

Tabler and Sänger (1984) first noted that this comparatively small increase in the amount of sequence duplication was accompanied by a substantial increase in infectivity and concluded that duplication of the additional five nucleotides (CCGGG, positions 93–97) was "essential" for PSTV cDNA infectivity. Such a conclusion is not consistent with the weak but reproducible infectivity of PSTV cDNAs having either *Bam*HI termini and only a six-nucleotide sequence duplication or *Ava*II termini and a five-nucleotide duplication. The infectivity of PSTV cDNAs whose termini are derived from positions 145–148 or 220–224 (Cress *et al.*, 1983; Tabler and Sänger, 1984) indicates that viroid RNA transcripts whose termini are not derived from the upper portion of the central conserved region can also be circularized.

Clearly less than a complete dimer of viroid cDNA is required for infectivity, but additional data were required to clarify the relationship between the infectivity of a particular viroid cDNA, the location of its termini, and the extent of sequence duplication. Two recent studies have supplied such evidence and refocused attention on the upper portion of the central conserved region as the site of processing for multimeric

replicative intermediates. Meshi *et al.* (1985) used *Bal*31 exonuclease digestion to generate a series of HSV cDNA constructs containing more than one but less than two full-length units of HSV sequence. Systematic comparison of the infectivities of these HSV cDNAs revealed that duplication of the upper portion of the HSV central region is required for infectivity. A similar conclusion can be drawn from infectivity studies with RNAs transcribed *in vitro* from partial multimers of PSTV cDNA (Tabler and Sänger, 1985).

Analysis of published data describing the effect of sequence duplication on the infectivity of PSTV cDNAs with *Bam*HI termini has allowed Diener (1986) to propose a model for the conversion of longer-than-unit-length viroid RNA transcripts into monomeric circular molecules during replication. The central feature of this model for the processing oligomeric replicative intermediates into monomeric circular progeny is the thermodynamically extremely stable base-paired conformation shown in Fig. 3C. This structure involves nucleotides from the central conserved region and secondary hairpin I, which are present in all viroids (see Fig. 1), and it can be formed by viroid RNAs that contain a duplication of the upper portion of the central conserved region. The opportunity for precise cleavage and ligation within the central helical region (positions 90–99) can explain the observed differences in the infectivities of tandemly repeated viroid cDNAs and those containing only partial sequence duplications. Although this pathway is almost certainly responsible for normal viroid replication, an alternative pathway is required to explain the weak infectivity of full-length viroid cDNAs whose termini are not derived from the upper portion of the central conserved region.

B. Site-Specific Mutagenesis of PSTV and CEV cDNAs

Three published studies describe mutant viroid cDNAs produced by site-specific mutagenesis that have proven valuable in efforts to develop and refine molecular models for viroid replication. Strategically located restriction sites have been used to target nucleotide deletions within HSV cDNA (Meshi *et al.*, 1985) and to introduce C → T transitions within PSTV cDNAs via bisulfite-catalyzed deamination of single-stranded deoxycytosine residues (Owens *et al.*, 1986). Because the use of deletion mutagenesis to determine the role of the central conserved region in HSV cDNA infectivity (Meshi *et al.*, 1985) was discussed above, it will not be considered further. Oligonucleotide-directed mutagenesis has been used in studies with CEV and PSTV cDNAs (Visvader *et al.*, 1985; Owens *et al.*, 1986). The unexpected abolition of PSTV cDNA infectivity by a single point mutation (Owens *et al.*, 1986) emphasizes our limited appreciation of the interrelationships between viroid structure and function.

Visvader *et al.* (1985) constructed a number of monomeric CEV cDNA clones in the *Bam*HI site of plasmid vector pSP64 and measured their

infectivity by inoculating tomato seedlings with RNA transcripts synthesized *in vitro*. Infectious clones contained the same 11-nucleotide sequence duplication of a portion of the central conserved region first described by Tabler and Sänger (1984). Oligonucleotide-directed mutagenesis of the 5′-terminus of the CEV cDNA clones produced two point mutants in which the duplicated sequence (positions 89–99) had been converted to GGATCCCC(A/T)GG, and these mutant cDNAs were infectious only when present within their pSP64 vector. The authors concluded that *in vivo* processing of longer-than-unit-length CEV occurs between CEV positions 97–99 and that the presence of a G at position 97 is important for normal viroid replication.

Owens *et al.* (1986) used sodium bisulfite-catalyzed deamination of single-stranded deoxycytosine residues and oligonucleotide-directed mutagenesis to create nucleotide substitutions within premelting region 2 of PSTV (see Fig. 1). The particular mutations investigated were chosen to have only minimal effects upon the native structure. In the particular cDNA construction used for bioassay, a C → T transition at position 92 appeared to be lethal. This mutation converts the 11-nucleotide sequence duplication described above (PSTV positions 87–97) to GGATC(T)CCGGG, and Owens *et al.* (1986) concluded that cleavage and ligation of longer-than-unit-length viroid RNAs can occur between positions 87 and 91 (albeit at a reduced rate).

Wild-type PSTV cDNA does not contain a recognition site for *Hin*dIII, but introduction of a single C → T transition at position 284 creates a recognition site that corresponds to those found in TASV, CEV, and CSV cDNAs. Position 284 lies just outside the lower portion of the central conserved region but just inside the left border of premelting region 2 (see Fig. 1). Although mutations at position 284 should not affect either the stems of secondary hairpins I–III or the sequence homologies with group I intron "boxes," a C → U transition at this position completely abolished infectivity (Owens *et al.*, 1986).

The equivalent of U_{284} in the native structures of CEV, CSV, and TASV is base-paired with an A rather than the G found at position 78 of PSTV. The substitution of a weak G:U base pair for the normal G:C base pair may impair replication by destabilizing premelting region 2 in the mutant PSTV. We have begun to examine the importance of structural stability within premelting region 2 by constructing pseudorevertants in which the G at position 78 in PSTV cDNA will be replaced by an A (Fig. 4).

C. Construction of Chimeric Viroid cDNAs

The various site-specific mutagenesis strategies discussed above are most appropriate for dissection of structure/function relationships previously suggested by comparative sequence analyses or physical–

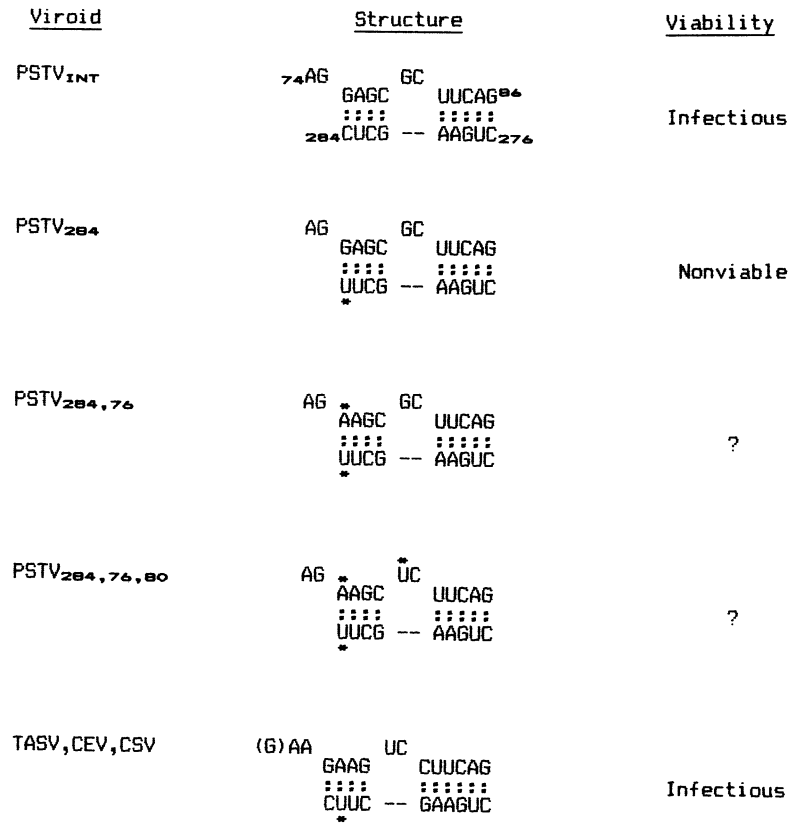

FIGURE 4. Potential pseudorevertants of $PSTV_{284}$. Homologous portions of the native structures of four infectious viroids ($PSTV_{INT}$, TASV, CEV, and CSV), one nonviable PSTV mutant, and two potential pseudorevertants are shown. Asterisks indicate positions of nucleotide substitutions in the PSTV mutants and the uridine residues in TASV, CEV, and CSV that are equivalent to U_{284} in $PSTV_{284}$.

chemical studies. In essence, the experimental protocol asks the inoculated plant to either accept (replicate) or reject (fail to replicate) the altered viroid RNA sequence, but conventional bioassays are not designed to differentiate between mutations that completely block viroid replication and those that severely reduce its rate. Site-specific mutagenesis is also laborious because mutations must be tested individually.

If, however, host plants are inoculated with viroid cDNAs that can give rise to a number of potentially viable RNA transcripts, individual infected plants might contain viroid clones whose sequence would vary from plant to plant. Characterization of these clones would identify nucleotide substitutions that do not destroy viability. One method that offers the host plant such a choice is inoculation with chimeric viroid cDNAs, i.e., full-length or longer cDNAs constructed from fragments derived from different viroids or viroid strains. Construction of viroid cDNA chimeras depends on the presence of homologous restriction sites in individual viroid cDNAs.

Digestion of TASV, CEV, or CSV cDNAs with *Bam*HI plus *Hin*dIII produces two fragments, one derived from the more stable right side of the native structure and another from the less stable left side (see Fig. 1). The *Bam*HI and *Hin*dIII sites in or near the central conserved region of CEV cDNAs can be used to investigate the relative contributions of the two pathogenesis-related regions to symptom expression (Visvader and Symons, 1985). Several other types of cDNA chimeras are also possible—mixed tandem multimers (or partial multimers) in which the two viroid cDNAs are joined at a common restriction site (Fig. 5A,B) and monomeric cDNAs in which restriction fragments from two viroids are combined (Fig. 5C). The number of possible constructions will increase as additional restriction sites are introduced into individual viroid cDNAs by site-specific mutagenesis.

Mixed viroid cDNA dimers constructed from full-length PSTV and TASV cDNAs are infectious (Owens *et al.*, 1986). Individual infected tomatoes contained either PSTV or TASV, but no evidence for either mixed infections or viroid chimeras could be obtained by nucleic acid hybridization analyses using full-length cDNA probes. Such a result is consistent with three previous observations: infectivity of the individual

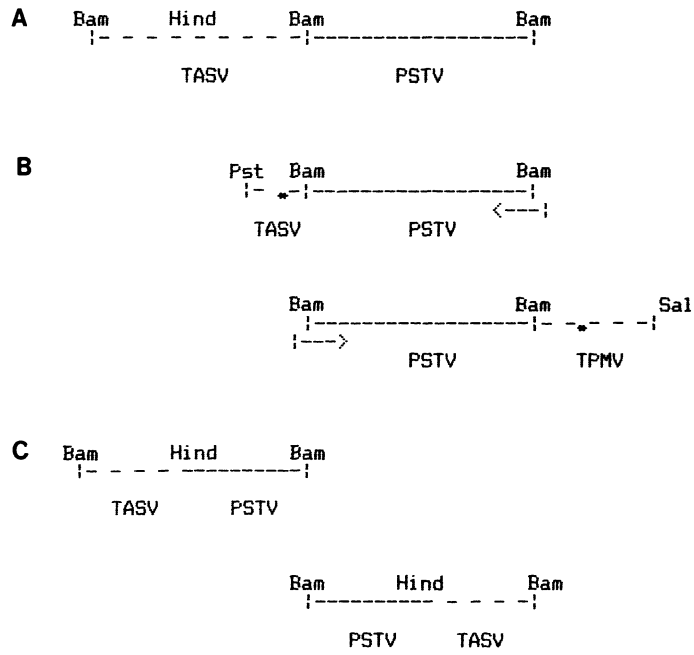

FIGURE 5. Construction of viroid cDNA chimeras. Three different types of chimeras are shown: mixed tandem dimers (A), mixed partial dimers (B), and chimeric "monomers" (C). Relative positions of the unique recognition sites for *Bam*HI, *Hin*dIII, *Pst*I, and *Sal*I used in the various constructions are indicated. Asterisks mark locations of the first nucleotide sequence difference between PSTV and either TASV or TPMV. Arrows give starting points for *Bal*31 deletions extending into PSTV cDNAs.

PSTV and TASV cDNAs; the probable location of the site for processing of oligomeric viroid replication intermediates within the overlapping *Bam*HI–*Sma*I site that joins the two full-length cDNAs (Diener, 1986); and the well-known phenomenon of viroid cross-protection (Niblett *et al.*, 1978). Because every inoculated cell simultaneously receives a copy of both infectious viroid cDNAs, such mixed tandem cDNA dimers should be useful in future studies of the molecular basis of cross-protection.

PSTV and TASV cDNA fragments produced by digestion with *Bam*HI plus *Hin*dIII can be used to construct full-length chimeric cDNAs in which the thermodynamically less stable left half of one viroid has been joined to the more stable right half of the second viroid (Fig. 5C). Although the secondary structure of their central conserved region and the ability of RNA transcripts to form a group I intron "core" structure appear to be unaltered, sequence divergence just outside the upper portion of the central conserved region prevents formation of two of the nine base pairs comprising the stem of secondary hairpin I. These PSTV–TASV cDNA chimeras contain either 354 or 365 nucleotides and are not infectious (Owens *et al.*, 1986).

It may be possible to avoid simultaneous disruption of several structural interactions by constructing partial cDNA multimers (Fig. 5B). Ligation of a full-length PSTV cDNA with *Bam*HI termini to the 125 base-pair *Bam*HI–*Sal*I fragment from TPMV cDNA creates a 490-base-pair chimeric viroid cDNA in which the first nucleotide difference occurs 30 nucleotides downstream from the *Bam*HI–*Sma*I site where the two fragments have been joined. Nucleotide sequences forming the stem of secondary hairpin I are identical in PSTV and TPMV, and the two viroids exhibit 83% overall sequence homology.

Wild-type PSTV progeny should appear following inoculation of tomato seedlings with the intact partial cDNA multimer, but cDNAs containing *Bal*31-generated deletions in the 5′-*Bam*HI site may produce chimeric viroid RNAs containing one or more TPMV-specific nucleotides. Indeed, progeny from infections initiated by cDNAs containing fewer than 359 PSTV-specific nucleotides must be chimeras that can be distinguished from wild-type PSTV by their failure to hybridize with selected PSTV-specific oligodeoxynucleotide probes (Wallace *et al.*, 1981; Owens *et al.*, 1986). If, however, deletions of less than 30 nucleotides are lethal, the model for the processing of oligomeric viroid replicative intermediates proposed by Diener (1986) would receive additional support (see Fig. 3C and Section II.A).

D. Characterization of Mutant Viroids

Rigorous characterization of the viroid progeny from all experiments involving mutant cDNAs is essential because the comparatively low fidelity of RNA synthesis may "rescue" single-base changes that are ac-

tually lethal (van Vloten-Doting *et al.*, 1985). Instability of recombinant plasmids within transfected cells may also lead to changes in viroid cDNA templates; nucleotide deletions/duplications, point mutations, and insertion of cellular DNA sequences have been reported (Calos *et al.*, 1983; Dixon and Hohn, 1985). There is, however, no obvious selection pressure for sequence reversion at the DNA level because viroid cDNAs appear not to replicate within the infected plant (Tabler and Sänger, 1984).

The appearance of wild-type PSTV progeny after *A. tumefaciens*-mediated inoculation with PSTV cDNA containing a C → T transition at position 284 (see Fig. 4) is probably a consequence of the high error frequency characteristic of RNA synthesis (Owens *et al.*, 1986). Data presently available suggest that sequence reversion at the RNA level will be less of a problem when recombinant plasmid or phage DNAs are used as inocula, but identification of mutations that severely impair viroid replication may depend upon the use of mutations that cannot be rescued by simple nucleotide substitutions. On the other hand, sequence alterations in less critical portions of the sequence will probably be stable. Ahlquist *et al.* (1984) have shown that a single nucleotide deletion in the noncoding region of BMV RNA-2 is preserved in viral RNA isolated from plants inoculated with RNA transcripts synthesized *in vitro*.

There is also a second situation in which careful characterization of viroid progeny is essential, i.e, when the mutations under study occur within sequence duplications present in the cloned viroid cDNA. Two groups have studied the effects of mutations within the upper portion of the viroid central conserved region on the infectivity of cDNAs with *Bam*HI termini (Visvader *et al.*, 1985; Owens *et al.*, 1986). Because the mutations were located within the 6- or 11-nucleotide sequence duplication generated by molecular cloning, wild-type progeny could be obtained under certain circumstances (see Section II.A).

Systemic viroid bioassays have two potential disadvantages. They may be unable to differentiate mutations that are actually lethal from those that severely inhibit viroid replication or intercellular transport. Second, the selective pressure in such assays favors the propagation of wild-type viroid generated by sequence reversion at the RNA level rather than the presumably less fit mutant. Therefore, we have begun to develop an alternative assay in which potentially infectious PSTV cDNAs are placed under the control of a functional promoter for RNA polymerase II and introduced into the host cell by means of the Ti plasmid of *A. tumefaciens* (Gardner *et al.*, 1986). Although Ti plasmid-mediated PSTV infections require only T-DNA transfer but not integration (Gardner and Knauf, 1986), transformed callus cultures or even intact plants can also be recovered. Such experimental systems maximize the opportunity for PSTV infection and can provide large amounts of tissue in which viroid replication need not be systemic to be detectable.

The use of this system to confirm the lethal effect of a single C → T transition at position 284 of PSTV cDNA was described above. We believe

that the same experimental approach will also be useful in the analysis of viroid replication. Gardner *et al.* (1986) have presented data suggesting that PSTV replication is blocked in turnips. Further analysis of total RNA preparations isolated from galls or transformed plants may identify the step at which replication is blocked, and large numbers of transformed cells containing RNA replicative intermediates are readily available for such studies. Similar analyses could be performed with nonviable mutants of PSTV in plants that are permissive for replication. Together these approaches may help to elucidate the various steps in the infection cycle of viroids.

III. VIROID TRANSCRIPTS AS SUBSTRATES FOR RNA RECOMBINATION

Apparent similarities between viroid biosynthesis and the mRNA splicing machinery of eukaryotic cells have led Zimmern (1982) to propose that viroids and RNA viruses may have originated from a novel class of eukaryotic RNA whose primary function is the intercellular exchange of genetic information (see also Branch and Robertson, 1984; Chapter 5, this volume). The origin, amplification, and extracellular exchange of these hypothetical regulatory RNAs would be dependent upon *intermolecular* RNA recombination, a poorly understood phenomenon first detected in studies of poliovirus and foot-and-mouth disease virus mutants (Cooper, 1977; King *et al.*, 1982). Additional support for this sort of speculation is provided by comparative sequence analyses suggesting that certain viroids may have arisen by recombination between two distinct viroid species coinfecting the same host (Keese and Symons, 1985) and the striking sequence homologies between group I introns and viroids (Chapter 7, this volume). This hypothesis for viroid origin/pathogenesis is particularly attractive because certain of its predictions can be tested experimentally.

Figure 6 illustrates one experimental approach to detect RNA recombination which takes advantage of the noninfectious PSTV cDNAs produced by site-specific mutagenesis studies. Mutants containing nucleotide insertions/deletions should be unable to serve as template for the synthesis of wild-type PSTV transcripts *in vivo*, but intermolecular RNA recombination might be able to generate wild-type PSTV following inoculation with a mixture of two defective cDNAs. Keese and Symons (1985) have pointed out that either direct cleavage/ligation or discontinuous transcription can provide plausible mechanisms for such RNA rearrangements.

This type of assay system to detect RNA recombination would be extremely sensitive because the desired product is capable of autonomous replication, but generation of wild-type PSTV cDNA templates by DNA

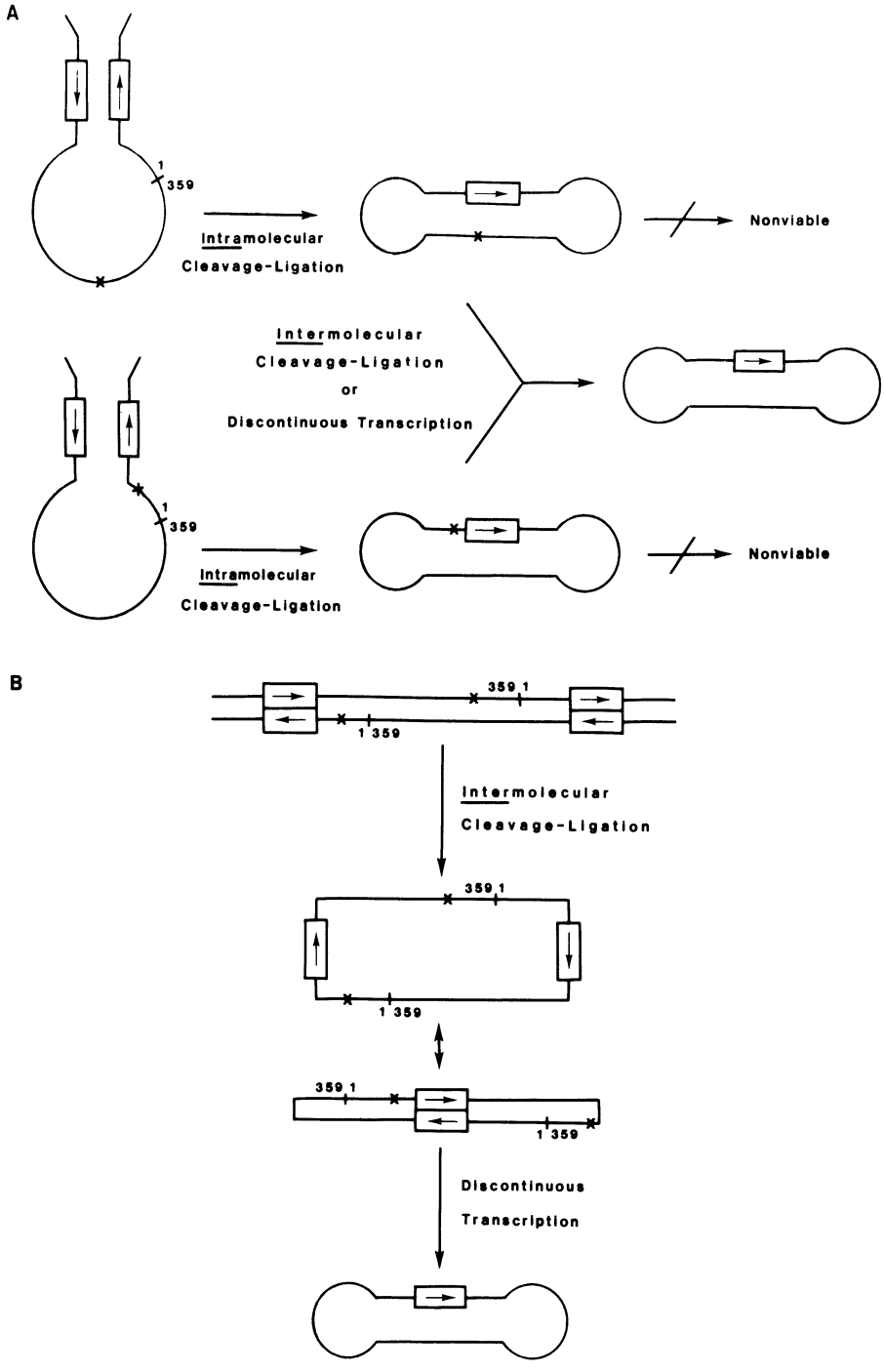

FIGURE 6. Viroid RNA transcripts as substrates for RNA recombination. Two possible pathways for the generation of wild-type viroid from two coinoculated defective viroid RNA transcripts are depicted. Both pathways may involve discontinuous RNA-directed RNA synthesis (Keese and Symons, 1985). The primary difference between the two pathways is the relative timing of the *intra*molecular and *inter*molecular cleavage–ligation (RNA recombination) reactions.

rearrangements *in vivo* (Calos *et al.*, 1983; Dixon and Hohn, 1985) must be excluded by appropriate control experiments, i.e., demonstration that appearance of wild-type PSTV also follows inoculation with RNA transcripts synthesized *in vitro*. The observed frequency of RNA recombination, which will probably be strongly dependent upon both the distance between the individual mutations and their specific locations within the viroid native structure, may also provide useful information about the molecular mechanism of viroid replication.

IV. CONCLUDING REMARKS

Site-specific mutagenesis of viroid cDNAs has already made a major contribution to our knowledge of structure/function relationships in these unusual plant pathogens. Selected alterations in infectious cDNAs have helped define the minimal requirements for viroid cDNA infectivity and support a proposed model for the cleavage/ligation of viroid replicative intermediates (Diener, 1986). Dramatic progress has also been made in related areas: comparative sequence analysis has identified portions of the PSTV and CEV sequence controlling symptom expression (Schnölzer *et al.*, 1985; Visvader and Symons, 1985); knowledge of the "rolling circle" mechanism responsible for viroid replication has increased (Hutchins *et al.*, 1985; Chapter 5, this volume); and physical–chemical studies have revealed potential interactions between portions of the viroid sequence that do not interact in the familiar native conformation (Riesner and Gross, 1985; Chapter 3, this volume). This information has been used to construct molecular models for pathogenesis that contain testable predictions about viroid structure/function relationships (e.g., Flores, 1984; Schnölzer *et al.*, 1985), and site-specific mutagenesis of infectious cDNAs provides the most direct method to test and refine such models.

Systematic investigations of several regions of PSTV and CEV (the "virulence modulating" and central conserved regions in particular) are under way as this chapter is being prepared. The basic strategy for these studies will be that underlying comparative sequence analysis, i.e., introduction of nucleotide changes that disrupt conserved elements of secondary structure followed by the creation of pseudorevertants in which the secondary structure interaction has been restored. Noller (1984) has discussed the successful application of this strategy to the analysis of structure/function relationships in ribosomal RNAs.

Bioassay of mutant viroid cDNAs containing single nucleotide substitutions may be complicated by reversion to wild type at the level of cDNA transcription or RNA replication. The limited data presently available suggest that the frequency of such reversion may depend upon the particular bioassay system used (Owens *et al.*, 1986), and rigorous characterization of the viroid progeny that appear following inoculation with mutant cDNAs is crucial. Because viroids appear not to code for poly-

peptides (reviewed by Sänger, 1982), the proportion of potential mutations that are phenotypically neutral may be much lower than for pathogens such as RNA viruses where evolution of structure/function relationships has been constrained by the necessity to retain mRNA activity.

Construction of viroid cDNA chimeras may prove as valuable as oligonucleotide-directed mutagenesis techniques for the identification of structural interactions. Chimeras constructed from cDNAs of mild and severe strains of CEV should be useful to estimate the relative contributions of nucleotide substitutions in the left and right sides of the native structure to symptom expression (Visvader and Symons, 1985), but a limited number of homologous restriction sites in viroid cDNAs necessitates substitution of large portions of the viroid sequence. Introduction of carefully chosen restriction sites via oligonucleotide-directed mutagenesis will dramatically increase the probability of obtaining viable chimeras from fragments of two different viroid species.

Detailed analysis of viroid structure/function relationships will demand quantitative *in vitro* assay systems for specific viroid functions to supplement the qualitative information provided by bioassay techniques. Hall and collaborators (Dreher *et al.*, 1984) have used the ability of mutant brome mosaic virus RNAs synthesized *in vitro* to act as a substrate for both tyrosyl-tRNA synthetase and BMV replicase to study structure/function relationships in the 3′-terminal region. This approach permits the generation of targeted mutations without regard to their viability *in vivo*. The first steps toward similar *in vitro* assays for individual viroid functions have recently been reported (Robertson *et al.*, 1985; Tsagris *et al.*, quoted in Tabler and Sänger, 1985; Chapter 5, this volume).

An *in vivo* RNA splicing assay for the self-splicing rRNA intron of *Tetrahymena thermophila* has also been reported (Waring *et al.*, 1985; Price and Cech, 1985). A DNA fragment containing the intron sequence was cloned into either a plasmid (pUC8) or phage (M13mp83) vector such that expression of the α-fragment of β-galactosidase is dependent upon intron excision from the mRNA precursor. Using such a system, a simple color assay can be used to screen for splicing-deficient mutants generated by either random or directed mutagenesis techniques as well as pseudorevertants of such primary mutations. Similar *in vivo* assays for individual viroid functions may also be possible; they would complement available viroid bioassay techniques and facilitate future studies of the role of specific structural interactions in replication and pathogenesis.

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CHAPTER 7

POSSIBLE VIROID ORIGIN

Viroids, Virusoids, and Group I Introns

GAIL DINTER-GOTTLIEB

I. INTRODUCTION

The coding regions of many eukaryotic genes are interrupted by apparently extraneous segments of DNA, termed *introns*. In order for the gene to be properly expressed, the introns must be removed from the RNA transcripts, and the residual exons ligated, in a reaction known as RNA splicing. Following excision, the intron may exist in a lariat structure (Grabowski *et al.*, 1984; Ruskin *et al.*, 1984; Domdey *et al.*, 1984), as a linear molecule (Burke and RajBhandary, 1982; Garriga and Lambowitz, 1984), or it may cyclize (Halbreich *et al.*, 1980; Grabowski *et al.*, 1981).

II. TYPES OF INTRONS

At least four types of introns have been identified, based on their splicing mechanisms and the structure of the RNA. The first type encompasses the introns found in nuclear mRNA. These contain a consensus sequence at the splice junctions.

A A G
CAGGTGAGT.....$(Py)_6$XCAGGT

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(Breathnach and Chambon, 1981; Sharp, 1981; reviewed by Cech, 1983). Their splicing mechanism appears to involve the small nuclear ribonucleoprotein (snRNP) U1 (Mount *et al.*, 1983; Tatei *et al.*, 1984; Kramer *et al.*, 1984). The mechanism entails the formation of a lariat structure (Padgett *et al.*, 1984; Ruskin *et al.*, 1984) involving the 5′ end of the intron in a 2′–5′ bond with the A nucleotide of a conserved sequence upstream of the 3′ end of the exon (Langford and Gallwitz, 1983; Pikielny *et al.*, 1983). Ligation of the exons and the release of the intron then occur. As yet, the enzymes (if any) involved in this reaction are unknown, although ATP and the U1 snRNP may be involved (Krainer, personal communication).

Nuclear tRNA introns, the second type, are excised through a mechanism whose enzymes are well characterized, involving an endonuclease, phosphatase activity, and ligase, with variations noted between yeast and HeLa cells (Konarska *et al.*, 1981; Peebles *et al.*, 1983; Greer *et al.*, 1983; Filipowicz and Shatkin, 1983). No consensus sequences are seen at the splice sites, nor within the intron.

A third class of introns comprises some of the mRNA introns of mitochondria and chloroplasts. These introns, called Group II by Michel and Dujon (1982), contain a conserved sequence within the last 100 residues at the 3′ end which can be folded into a 14-bp hairpin, with a constant GAAA terminal loop. The mechanism for their splicing has begun to be delineated (Peebles *et al.*, 1986). It involves lariat formation, like the nuclear mRNA, and self-splicing, like the Group I introns, discussed below.

The fourth intron class is recognized by conserved sequence and structural features, and is found among mitochondrial messenger and ribosomal RNA genes, chloroplast RNA genes, and nuclear RNA genes, a surprisingly broad distribution (Burke and RajBhandary, 1982; Davies *et al.*, 1982; Michel *et al.*, 1982; Anziano *et al.*, 1982; De La Salle *et al.*, 1982; Netter *et al.*, 1982; Weiss-Brummer *et al.*, 1982; Steinmetz *et al.*, 1982; Bonnard *et al.*, 1984). The hallmark of this intron class is a 16-nucleotide phylogenetically conserved sequence (Burke and RajBhandary, 1982), the Group I consensus sequence (Fig. 1A). Twelve bases of this sequence, termed Box 9L, comprise a region of *cis*-dominant mutations in the splicing of the yeast mitochondrial cytochrome *b* intron. Elsewhere in the intron is Box 2, which also affects splicing upon mutation (De La Salle *et al.*, 1982), and is complementary to Box 9L. Another pair of sequence elements, called A and B (or P and Q), is less conserved as to sequence, but located upstream of Box 2, and rich in GC (Waring *et al.*, 1982). Although no splicing-defective mutants have been found *in vivo*, mutations introduced into this region in the *Tetrahymena* intervening sequence cDNA cloned into *E. coli* cause defective splicing of the RNA transcript (Waring *et al.*, 1985). A third pair of sequences, Box 9R and 9R′, although not conserved as to sequence, are conserved in location. 9R is located just downstream of Box 9L, and 9R′ is just upstream of A

and B. The ability of 9R and 9R′ to base pair has been implicated in yeast cytochrome *b* gene splicing, since a point mutation at the base of the helix destroys splicing, while a double mutation, restoring pairing, also restores splicing of the intron (Weiss-Brummer *et al.*, 1983).

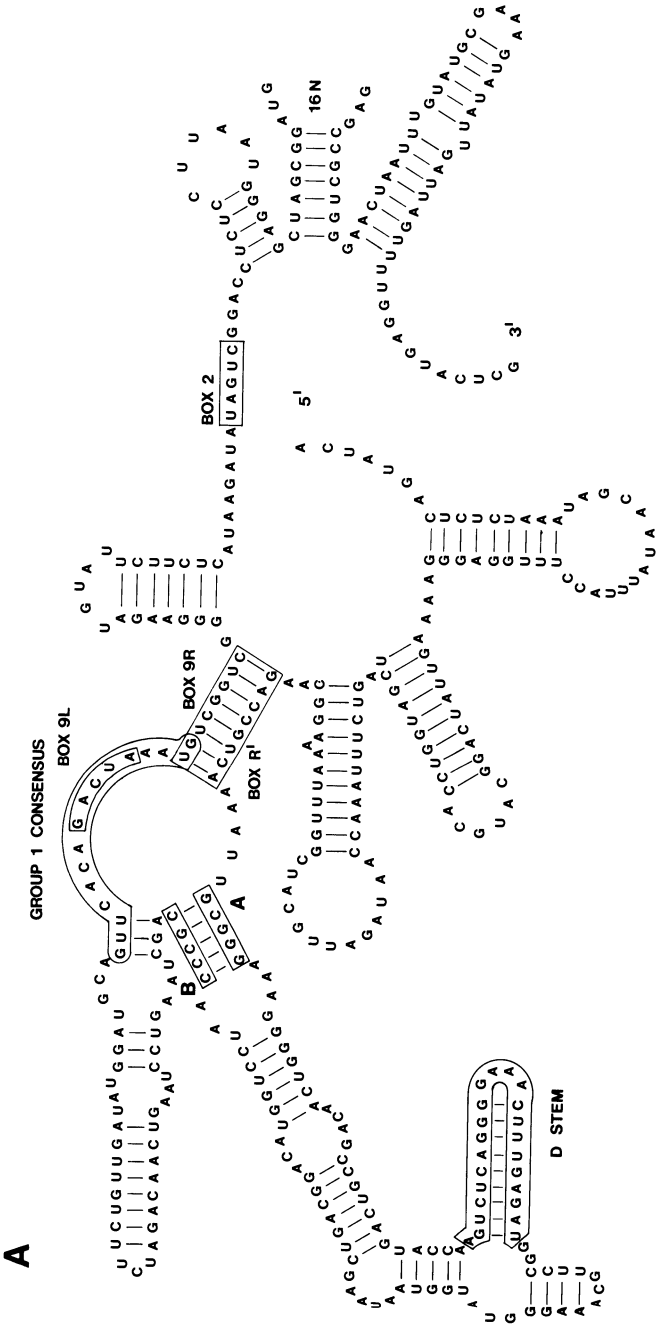
The pairing of the complementary boxes, and the order in which they occur within the intron, 9R′, A, B, 9L, 9R, and 2, force the RNA into a characteristic structure (Fig. 1A). In this case, the Box 9L:Box 2 pairing would be at the level of tertiary structure.

The phenomenon of self-splicing has been described *in vitro* for three Group I introns, the nuclear RNA intron of *Tetrahymena thermophila* (Cech *et al.*, 1981; Kruger *et al.*, 1982, Zaug *et al.*, 1983), and the *Neurospora* mitochondrial cytochrome intron 1 and yeast large rRNA and mRNA introns (Garriga and Lambowitz, 1984; Tabak *et al.*, 1983; van der Horst and Tabak, 1985). No enzymes or other proteins are required for these reactions *in vitro*, the sole components being GTP, a monovalent cation, and a divalent cation, usually magnesium. A series of transesterification reactions occurs, first as guanosine becomes covalently bonded to the 5′ end of the excised intron, then as the 3′ terminal G-OH attacks a specific bond 4 to 19 bases from the 5′ end of the intron, thus creating a covalently closed circle and releasing a short oligonucleotide. Both the *Tetrahymena* and the yeast rRNA introns cyclize, but no circular form has been found for the *Neurospora* intron. Based on similarities in intron structure, it seems reasonable that all Group I introns splice through a similar mechanism (Cech *et al.*, 1983). In some cases, however, proteins may be necessary for stabilization of the structure and maintaining the base-pairing of the boxes.

III. VIROIDS AS INTRONS

Since viroids are single-stranded, circular RNAs, similar in size to some of the circular introns, comparisons have been drawn between the two types of molecules. Roberts (1978) and Crick (1979) proposed that introns, and viroids, might function as regulatory molecules within the cell. Diener (1981) noted the similarities between the RNAs and, based on homologies between the negative strand of the viroid and the U1 snRNP, proposed that the viroids might be escaped introns. Other homologies with the positive and negative strand viroids were noted by Dickson (1981) and Gross *et al.* (1982). As the sequences of the viroids became available, further comparisons between the viroids and the various intron types were possible.

A search of the viroid sequences revealed that the 16-nucleotide Group I consensus sequence is present in all of the viroids (Dinter-Gottlieb and Cech, 1984). In fact, this sequence represents the lower portion of the "central conserved region" of the viroids (Kiefer *et al.*, 1983). This region is centrally located when the structure is written in the canonical rod


A
GROUP 1 CONSENSUS
BOX 9L
BOX 9R
BOX R'
BOX 2
16N
5'
3'
D STEM
A
B

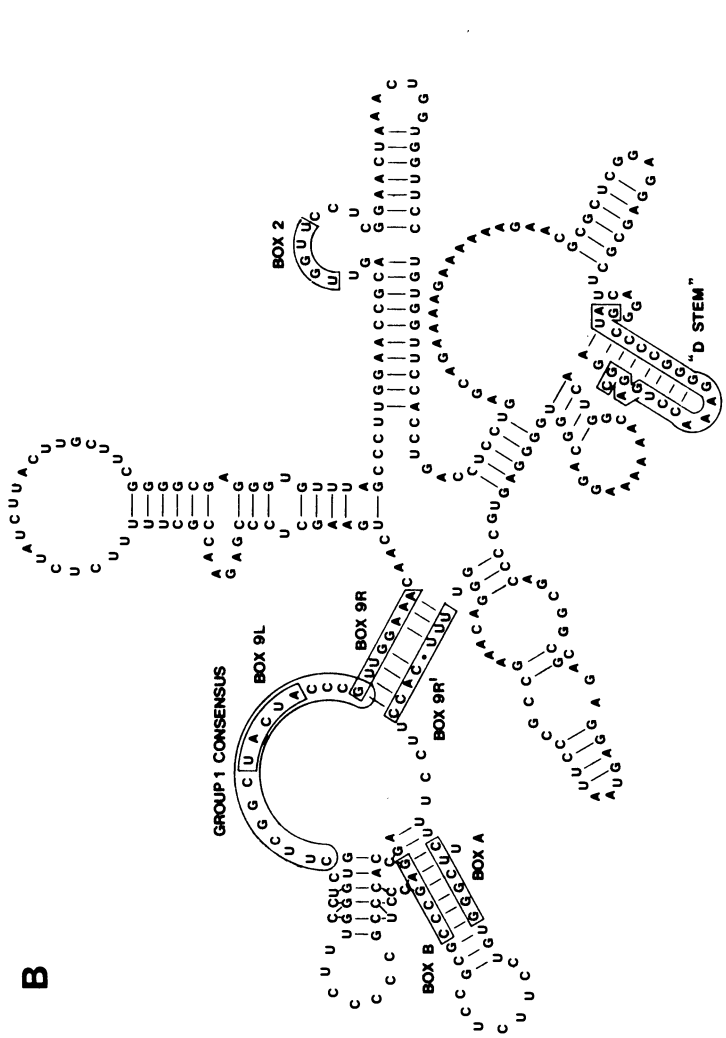


FIGURE 1. (A) The secondary structure of a Group I intron (exemplified by the self-splicing intron of *Tetrahymena*) generated by pairing Box 9R with Box 9R′, and Box A with B in the secondary structure, and Box 9L pairs with Box 2 at the level of tertiary structure (after Michel and Dujon, 1982). (B) PSTV structure derived from pairing the conserved sequence elements that are also found in Group I introns.

form (Gross *et al.*, 1978; Fig. 2A). As shown in Fig. 2B, the sequence is present even in viroids such as CCCV, which shares only 11% sequence homology with the PSTV group (Haseloff *et al.*, 1982). Surprisingly, it is found in virusoids (see Chapter 8, this volume) as well. Two of the virusoids that have been sequenced, velvet tobacco mottle RNA 2 (VTMoV) and *Solanum nodiflorum* mottle (SNMV) RNA 2 (Haseloff and Symons, 1982), contain the Group I consensus sequence and box sequences (Fig. 2B,C), although the virusoids contain little sequence homology with the viroids.

The conserved sequence elements of Group I introns also occur in the viroids (Dinter-Gottlieb and Cech, 1984). Box 9L, a portion of the consensus sequence, is present in all viroids, although there is one base change in all viroids except TASV and TPMV. The initial G has been changed to a U or A. A Box 2 region has been located, in some cases it contains a single base change such that its ability to form five base pairs with Box 9L is preserved (Fig. 2C). Such compensatory base changes provide evidence that sequence elements are paired in folded RNA structures (Noller and Woese, 1981). Boxes 9R and 9R′ are also present, as well as the GC-rich Boxes A and B. Significantly, the 5′-to-3′ order of the sequence elements, 9R′, A, B, 9L, 9R, and 2, is the same as in the Group I introns.

This order is important, because the pairing of the sequence elements determines the structure of the Group I intron (Fig. 1A). In this case, the secondary structure would involve 9R:9R′ and A:B pairing, while 9L and 2 would pair at the tertiary level. When the boxes in PSTV are paired, a viroid structure is generated that is strikingly similar to that of Group I introns in the region of the boxes (Fig. 1B). The calculated free energy of this molecule, approximately −100 kcal, is not nearly as favorable as that calculated for the rod structure, −209.8 kcal, yet it is possible that a structure such as this might be stabilized by proteins *in vivo*.

The self-splicing intron of *Tetrahymena* rRNA requires no proteins for splicing, or to maintain its structure, but other introns, with less stable base pairing or less complementarity in the boxes, might well require proteins to maintain an active structure. Such a situation appears to occur with the nuclear rRNA intron of *Neurospora crassa*. Although the six boxes are present, they do not pair to give the core structure in deproteinized RNA because stronger alternate base pairing forces the region into a rod structure. *In vivo*, however, the intron exists as a ribonucleo-

FIGURE 2. (A) The "central conserved region" of PSTV contains a 15/19 nucleotide homology with the "d stem" of the *Tetrahymena* self-splicing intron, and a 12/16 nucleotide homology with the Group I consensus sequence, found in all Group I introns. (B) Homology with the Group I consensus sequence is present in both viroids (PSTV and CCCV are shown) and virusoids (VTMoV). (C) A series of conserved sequence elements that pair and delineate a characteristic structure for the Group I introns are also found in the same 5′-to-3′ order in viroids and virusoids.

A

```
        "d STEM"

85      GGA          GAAAC         109
      AG   UCC.CCGGG      CUGGAGC
      ..   ... .....      ......
277   UC   AGGUGGCCC      GGCUUC   249
       AACAA         AUCAUC

    "GROUP I CONSENSUS"
```

B

```
                        C     GA
Group I Consensus:      GPyUCAACGACUACANG
                        U
```

Zea mays mitochondrial mRNA intron CO II Il: CGUCGGAGACUAAAAG

Vicia faba chloroplast tRNA leu intron: GUGCAGAGACUCAAUG

Tetrahymena thermophila nuclear rRNA intron: GUUCACAGACUAAAUG

PSTV: CUUCGGCUACUACCCG

CCCV: UGGGAGAGACUACCCG

VTMoV: GUAAACGUACUACAGA

C

	Box 9L:	Box 9R:	Box A:
	Box 2	Box 9R'	Box B
Tetrahymena rRNA intron:	GACUA ..... CUGAU	UGUCGGUC .. .. ACUGCCAG	UGCGGG ..... ACGCCC
PSTV	UACUA .... UUGGU	GGUGGAAA .. .... CCCACUUU	UUCGGG . .... ACGCCC
CCCV	GACUA ..... CUGGU	GGUGGAAA .. ... . CCUCCUCU	CUUCUGG .... GAAGGCC
VTMoV:	UACUA .... UUGGU	CAGAGCUA . .... CUAGUGAU	GGGAGG . . .. CACUCC

protein, and psoralen cross-linking studies indicate a structure that is consistent with pairing of the conserved sequence elements (Wollenzein, personal, communication).

Recent evidence indicates that PSTV is found in a ribonucleoprotein complex *in vivo* (Schumacher *et al.*, 1983; Wolff *et al.*, 1985) and this offers the intriguing possibility that viroids, found as rods in the deproteinized state (Riesner *et al.*, 1979; Henco *et al.*, 1979; Gross *et al.*, 1982), may yet assume other foldings when stabilized by proteins in the cell.

IV. VIROIDS AND THE SELF-SPLICING INTRON OF *TETRAHYMENA*

The homologies between Group I introns and viroids prompted a search for other similarities with the self-splicing intron of *Tetrahymena thermophila*. The two circular RNAs are similar in size, PSTV is 359 bases in length, and the *Tetrahymena* intron is 399 bases in its circular form. The similarities between the two classes of RNAs in their secondary and tertiary structures as Group I introns have already been noted. Surprisingly, a strong homology between the "d stem" of the *Tetrahymena* intron and the viroid central conserved region, comprising 15/19 bases, was found (Diener and Hadidi, personal communication; Fig. 3A). This is the portion of the viroid that is shown paired, in the central conserved region of the rod structure (Fig. 2A), with the 16-nucleotide consensus sequence found in all Group I introns. This region has been proposed as a recognition site for a polymerase involved in viroid replication (Kiefer *et al.*, 1983), or as a signal for initiation, elongation, or termination of replication (Gross *et al.*, 1982). The virusoids, which contain the 16-nucleotide region, but contain a degenerate "d stem" sequence (Fig. 3A), are unable to replicate without a specific helper virus present (Haseloff and Symons, 1982). Experiments in our laboratory have shown that the "d stem" portion of the *Tetrahymena* intron can be deleted with little or no effect on the self-splicing activity of the molecule (G. Dinter-Gottlieb, L.A.H. Dokken, and T.R. Cech, in preparation). Thus, it must serve another function in the RNAs in which it is conserved.

Concatemer linear forms of viroids (see Chapter 5, this volume) and virusoids (Bruening *et al.*, 1982) have been detected *in vivo*. These are believed to be replication intermediates that are synthesized in a rolling circle-type mechanism by unidentified plant polymerases (Owens and Diener, 1982; Branch and Robertson, 1983; Chapter 5, this volume). Existence of oligomeric replication intermediates requires that monomers are produced by specific cleavage of oligomers and ligation of the monomers to form covalently closed circular molecules. Although the enzymes for these reactions have not been identified, an RNA ligase activity from wheat germ can cyclize the natural PSTV linear monomers (Branch *et al.*, 1982).

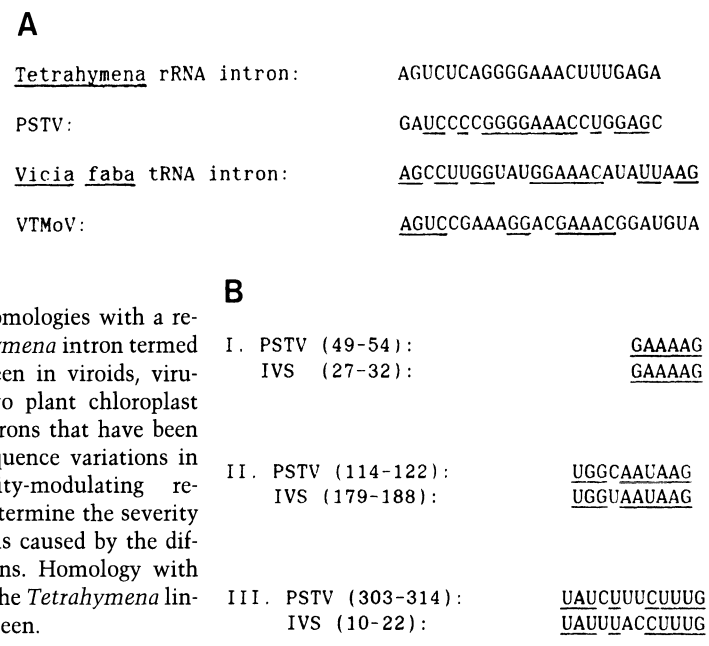


FIGURE 3. (A) Homologies with a region of the *Tetrahymena* intron termed the *d stem* are seen in viroids, virusoids, and the two plant chloroplast tRNA Group I introns that have been sequenced. (B) Sequence variations in the "pathogenicity-modulating regions" of PSTV determine the severity of viroid symptoms caused by the different viroid strains. Homology with these regions and the *Tetrahymena* linear intron is also seen.

Yet linear concatemers can be formed in a nonenzymatic fashion. The linear *Tetrahymena* intron is capable of concatemerization *in vitro*. In this case, the 3′ terminal G-OH attacks the junction between nucleotides 15 and 16 in another linear molecule, covalently attaching, and releasing a 15-mer oligonucleotide, in a reaction analogous to the cyclization reaction. Dimer, trimer, and larger linear molecules may be produced, as well as their circular forms. Under cyclization conditions, these will then form monomeric circles (Zaug and Cech, 1985). Cloned viroid transcripts might be assessed for such activity as well. A similar model for minus-strand concatemerization and plus-strand processing for the peanut stunt virus-associated RNA 5 (PARNA 5), which also contains intronlike box sequences, as well as a noncoded 3′ terminal guanosine in the minus strand, has recently been proposed (Collmer *et al.*, 1985).

Finally, comparisons were made between the pathogenicity-modulating regions of PSTV and the *Tetrahymena* intron. The differences in symptom severity caused by mild, intermediate, and severe strains of PSTV depend upon the nucleotide sequences in three regions of the molecule (see Chapter 5, this volume). Figure 3B reveals that homologies are found between the *Tetrahymena* linear intron and each of the pathogenicity-modulating regions of the mild PSTV strain. The region of homology in the IVS from nucleotides 10 to 22 contains the site of cyclization of the linear molecule (Zaug *et al.*, 1983). Since even minor changes in the sequence of PSTV may eliminate replication and infectivity (Owens *et al.*, 1986), it is difficult to ascertain whether these similarities have any

significance, but they further reinforce the relationship between the two molecules and emphasize the conservation of significant sequences across species.

V. DISCUSSION AND DIGRESSIONS

In summary, the hypothesis that viroids might be related to introns has been further supported by numerous sequence and structural homologies between Group I introns as a class, and specifically with the self-splicing intron of *Tetrahymena thermophila*. In fact, based on the crucial sequence similarities, the viroids and virusoids appear to be closely related to Group I introns. The question still remains as to whether viroids evolved from introns, or whether both evolved from a common ancestor molecule. The consensus sequence of 16 nucleotides appears to be an integral part of the Group I intron structure, and its phylogenetic conservation attests to its importance.

Fortunately, it is possible to approach some of the pertinent questions on an experimental level. To begin, can viroids self-cleave and autocyclize? The cloning of PSTV cDNA (Cress *et al.*, 1983) allows *in vitro* transcription of viroid molecules, and possible precursor molecules can be isolated and placed under splicing and cyclization conditions, in reactions analogous to those seen for the *Tetrahymena* self-splicing intron (Grabowski *et al.*, 1981; Zaug and Cech, 1982; Zaug *et al.*, 1983). A possible drawback here concerns the *in vitro* structure of the viroid transcript, which, once deproteinized, will be in the rod form, and presumably inactive. However, the existence of metastable forms (Henco *et al.*, 1979; Gross *et al.*, 1982) might contribute a population of active molecules with base-pairing similar to Group I introns.

Multimers of PSTV and of the satellite RNA of tobacco ringspot virus have recently been reported to produce monomeric-length molecules containing a 2′,3′ cyclic phosphate *in vitro*, under conditions similar to those used for *Tetrahymena* intron self-splicing (Robertson *et al.*, 1985; Prody *et al.*, 1986). If this reaction proves to be relevant to the *in vivo* replication cycle of these molecules, it would indicate a different cleavage and ligation mechanism than that seen for the *Tetrahymena* intron.

Another question concerns the natural linear viroid molecules arising from the viroid circles upon storage (Sänger *et al.*, 1979). Attempts have been made to identify the ends of these natural linear molecules, but the results have been only partially in agreement (Kikuchi *et al.*, 1982; Palukaitis and Zaitlin, 1983). When the circular *Tetrahymena* intron is incubated in a Mg^{2+}-containing buffer, a unique bond is broken, at the same site at which the circle was formed. This has been termed *autoreopening* or *site-specific hydrolysis* (Zaug *et al.*, 1984). The reaction rate increases rapidly with pH in the pH range 7.5–9.5 (Zaug *et al.*, 1984). It should be possible to subject the viroid circles to similar conditions, in order to see if such a specific bond might be selected.

The discovery that the "central conserved region" of the viroids is not unique to viroids, but that one portion of it exists in all Group I introns as the Group I consensus, and another portion is found in some introns as the "d stem" (Fig. 2A), raises further questions about the function of this region. So far the "d stem" region has been found to be well conserved in the *Tetrahymena* intron, and present in degenerate form in the virusoids and in the two plant chloroplast Group I tRNA introns that have been sequenced (Steinmetz *et al.*, 1982; Bonnard *et al.*, 1984; Fig. 3A). While this region is not necessary for self-splicing of the *Tetrahymena* intron, it may serve as a recognition site for structural proteins, or, as previously suggested, be essential for replication of the viroids.

The function of introns is still unknown. It has been theorized that they might contribute to evolutionary diversity (Gilbert, 1978), but since such a contribution can only be seen in retrospect, experimental work has centered on finding functions for specific introns. A function in gene expression has been found for the yeast tRNA tyr intron, SUP 6 (Wallace *et al.*, 1980; Johnson and Abelson, 1983), but no similar function was detectable for the yeast actin gene (Ng *et al.*, 1985). In *Tetrahymena pigmentosa*, not all strains contain an intron in the nuclear rDNA (Wild and Gall, 1979).

The Group I introns of the yeast mitochondrial cytochrome *b* gene contain open reading frames that can code for maturase, a protein involved in the splicing of the intron (Lazowska *et al.*, 1980; De La Salle *et al.*, 1982). The splicing of the intron destroys its capacity to code for the maturase, so a feedback regulatory loop is established in which the intron controls its own splicing.

In the case of the viroids, it appears that an intron can display selective pathogenic activity. Is it possible that other introns may behave as pathogens? No similar phenomena have been reported, and unique features of the viroid may make it unlikely. While the half-life of the *Tetrahymena* intron is 6 sec following excision *in vivo* (Brehm and Cech, 1983), viroids may remain stable for months due, presumably, to their unusually stable structure. In fact, the rod structure might serve as a storage form while a viroid ribonucleoprotein complex is active during replication.

Recent results from this laboratory indicate that the linear form of the excised *Tetrahymena* intron can act as an RNA polymerase (Zaug and Cech, 1986). It would be intriguing to test whether viroid molecules have such an activity. Its presence might account for some of the pathogenic effects of the viroid molecules in plants.

Individual viroids have evolved to an exquisite precision in their ability to replicate in certain plants, yet the existence of different viroid "species," differing in sequence and host specificity, may indicate that other introns might be pathogenic once their proper host is found.

Viroids are the smallest pathogens known. Their small size and composition have made them appealing to study, but recent findings and the

questions they raise suggest that we are only beginning to understand the complexities of their pathogenicity and origin.

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CHAPTER 8

POSSIBLE VIROID ORIGIN
Encapsidated Viroidlike RNA

R. I. B. FRANCKI

I. INTRODUCTION

Although showing many characteristics of the Sobemovirus group, four recently described viruses from Australia have unusual RNA complements (Francki *et al.*, 1985). In addition to RNA similar to that of true Sobemoviruses, the viruses also encapsidate large amounts of low-molecular-weight RNA reminiscent of viroid RNA, which has been referred to as viroidlike RNAs by some workers (Francki *et al.*, 1985) and virusoids by others (Symons *et al.*, 1985).

The main features of viroidlike RNAs have recently been reviewed (Francki *et al.*, 1985) as has their possible mode of replication (Symons *et al.*, 1985). Here, I wish to present a brief overview of the properties of viroidlike RNAs, their biological significance, mode of replication, and possible origin and evolution. Throughout this review I have emphasized the similarities and differences between the viroidlike RNAs and the viroids.

II. VIRUSES THAT ENCAPSIDATE VIROIDLIKE RNAs

Velvet tobacco mottle virus (VTMoV) was isolated from *Nicotiana velutina* (velvet tobacco), a wild native plant growing in the desert zone of southcentral Australia (Randles *et al.*, 1981), and *Solanum nodiflorum* mottle virus (SNMV) from the common weed *Solanum nodiflorum* growing along the northeastern coast of Australia (Greber, 1981). The two viruses were shown to be serologically closely related (Randles *et al.*,

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1981; Chu and Francki, 1983) and to have narrow host ranges confined almost entirely to the Solanaceae (Francki *et al.*, 1985). The viruses have many characteristics of the Sobemoviruses (Fig. 1) but each was found to have an unusual RNA complement. In addition to a single-stranded (ss) RNA about 4500 nucleotides long (RNA 1) and two apparently subgenomic RNAs (RNA 1a and RNA 1b) similar to those of southern bean mosaic virus [the type member of the Sobemovirus group (Rutgers *et al.*, 1980)], each of the viruses also encapsidates two low-molecular-weight RNAs (Randles *et al.*, 1981; Gould, 1981; Gould and Hatta, 1981). One of these is a covalently linked circle (RNA 2) whereas the other is of the same size and base sequence but is linear (RNA 3). RNAs 2 and 3 have no base sequence homology with RNA 1 (Gould, 1981; Gould and Hatta, 1981). Separation of these RNAs, isolated from VTMoV capsids, by PAGE and their appearance in the electron microscope are shown in Fig. 2. RNAs 2 and 3 have many characterisitics of viroids (Diener, 1983).

Viroidlike RNAs have also been detected in capsids of lucerne transient streak virus (LTSV) (Tien *et al.*, 1981), a virus originally isolated from lucerne (*Medicago sativa*) in southeastern Australia (Blackstock, 1978) but subsequently also found in New Zealand (Forster and Jones, 1979) and Canada (Paliwal, 1983). No serological relationships were detected between LTSV and either VTMoV or SNMV. However, there appears to be a very distant relationship between LTSV and subterranean clover mottle virus (SCMoV) isolated from two clover species (*Trifolium subterraneum* and *T. globerata*) in southwestern Australia (Francki *et al.*, 1983b). Viroidlike RNA has also been detected in capsids of SCMoV (Francki *et al.*, 1983b).

III. ARE VIROIDLIKE RNAs SATELLITE RNAs?

A satellite (sat) RNA requires a helper virus both for its replication and encapsidation (Murant and Mayo, 1982; Francki, 1985). There is no doubt that the viroidlike RNA of LTSV is a sat-RNA because Jones *et al.* (1983) demonstrated that LTSV RNA 2 or 3 could not replicate autonomously but that RNA 1 could. A culture of LTSV free of RNAs 2 and 3 was maintained for many passages, which induced chlorotic instead of necrotic lesions on the inoculated leaves of *Chenopodium amaranticolor*; a property that was reversed by adding RNA 2 from LTSV to the inoculum (Jones *et al.*, 1983). The sat-RNA nature of LTSV RNA 2 is also supported by the demonstration that it can be helped by either sowbane mosaic virus (Francki *et al.*, 1983a) or southern bean mosaic virus (Paliwal, 1984) and also possibly by turnip rosette virus (Jones and Mayo, 1984), all of which probably belong to the Sobemovirus group but are serologically unrelated to LTSV (Matthews, 1982). None of these viruses had been originally isolated with viroidlike RNAs.

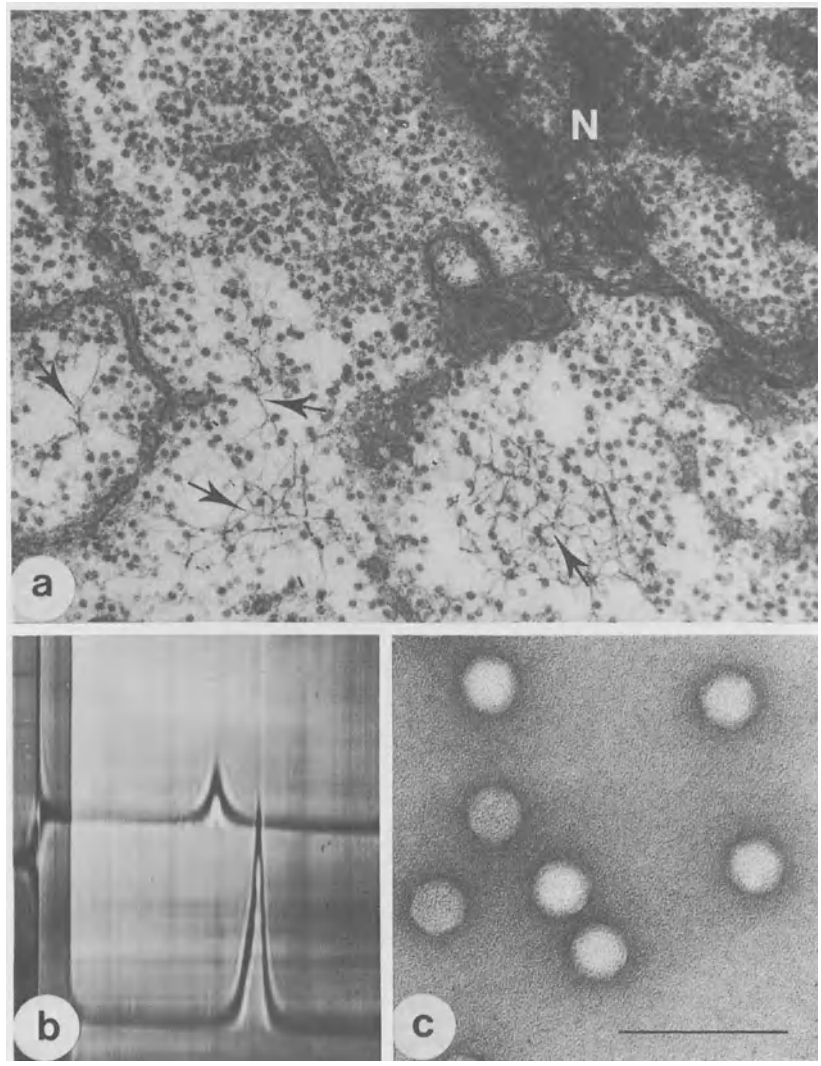

FIGURE 1. Properties of velvet tobacco mottle virus (VTMoV). (a) Electron micrograph showing VTMoV particles in a thin section of a leaf cell from infected *Nicotiana clevelandii*. The tissue had been cleared of ribosomes by RNase treatment as described by Hatta and Francki (1981); hence, all the small polyhedral particles, both in the cytoplasm and in the nucleus (N), are virus particles. The section also shows dense fibrillar material in the ground cytoplasm (arrows), which has been identified as virus-specific dsRNA (R. I. B. Francki, unpublished results). 2.5 cm = 500 nm. (b) Analytical ultracentrifugation showing the Schlieren pattern from a preparation of 2 mg/ml VTMoV (above) and a mixture of 2 mg/ml VTMoV and 2 mg/ml southern bean mosaic virus (below) indicating that both viruses sediment at the same rate (115 S). The photograph was taken at a bar angle of 50°, 12 min after the Spinco AnD rotor had reached a speed of 33,450 rpm. Sedimentation is from left to right. (c) Electron micrograph of a purified preparation of VTMoV particles negatively stained with uranyl acetate. 2.3 cm = 100 nm.

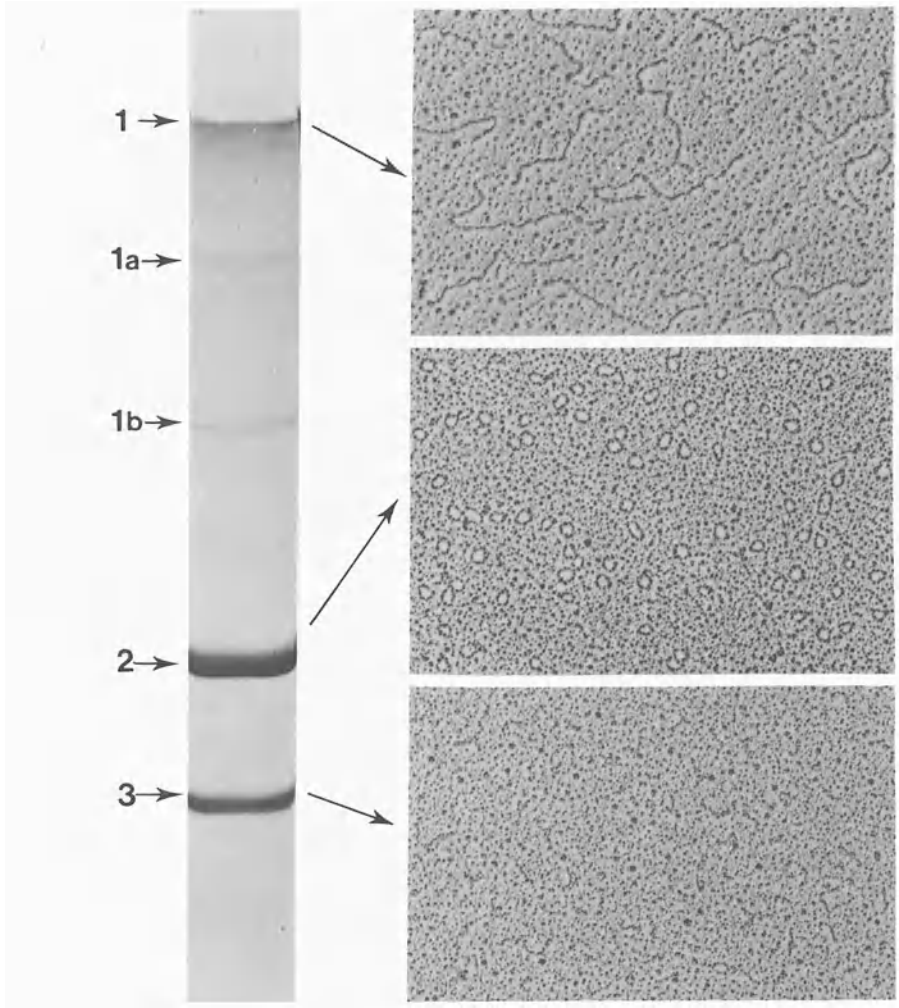

FIGURE 2. Properties of velvet tobacco mottle virus (VTMoV) RNA. RNA isolated from purified VTMoV was electrophoresed in polyacrylamide gel under denaturing conditions (Gould, 1981) as shown on the left. The fractions, 1, 2, and 3 (indicated by arrows) were recovered from the gels and examined by electron microscopy as described by Randles *et al.* (1981). Bar = 500 nm.

Like LTSV, the RNA 2 of SNMV also appears to be a sat-RNA. Jones and Mayo (1983, 1984) established a culture of SNMV devoid of RNA 2 and showed that the particles produced were serologically indistinguishable from those normally produced by SNMV. They observed that the symptoms produced on the inoculated leaves of *Nicotiana debneyi* were similar whether inoculated with SNMV containing RNA 2 or devoid of it. It was shown that SNMV RNA 2 could be helped by LTSV RNA 1 but

LTSV RNA 2 could not be helped by SNMV RNA 1. This indicates that there is some specificity among viroidlike RNAs and their helpers.

Contrary to some of the findings cited above, Gould *et al.* (1981) were unable to demonstrate the ability of either SNMV RNA 1 or VTMoV RNA 1 to replicate without their homologous RNAs 2. This led them to conclude that with both VTMoV and SNMV, RNAs 1 and 2 are indispensable for infection and that the interdependence is highly specific. At present it is difficult to reconcile these data with those indicating that the RNAs of LTSV and SNMV are sat-RNAs (Jones and Mayo, 1984). A similar discrepancy of data has emerged in the case of VTMoV, because recently an isolate of VTMoV devoid of RNAs 2 and 3 has been obtained in our own laboratory (Francki *et al.*, 1986). The virus was isolated from a *Nicotiana clevelandii* plant inoculated by a single viruliferous *Cyrtopeltis nicotianae,* the mirid vector of VTMoV (Randles *et al.*, 1981). This virus isolate (KI) has been passaged six times by mechanical inoculation and shown to retain its freedom from RNAs 2 and 3 and its antigenic identity with VTMoV. The KI isolate did not produce necrotic lesions on the inoculated leaves of *N. clevelandii.* However, after adding viroidlike RNA from normal VTMoV, the KI virus was shown to support viroidlike RNA and reverted to producing necrotic lesions on the inoculated leaves of *N. clevelandii* and systemic mosaic indistinguishable from that induced by normal VTMoV (Francki *et al.*, 1986). This indicates that RNA 1 of VTMoV, like those of LTSV and SNMV, is capable of autonomous replication and that VTMoV viroidlike RNA is a sat-RNA. However, the possibility that RNAs 1 of some isolates of VTMoV and SNMV (Gould *et al.*, 1981), or LTSV for that matter, require viroidlike RNAs for their replication cannot be disregarded.

Two distinct species of RNAs 2 and 3 have been detected in isolates of SCMoV and either one or both have been shown to be present in different field isolates of the virus (Francki *et al.*, 1983b). However, the relationship of their replication to RNA 1 has not been investigated.

Further research is needed to establish beyond doubt the exact relationships and specificities of all the viroidlike RNAs described with the RNA 1 of the virus that encapsidates them.

IV. THE DEPENDENCE OF VIROIDLIKE RNA ON VIRAL RNA 1

All the available data on VTMoV, SNMV, and LTSV indicate that when inoculated to plants alone, their viroidlike RNAs do not cause any symptoms and do not appear to multiply (Gould *et al.*, 1981; Francki *et al.*, 1983a; Jones and Mayo, 1983, 1984; Jones *et al.*, 1983). Although this suggests that RNAs 2 and 3 are dependent on RNA 1 for their replication, it is conceivable that the viroidlike RNAs do multiply in the inoculated

cells but are unable to egress from them, and that this function is fulfilled by RNA 1. In experiments where whole plant leaves are inoculated mechanically, only very few cells are infected directly and hence unless infection spreads to adjoining cells, any replication would be difficult to detect. In order to determine if RNAs 2 and 3 replicate in cells without the addition of RNA 1 to the inoculum, an experimental system is needed in which high multiplicity of infection can be achieved. The use of protoplasts (Takebe, 1977) may be suitable for this.

There are other benefits that viroidlike RNAs derive from RNA 1 besides possible help in their replication or intercellular movement, or both. Evidence from *in vitro* translation studies indicates that the capsid protein genes of LTSV, VTMoV, and SNMV reside on their RNAs 1 and are translated from their partial transcripts (Morris-Krsinich and Forster, 1983; Kiberstis and Zimmern, 1984; Francki *et al.*, 1985). Encapsidation of an infectious RNA not only offers it protection from nucleolytic enzymes, but can also ensure its vector transmission from plant to plant and may help its long-distance systemic spread within the plant. It seems clear that viroidlike RNAs gain much from their association with their RNAs 1. At present, however, it is more difficult to conclude about any potential advantages that RNAs 1 may derive from their association with viroidlike RNAs.

V. STRUCTURE OF VIROIDLIKE RNA

The entire nucleotide sequences of VTMoV, SNMV, LTSV, and SCMoV RNAs 2 have been determined. They are presented in Fig. 3 as models with their predicted secondary structures.

Haseloff and Symons (1982) found that preparations of VTMoV RNA 2 consisted of two approximately equimolar species, one of 365 and the other with an additional U residue in position 108. The primary structure of SNMV RNA 2 is remarkably similar to that of VTMoV (Fig. 3). It is a little larger in having 377 residues, but 92% of SNMV RNA 2 is homologous with VTMoV RNA 2 and 95% of VTMoV RNA 2 is homologous with that of SNMV RNA 2 (Haseloff and Symons, 1982). This is not surprising because VTMoV and SNMV have many properties in common, including substantial RNA homology of their RNAs 1 (Gould and Hatta, 1981) and they are closely related serologically (Chu and Francki, 1983; Francki *et al.*, 1985). Except for a few short sequences, there is very little homology between the RNAs 2 of either VTMoV or SNMV and LTSV (Keese *et al.*, 1983). Similarly, the two RNAs 2 of SCMoV appear to have few sequences in common with any of the other viroidlike RNAs. However, the sequences of the two SCMoV RNA 2 species show interesting differences. More than half the molecules show remarkable homology (left part of molecules in Fig. 3), whereas the remaining parts appear to be quite unrelated.

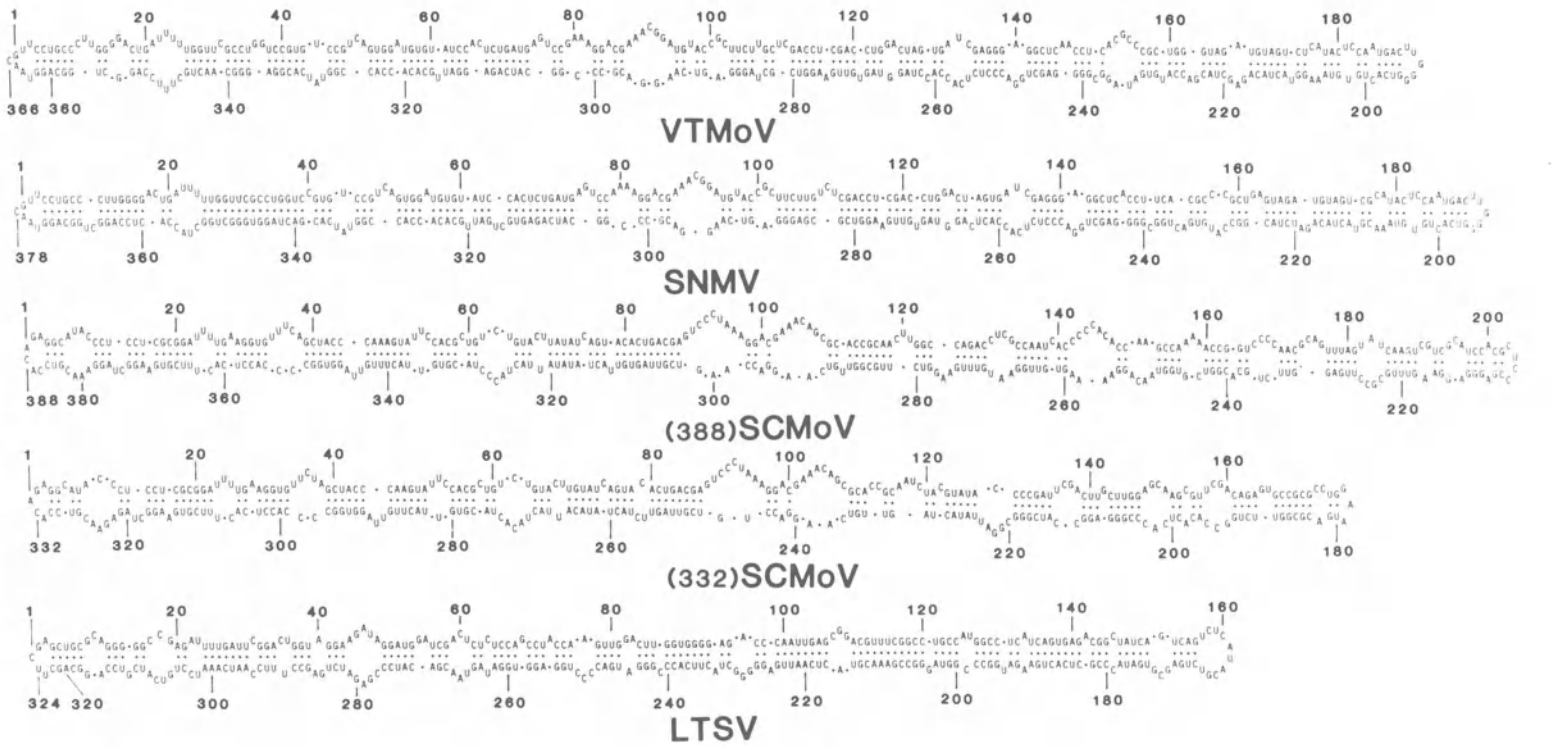

FIGURE 3. Structure of viroidlike RNAs. The primary and predicted secondary structures of viroidlike RNAs 3 from VTMoV, SNMV, SCMoV (the 388- and 332-nucleotide variants, respectively), and LTSV (New Zealand isolate) based on data from Haseloff and Symons (1982), Keese *et al.* (1983), Kiberstis *et al.* (1985), and J. Haseloff, C. Davies, and R. H. Symons (unpublished results).

The RNAs 2 of both the Australia (A) and New Zealand (N) isolate of LTSV (Fig. 3) are 324 nucleotides long with 98% sequence homology (Keese *et al.*, 1983). Again, this is not surprising because the virus isolates are serologically closely related although the degree of relationship has not been determined precisely (Francki *et al.*, 1983b). The two LTSV isolates are also similar in many other respects although they can be distinguished by their host ranges (Francki *et al.*, 1985).

Recently, Kiberstis *et al.* (1985) have detected in the nuclease PI digests of the RNAs 2 of both VTMoV and SNMV, a ribonuclease-resistant dinucleotide of unusual properties indicating that it has a 2′-phosphomonoester group with a core structure C3′p5′A. The C residue carrying the phosphomonoester was mapped to position 49 on the molecule (Fig. 3), a nucleotide that was overlooked from the VTMoV and SNMV RNA 2 sequences published by Haseloff and Symons (1982). This is not surprising because the 2′-phosphomonoester would have prevented both enzymatic and alkaline cleavage between the C and A residues of this core during the direct RNA sequencing methods used. The presence of this additional C residue has been confirmed when cDNA clones were sequenced (Kiberstis *et al.*, 1985). The significance of the C residue carrying the 2′-phosphomonoester has not been elucidated but it seems most likely that it is the site at which the viroidlike RNA molecules are litigated. It will be interesting to know if similar 2′-phosphomonoester-carrying residues can be detected in the other viroidlike RNAs.

The sedimentation properties and thermal denaturation kinetics of VTMoV and SNMV RNAs 2 have been investigated by Randles *et al.* (1982) and Riesner *et al.* (1982). The data indicate that the structure of these RNAs is essentially similar to that of viroids. However, the viroidlike RNAs sediment slightly faster, are thermally more stable, and melt with lesser cooperativity. It was concluded that the viroidlike RNAs are hydrodynamically more flexible than viroids. The biological significance of the physical differences between the two types of circular RNAs remains obscure.

VI. REPLICATION OF VIROIDLIKE RNA

The limited amount of work that has been done on the replication of viroidlike RNA is confined almost exclusively to that of VTMoV. Hence, the discussion here will be confined to the RNAs associated with that virus.

VTMoV multiplies profusely in *N. clevelandii*, an experimental host, reaching concentrations in excess of 3 and 10 mg/g fresh weight of inoculated and systemically infected leaf tissues, respectively (Chu *et al.*, 1983). About 80% of the RNA in the virus particles isolated from such plants can be accounted for by the viroidlike RNAs 2 and 3 (Randles *et al.*, 1981). Furthermore, it has been found that from one-third to one-half of the total RNA in fully infected leaves can be accounted for by the

viroidlike RNA (Randles *et al.*, 1981). Studies on *N. clevelandii* leaves inoculated with VTMoV have also revealed the appearance of virus-specific dsRNA and RNA-dependent RNA polymerase (Chu *et al.*, 1983; Francki *et al.*, 1985).

A. *In Vivo* Studies

PAGE profiles of RNA preparations extracted from healthy and VTMoV-inoculated leaves revealed the presence of several virus-specific RNAs in the infected tissues (Chu *et al.*, 1983). Two days after inoculation, small amounts of RNAs 2 and 3 were already detectable and their concentrations increased thereafter. With increasing time of infection, the ratio of RNA 2 concentration to that of RNA 3 increased significantly, suggesting that RNA 3 may be a precursor of RNA 2. Such a chain of events would require the replication of RNA 3 followed by its ligation to produce RNA 2. A ligation reaction converting RNA 3 to RNA 2 has been demonstrated *in vitro* by T4 RNA ligase (Chu *et al.*, 1983). The recent demonstration that one C residue of VTMoV RNA 2 carries a 2′-phosphomonoester (Kiberstis *et al.*, 1985) suggests that it is the point of ligation via a 2′-phosphomonoester, 3′,5′-phosphodiester bond catalyzed by an enzyme akin to the RNA ligase found in wheat germ and *Chlamydomonas* (Konarska *et al.*, 1981; Kikuchi *et al.*, 1982). The enzyme from wheat germ has been shown to be capable of circularizing viroids (Branch *et al.*, 1982).

Two distinct dsRNAs of M_r about 3.6×10^6 and 2.8×10^6, respectively, have been detected in VTMoV-inoculated leaves. Molecular hybridization experiments indicate that the smaller dsRNA was a duplex of VTMoV RNA 1 and the larger consisted of a single strand of RNA with base sequences complementary to RNA 3 paired to various lengths of RNA with base sequences of RNA 3 (Chu *et al.*, 1983). It appears that this large molecule (M_r 3.6×10^6) consists of a tandem of about 15 negative-strand RNA 3 molecules paired to various polymers of positive RNA 3. It was suggested that perhaps the smaller dsRNA is a replicative form of RNA 1 and the larger, a complex for the replication of RNA 3, and hence following ligation, also RNA 2 (Chu *et al.*, 1983; Francki *et al.*, 1985). However, both dsRNAs were shown to accumulate with time of infection, reaching their highest concentration when the rate of virus synthesis was already declining (Chu *et al.*, 1983). This suggests that both virus-specific dsRNAs are, at least in part, by-products of virus synthesis.

B. *In Vitro* Studies

The RNA polymerase detected in VTMoV-infected *N. clevelandii* leaves has been isolated together with its endogenous template from the cytoplasmic fraction of tissue extracts, and used for *in vitro* RNA syn-

thesis experiments (Francki *et al.*, 1985; Rohozinski *et al.*, 1986). The enzyme–template complex was shown to synthesize dsRNA only. The results of molecular hybridization experiments with ssDNA probes (cloned in phage M13) containing positive- and negative-sense inserts specific to VTMoV RNAs 1 and 2, established that only positive strands of the dsRNA had been synthesized *in vitro* (Rohozinski *et al.*, 1986). The synthesis was unaffected by actinomycin D or DNase, indicating that the polymerase was not DNA-dependent. The *in vitro* reaction was also unaffected by α-amanitin at concentrations between 10^{-8} and 10^{-5} M, indicating that RNA polymerase II or III was not involved in the synthesis of the RNA product. It was concluded from these studies that the viroidlike RNA utilizes an RNA-dependent RNA polymerase in the cytoplasm of VTMoV-infected cells. This is unlike the viroids, which appear to be synthesized in the nuclei of plant cells with the help of host RNA polymerases, probably mainly RNA polymerase II (Rackwitz *et al.*, 1981; Semancik and Harper, 1984).

The dsRNA synthesized *in vitro* by the VTMoV-specific enzyme–template complex was separated into two fractions by PAGE, a larger dsRNA of M_r about 3.6×10^6 and a smaller one of about 0.72×10^6. The larger product was initially thought to correspond to the two dsRNAs isolated from virus-infected tissues (Francki *et al.*, 1985), but subsequent experiments have provided data inconsistent with this conclusion (Rohozinski *et al.*, 1986).

The larger dsRNA (M_r 3.6×10^6) synthesized *in vitro* appears to have an electrophoretic mobility slightly slower than either of the dsRNAs isolated form infected leaf tissues. On melting in formamide and formaldehyde, it yielded material with even slower mobility, indicating that it is RNA 1-specific. The smaller dsRNA (M_r 0.72×10^6) when melted, however, yielded mainly ssRNA with the electrophoretic mobility of RNA 3 (Rohozinski *et al.*, 1986). This suggests that the viroidlike RNA is synthesized on a template about three times its length. All attempts to detect the synthesis of a dsRNA of this size in infected leaves by labeling strips of tissue with [^{3}H]uridine were unsuccessful (Rohozinski *et al.*, 1986). The possibility that the dsRNA synthesized *in vitro* was a degradation product of the larger RNA 3-specific dsRNA detected in leaf tissues (Chu *et al.*, 1983) was considered, but seems unlikely (Rohozinski *et al.*, 1986).

C. How Is Viroidlike RNA Synthesized?

With the limited data available at present, it is probably premature to speculate in any detail about the mechanism of viroidlike RNA replication. The apparent discrepancy between the results of the *in vitro* experiments using the RNA-dependent RNA polymerase-template preparation and those with intact leaf tissue is somewhat puzzling and requires elucidation. One possibility is that the smaller dsRNA detected

in the *in vitro* experiments is a very short-lived intermediate in the synthesis of RNA 3 which does not reach sufficient concentrations for long enough to be detected by *in vivo* labeling. On the other hand, the larger RNA 3-specific dsRNA detected in leaf tissues may be a dead-end product of RNA 3 replication and not an intermediate.

Even the limited data available at present, point to some similarities in the replication of viroidlike RNA to that of viroids for which rolling-circle models have been proposed (Branch and Robertson, 1984; Ishikawa *et al.*, 1984; Symons *et al.*, 1985), although the cellular sites of synthesis and the enzymes involved appear to be different. This conclusion is supported by the following observations:

1. That VTMoV RNA 3 synthesized *in vitro* by virus-specific polymerase-template preparations was detected as a greater-than-unit-length dsRNA complex (Rohozinski *et al.*, 1986).
2. That greater-than-unit-length positive- and negative-sense transcripts of VTMoV viroidlike RNA were detected in infected leaf tissues (Chu *et al.*, 1983).

VII. POSSIBLE RELATIONSHIPS OF VIROIDLIKE RNAs TO VIROIDS

The viroidlike RNAs resemble viroids in that they are circular ssRNAs of similar size and high degree of base pairing (Haseloff and Symons, 1982; Keese *et al.*, 1983); also, both lack *in vitro* messenger activity (Kiberstis and Zimmern, 1984; Francki *et al.*, 1985). These striking similarities raise the question as to whether one of these pathogenic RNAs evolved from the other. Francki *et al.* (1985) actually suggested that viroidlike RNAs may have originated from viroids by the coinfection of plants with viruses and viroids. It was postulated that such an association may have led to the loss of the viroid's ability to replicate autonomously and acquiring dependence on the virus for both its replication and encapsidation. It must be stressed, however, that before such a hypothesis is embraced too enthusiastically, it is essential also to consider the important differences between viroidlike RNAs and viroids.

A very significant difference between a viroidlike RNA such as that of VTMoV and viroids like PSTV or CEV is their apparent sites of synthesis in cells. *In vitro* replication studies of VTMoV (Francki *et al.*, 1985; Rohozinski *et al.*, 1986) indicate that the viroidlike RNA replicates in the cytoplasm, utilizing an RNA-dependent polymerase. Also, viroidlike RNA reaches very high concentrations in infected plants (Randles *et al.*, 1981). On the other hand, viroids appear to be largely confined to the nucleoli of infected cells where they occur in low concentrations (Schumacher *et al.*, 1983). Moreover, viroid replication appears to utilize nuclear RNA polymerases, probably predominantly RNA polymerase II (Rackwitz *et al.*, 1981; Semancik and Harper, 1984). Thus, the origin of

viroidlike RNAs from viroids would involve not only a loss of the viroid's ability to replicate autonomously and cement an association with a virus, but it would also have to change its exploitation of the host's nuclear RNA-synthesizing machinery to that of a virus-induced system in the cytoplasm. At present, it is difficult to assess the likelihood of such evolutionary changes having taken place.

Recent evidence from sequence data has established some striking similarities between viroids and plant virus satellite RNAs with linear molecules such as those of peanut stunt and tobacco ringspot viruses (Collmer *et al.*, 1985; Buzayan *et al.*, 1986). This suggests that some satellites other than those with circular molecules (viroidlike RNAs) may also have affinities with the viroids.

Numerous suggestions have been made about the possible origins of viroids (Sänger, 1982). Perhaps the most plausible of these is that they evolved from spliced out introns that became circularized (Roberts, 1978; Crick, 1979; Diener, 1979, 1981; Gross *et al.*, 1982; Zimmern, 1982). This idea is supported by the similarities between the conserved regions of viroid molecules and those of transposable elements (Kiefer *et al.*, 1983). It may be worth considering the possibility that the viroidlike RNAs as well as some other satellite RNAs had similar origins. The viroids may be the descendants of those circular RNAs that parasitized plant nuclei and the satellite RNAs those that escaped into the cytoplasm to become associated with viruses that became their helpers.

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PART II

SPECIAL SECTION

CHAPTER 9

Potato Spindle Tuber

T. O. DIENER

Of all plant diseases now known to be caused by viroids, the potato spindle tuber disease was the first to be recognized and studied by plant pathologists. Also, it was during efforts to purify a putative virus from potato spindle tuber-affected plants that the aberrant properties of the causative agent were recognized, the first viroid isolated, and the viroid concept developed. Thus, the potato spindle tuber disease truly represents the prototype of all viroid diseases.

I. HISTORICAL

The spindle tuber disease of potato was first described by Martin (1922), who reported that this "new potato trouble made its appearance in South Jersey in fields of late planted Irish Cobblers grown for seed purposes." Martin noted that the name *spindle tuber* originated with local growers and that in almost every instance, affected plants were found in fields planted with potatoes grown in Maine the previous year. Although he had no positive evidence to the effect that spindle tuber was an infectious disease (such as was known to be the case at that time for potato leaf roll and mosaic disease), Martin considered this to be likely because by the fall of 1922 some strains of Irish Cobblers that had been grown in New Jersey for a number of years developed the disease (Martin, 1922).

Evidently, the spindle tuber disease was imported into New Jersey from Maine and in the following year, Schultz and Folsom reported on their extensive investigations of the disease in Maine. They stated (Schultz

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and Folsom, 1923) that the disease had been recognized for many years by growers and others by various names, such as "running out," "running long," "off shape," "poor shape," "reversion," or "senility." The authors described their observations and experimental work from 1917 to 1921 that led to the conclusion that the disease spread in the field, was infectious, and therefore properly belonged to the group of diseases that were at that time known by the term *degeneration disease.* From a historical standpoint, it is interesting to recall that these degeneration diseases had long been blamed on senility, "reversion," or loss of vigor caused by "unnatural" prolonged asexual reproduction of potatoes. At the time of the first report of Schultz and Folsom (1923), however, the infectious nature of these diseases was recognized.

In the same year, potato spindle tuber disease was reported from Vermont and further reports on the disease appeared. For a number of years, research on spindle tuber disease was centered in New England and Nebraska (discussed in Diener, 1979).

II. GEOGRAPHIC DISTRIBUTION

The disease is common in the potato-growing regions of the northern and northeastern United States and Canada (Diener and Raymer, 1971). Potato "gothic" virus, described from the USSR, has been reported to be a combination of "necrotic tuber spot, leaf roll, black peel, and the spindle tuber virus of America" (Leont'eva, 1964). Thus, the potato spindle tuber disease appears to be present in the USSR. Fernandez Valiela and Calderon (1965) reported that the disease occurred in potato-growing areas of Argentina and Li (1983) that it is widespread in China.

III. SYMPTOMATOLOGY

Depending on the potato cultivar and on environmental conditions, symptoms of the disease may vary considerably. Foliage symptoms are obscure in many instances and the plants may be severely or not at all stunted. From late spring to midsummer, the foliage often turns slate gray with dull leaf surface. The tubers characteristically are elongated with prominent bud scales ("eyebrows") and sometimes have severe growth cracks (Diener and Raymer, 1971) (Fig. 1).

In their original publications, Schultz and Folsom noted that in the cultivar Green Mountain, the disease was characterized by spindliness and uprightness of potato plants, by more erect and often somewhat darker green leaves than healthy ones, and by slight rugosity of leaves. The tubers were "abnormally spindling, spindle shaped, cylindrical and supplied with conspicuous eyes" (Schultz and Folsom, 1923).

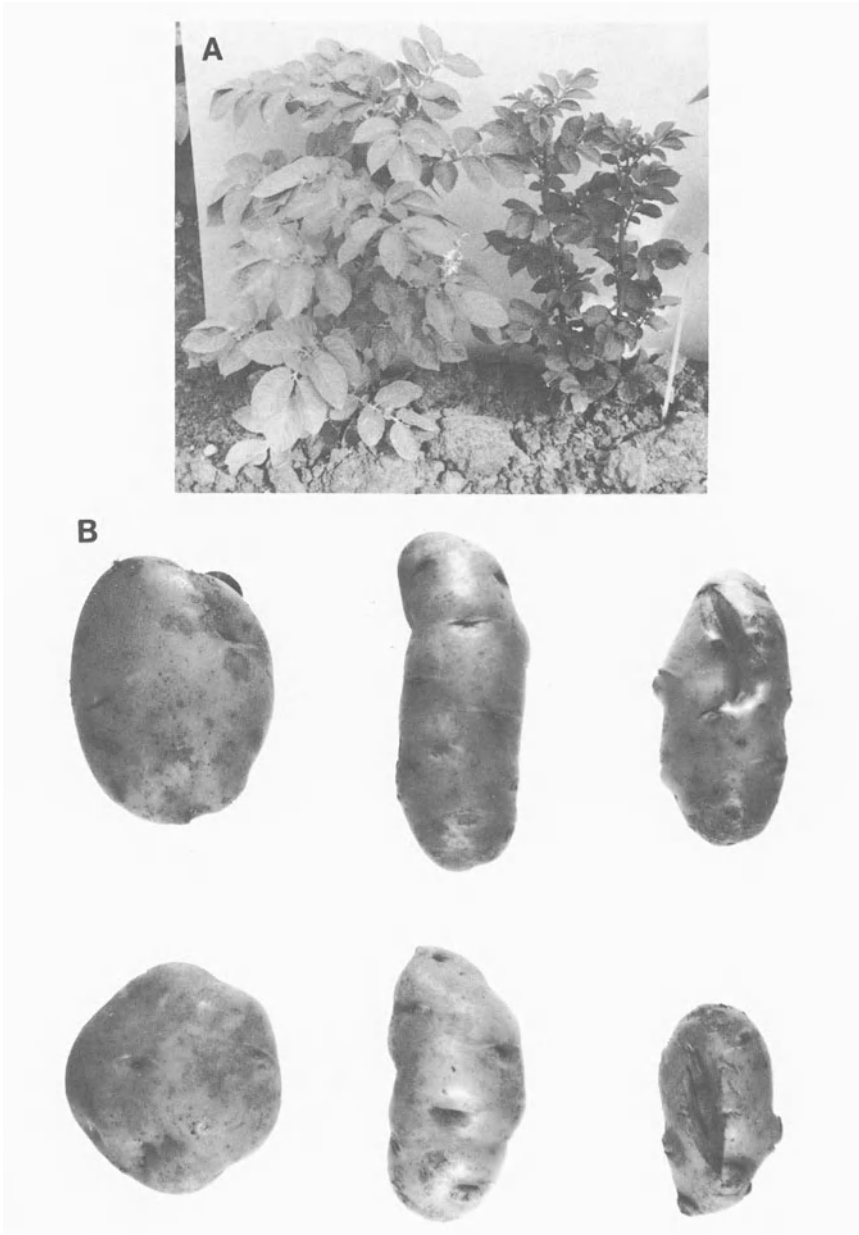

FIGURE 1. Potato spindle tuber disease. Symptoms of PSTV in potato (*Solanum tuberosum*). (A) Foliage symptoms in cv. Irish Cobbler. Healthy plant at left. (B) Tuber symptoms. Upper row: cv. Saco; lower row: cv. Kennebec. Left: healthy; center: infected with the type strain; right: infected with unmottled curly dwarf strain. Courtesy of U.S. Department of Agriculture, Beltsville, Md.

In Irish Cobbler plants, the disease is manifested by vines that grow upright, branch but little, and are smaller than normal (Martin, 1922). The affected vines die earlier than healthy ones and the leaves are much more narrow and pointed than typical leaves. Tubers from affected plants are long, narrow, smooth skinned, show more eyes than the true type cobblers, and the eyes are sometimes borne on knoblike protuberances (Martin, 1922).

Early investigators already noticed the marked influence of environmental factors on symptom development. Thus, Goss (1924) reported that cool weather during the early growth of plants masked the symptoms of spindle tuber so that even with high temperatures later in the season the plants did not have severe symptoms. On the other hand, plants that started growth under high-temperature conditions, such as occurred with the late-planted lots, showed severe symptoms throughout the season. Similarly, tuber symptoms of plants grown in the greenhouse at a temperature of 25°C were more severe than at 15°C.

The importance of temperature on symptom expression was confirmed in a study in which individual lots of tubers were divided, with one portion being planted in Maine and the other portion in Florida (Gratz and Schultz, 1931). In Florida, the spindle tuber plants never attained a height greater than from one-third to one-half that of normal plants, while in Maine the diseased plants were about two-thirds to three-fourths as large as those from healthy stock. Otherwise, however, the foliage symptoms were practically the same in both locations.

IV. FIELD TRANSMISSION AND SPREAD

Conclusive evidence for the infectious nature of the spindle tuber disease was first presented by Schultz and Folsom (1923), who noticed that if strains free from this malady were planted near stock with a high percentage of diseased plants, a considerable percentage of spindle tuber resulted among the healthy plants "in a few years."

Experiments in which healthy plants were allowed to grow at various distances from spindle tuber plants indicated that the percentage of spindle tuber decreased as the distance from diseased hills increased, and that a higher percentage of infection of healthy hills occurred as the percentage of spindle tuber increased (Schultz and Folsom, 1923).

In transmission experiments, both in the greenhouse and in the open field, infection was obtained with tuber and vine grafts, with a "leaf mutilation" technique, and with aphids (species not indicated) (Schultz and Folsom, 1923).

On the basis of these results (and because of the demonstrated perpetuation of the disease in the tubers), the authors characterized spindle tuber as a degeneration disease. Later results by other workers demon-

strated that spindle tuber was readily transmitted by contact of diseased with healthy plant parts, including foliage.

Although mechanical transmission of spindle tuber through the foliage had thus been shown to occur, for many years this method was not considered to be important under field conditions. It was only when Bonde and Merriam (1951) demonstrated that relatively high percentages (33 to 50%) of transmission could be obtained either by rubbing together freshly cut surfaces of healthy and diseased seed pieces or by bruising young sprouts of healthy plants and contaminating them with sap from infected tubers that the concept of mechanical transmission as an important means of spindle tuber spread under field conditions gained acceptance.

Further detailed studies on the mechanical transmission of the disease under field conditions were reported by Manzer and Merriam (1961). They concluded that the disease could be disseminated very readily in potato fields by contact of healthy vines with contaminated cultivating and hilling equipment. In the Katahdin and Kennebec varieties, nearly 100% transmission was recorded in tests that simulated excessive contact of large vines with contaminated equipment, whereas under conditions of less severe vine contact, a lesser amount of transmission was observed.

In summary, the importance of mechanical transmission for the spread of potato spindle tuber disease in the field appears to be well established, whereas the role of arthropod species as vectors of the disease agent is equivocal.

Experimentally, PSTV can be transmitted through both the seed and pollen of infected plants (Fernow *et al.*, 1970). Although transmission through the seed from open-pollinated female parents to the seedlings occurred frequently (average 31%), it varied in individual collections from 0 to 100%. The amount of transmission did not appear to be correlated with variety or with age of the seed (Fernow *et al.*, 1970). Whether seed or pollen transmission of PSTV is epidemiologically important is not known, but seems unlikely in view of the asexual propagation of potatoes.

V. ECONOMIC LOSSES

The potato spindle tuber disease poses a potentially serious threat in seed production, germ plasm collections, and potato cultivation. Foliage symptoms are often obscure, and severity of the characteristic tuber symptoms—elongation with the appearance of prominent bud scales and growth cracks—depends upon temperature and length of infection. While the disease causes neither total crop destruction nor storage losses, it can cause a serious reduction in total production.

Singh *et al.* (1971) reported that in field trials, three isolates of the mild strain of the spindle tuber agent reduced yield in the variety Saco by 17, 24, and 24%, respectively, and the severe strain reduced yield by

64%. The authors estimated an incidence of 3.8% of potato spindle tuber disease among the three major varieties Kennebec (3.3%), Katahdin (2.5%), and Netted Gem (Russet Burbank) (4.6%). On the basis of these estimates, the ratio of mild to severe strains observed, and the measured yield reduction with each strain, Singh *et al.* (1971) calculated a hypothetical loss of about 1% of the potato crop.

These studies emphasize the importance of control of spindle tuber. As long as control is effective in keeping the incidence of the disease low, yield losses are of little consequence. Inadequate control, however, can lead to catastrophic losses in a relatively short time.

When potatos are grown at high temperatures, PSTV causes severe damage and total crop loss may ensue. Because of the potato's importance as a food source, major efforts are under way to supplement the world's food supply by adapting the potato to growth in subtropical and tropical climates. A lowland tropical potato cultivar has already been developed (Sawyer, 1979). Under such environmental conditions, PSTV poses a particularly serious threat and effective means of excluding the viroid from "seed" stocks are essential.

VI. HOST RANGE

In nature, potato is the only known host of PSTV. No weed species that might serve as reservoirs of the viroid have been reported.

Systematic work on the properties of PSTV became feasible only after Raymer and O'Brien (1962) had discovered that the agent is able to infect tomato (*Lycopersicon esculentum* Mill.) and to produce in certain cultivars of this host a characteristic syndrome that cannot be confused readily with that of any other known potato or tomato pathogen (except bunchy top of tomato, see below). In the cultivar Rutgers, first symptoms consist of epinasty, rugosity, and lateral twisting of leaflets formed after inoculation. Later, necrosis of midribs and lateral veins of these rugose leaflets becomes evident (Raymer and O'Brien, 1962). This necrosis of the vascular tissue gradually becomes more extensive and causes the leaves at 3–5 nodes to become first yellow and then brown. Small, mildly rugose leaves with less severe vascular necrosis and epinasty continue to develop above the necrotic region. At this stage, lower leaves of the plants are normal in appearance. Infected plants are severely stunted when compared with healthy ones. No apparent interference with flower or fruit development occurs, except that the fruits are considerably smaller than those produced on healthy plants (Raymer and O'Brien, 1962).

Later tests showed that the viroid concentration in Rutgers tomato plants is comparable to that in infected potato plants (Raymer *et al.*, 1964). Thus, this plant is well suited as a propagation host for PSTV.

Later, many other hosts of PSTV were discovered (reviewed in Diener, 1979). Most of these are solanaceous species and no disease symptoms

develop. The fact that PSTV had infected these species and had replicated could be determined only by back inoculation to tomato or potato, in which the typical symptoms of the disease again developed.

Singh (1973) assembled a list of 94 species in 31 families that were found to be resistant to PSTV. Interestingly, these include many solanaceous species, some of which belong to genera that include susceptible species, such as *Capsicum*, *Datura*, and *Solanum*. A complete list of known suscepts of PSTV is given by Diener (1979).

VII. STRAINS

A. Unmottled Curly Dwarf Strain

In addition to the type strain [as maintained under the name *spindle tuber* in the Schultz Potato Virus Collection (Webb, 1958)], Schultz and Folsom (1923) described another potato disease with similar, but more severe, symptoms. This condition was named *unmottled curly dwarf* because of its similarity to "curly dwarf" of potato described earlier but lacking mottling of leaves. Because this condition remained true to type for 3 years, Schultz and Folsom (1923) considered it a single disease, not a combination of diseases. Symptoms consisted of "pronounced dwarfing, spindliness, dark green color of the foliage early in the season, wrinkling, burning, somewhat premature death, and spindling, gnarled, and cracked tubers" (Schultz and Folsom, 1923). Later, Folsom (1926) pointed out that the symptoms of spindle tuber and unmottled curly dwarf were similar and that the difference was chiefly one of degree, with a few additional related symptoms characterizing unmottled curly dwarf, particularly the appearance of serious lengthwise growth cracks in the tubers (Fig. 1). Today, unmottled curly dwarf is generally considered to be a severe strain of potato spindle tuber (Diener and Raymer, 1971).

B. Mild Strains

The existence of mild strains of the potato spindle tuber agent became evident only after tomato was used as a test plant for the pathogen. Fernow (1967) reported that use of tomato resulted in the detection of only about 13% of the infected clones tested and attributed the poor results to the existence of a strain that produced symptoms in tomato so mild that they were easily overlooked. The author, after ruling out other explanations for these results, such as the presence of inhibitors of infection in some clones, adopted and subsequently confirmed a working hypothesis that there are two strains (or groups of strains) of the spindle tuber agent in potatoes. One of these, which can be designated as "severe" in tomato, induces extreme shortening of internodes, causing a rosette appearance,

severe epinasty and downward curling of leaves, severely wrinkled leaves, and shortening of petioles and midribs. Severe necrosis of stems, petioles, and midribs often occurs, but usually 5 to 7 days after the other symptoms. This type was found only rarely in inoculations from field-grown potatoes. The other type, designated as "mild" in tomato, induces symptoms in tomato that are slow to develop and are so mild that they are easily overlooked. They consist of slight epinasty and twisting of terminal leaflets and a general reduction in growth. Necrosis does not develop with the mild strain (Fernow, 1967). The author found that cross protection by this mild strain against the severe strain occurred, but that it was usually partial and temporary. The author developed a double-inoculation technique and demonstrated its validity and value for the elimination of spindle tuber from seed stocks before planting (Fernow *al.*, 1969).

Singh (1970) and Singh *et al.* (1970) established by use of Fernow's double-inoculation technique that, of field samples found to be infected, 86 to 92% harbored the mild strain, and only 8 to 14% the severe strain. Greenhouse and field tests indicated that the mild strain, so named because of mild symptoms on tomato, was mild in potato as well.

More recently, PSTV strains have been isolated and characterized that differ in symptom expression in tomato from essentially symptomless to mild, to intermediate, to severe, to almost lethal and their nucleotide sequences have been determined (Schnölzer *et al.*, 1985) (see below).

VIII. EXPERIMENTAL TRANSMISSION

PSTV is readily transmitted by conventional mechanical inoculation from potato to potato and tomato, as well as from tomato to tomato.

IX. DIAGNOSIS

A. Bioassay

Before the physical/chemical nature of the potato spindle tuber agent had been recognized, bioassay on suitable indicator plants was the only means of detection and assay available.

Although indexing on Rutgers tomato greatly facilitated the task of PSTV diagnosis, some workers encountered difficulties in that they were unable to identify all infected plants by indexing on tomato. For example, Fernow (1967) detected only about 13% of the infected clones tested. These poor results were attributed to the existence of a strain that produced symptoms in tomato so mild that they were easily overlooked. To overcome this difficulty, the author developed a double-inoculation tech-

nique that made possible the ready detection of either the mild or the severe strain. With this procedure, PSTV-infected tubers were effectively eliminated from "seed" stocks before planting (Fernow *et al.*, 1969). A further improvement in the indexing procedure was implemented by Singh *et al.* (1970), who used RNA, extracted from PSTV-infected plants by a simplified procedure, as inoculum in Fernow's cross-protection test.

Mild strains of PSTV produce few or no symptoms in Rutgers tomato plants grown in low light intensities during winter months, but well-fertilized plants inoculated at an early stage of development and grown under adequate light intensities at reasonably high temperatures will develop diagnostic symptoms in response to inoculation with mild strains (Diener and Raymer, 1971).

For the semiquantitative determination of PSTV, assay on Rutgers tomato is suitable.

To determine the relative infectivity titer of several PSTV preparations, it is necessary to make a series of tenfold dilutions of each preparation to be bioassayed and to inoculate each dilution to three to five plants. To obtain accurate results, cross-contamination must be carefully prevented and the assay plants must be inoculated with as uniform a technique as possible.

Several criteria may be used to evaluate results. The least desirable of these is the dilution end point because a large sampling error is associated with the small number of plants infected at the dilution end point (Brakke, 1970). Other criteria are percentage of inoculated plants that become infected at each dilution and time of symptom appearance.

For the assay of PSTV in Rutgers tomato, Raymer and Diener (1969) developed an empirical "infectivity index" that takes into consideration the dilution end point, the percentage of plants infected at each dilution, and the time required for symptom expression. These three factors are used in the index as a means of estimating relative viroid concentration.

As soon as the first plant in an experiment begins to develop symptoms, readings are initiated and are continued at 2-day intervals until no further increase in the number of plants with symptoms has occurred for two or three consecutive readings. The experiment is then terminated.

The more concentrated the viroid preparation, the earlier symptoms appear. The index is computed by adding together the number of plants infected at each dilution over the entire recording period, multiplying this figure by the negative log of the dilution, and adding together these products for all the dilutions tested. This index permits discrimination between treatments that might achieve the same dilution end points but differ both in the earliness with which symptoms are expressed and in the number of plants infected. Undoubtedly, the difference in viroid titer is underestimated with this method, since a tenfold difference in dilution is represented by a difference of only one in the multiplier. On the other hand, the infectivity index gives relatively little weight to individual

plants that express symptoms late, after having been inoculated with a highly diluted preparation. Thus, much of the variability inherent in an assay of this type is "dampened out."

With the elucidation of the physical/chemical properties of viroids, diagnostic tests that are based on these properties have been developed and have largely replaced bioassay as a diagnostic tool.

B. Polyacrylamide Gel Electrophoresis

Morris and Wright (1975) developed a procedure for the detection of PSTV in small amounts of potato and tomato tissue. The method is a simplified version of a viroid purification scheme and involves extraction of cellular nucleic acids, their separation by PAGE, and staining of the nucleic acid bands. Nucleic acid preparations from infected plants are characterized by the appearance in the gel of an extra band, which is due to the presence of PSTV. Both mild and severe strains of PSTV are detected. Use of the test in a routine potato-indexing program showed that it allows rapid and reliable diagnosis of the disease. This test appears well suited for the elimination of PSTV from elite, or basic seed stocks in certification programs (Morris and Smith, 1977).

In addition to distinguishing viroid-infected from healthy tissue, such a test is capable, in some cases, of distinguishing among different viroids. Such a distinction is possible, for example, between PSTV and CSV, because these two viroids differ significantly in their electrophoretic mobilities (Diener and Lawson, 1973). With viroids that do not significantly differ in this property, such as PSTV and CEV, additional criteria must be used for positive identification.

Many variations of procedures employing PAGE have been proposed. All such tests, however, are laborious and, therefore, expensive; they are not suitable for the assay of thousands of samples, as is required in practical agriculture.

C. Molecular Probes

One possible alternative is the use of specific molecular probes. A practical diagnostic test for the detection of PSTV that is based on nucleic acid hybridization has been developed (Owens and Diener, 1981). This test is based on the hybridization of highly radioactive DNA complementary to PSTV (PSTV cDNA), obtained by recombinant DNA technology, with PSTV bound to a solid support and autoradiographic detection of the resulting DNA–RNA hybrids. Because the PSTV cDNA is cloned, it can be obtained inexpensively, and in virtually unlimited quantities. Also, clarified sap rather than purified nucleic acid is suitable, thus greatly expediting and simplifying sample preparation.

Nucleic acid hybridization tests in many variations are now widely used for the detection not only of PSTV, but of other viroids and viruses. These tests have almost completely supplanted previous diagnostic techniques, such as bioassay or detection by PAGE.

X. INTERACTION WITH OTHER PATHOGENS

No synergisms or antagonisms in plants doubly infected with a viroid and a plant virus appear to have been noted. In doubly infected tomato plants, tobacco mosaic virus and PSTV replicate independently, and the characteristic symptoms of both pathogens are expressed as well as in singly infected plants (T. O. Diener, unpublished observations).

However, viroids share with viruses the phenomenon of cross-protection, in which infection of plants with a mild strain of a viroid protects the plants from the effects of superinfection with a severe strain of the same viroid. As with viruses, cross-protection is believed to occur only if the mild and superinfecting pathogens are related (Fernow *et al.*, 1969; Niblett *et al.*, 1978). The molecular mechanism responsible for cross-protection is unknown.

XI. PURIFICATION

Viroids constitute only a small portion of the total RNA extractable from infected tissue. Originally, to identify the infectious RNAs by physical means, it was necessary to isolate them from relatively large amounts of tissue. This was first achieved with PSTV (Diener, 1972) with the demonstration after PAGE of a prominent, UV-absorbing peak that coincided with infectivity distribution in the gel and that was not detectable in identically prepared extracts from healthy leaves. Further improvements in the methods of viroid separation and purification made it possible to obtain viroid preparations essentially free of contaminating host nucleic acids, thereby allowing viroid purification in amounts sufficient for biophysical and biochemical characterization. Details of the appropriate procedures have been described (Diener *et al.*, 1977).

Numerous variations of purification schemes have been proposed. Most are adaptations of more or less routine procedures used in nucleic acid research. Usually, they consist in the grinding of infected tissues (often frozen and/or pulverized in the presence of dry ice) in an aqueous buffer of relatively high ionic strength (such as 0.1 or 0.5 M potassium phosphate), pH 7.5–8.5, containing nuclease inhibitor (e.g., bentonite). In most procedures, the initial grinding of infected tissue is performed in the presence of phenol (often supplemented with chloroform and/or sodium dodecyl sulfate). In other procedures, phenol is added to the clarified tissue homogenate and the mixture shaken or stirred at 0–5° C. In either

procedure, the aqueous and organic phases are separated by low-speed centrifugation. Often, treatment with phenol is repeated one or more times. Nucleic acids are recovered from the final aqueous phase by ethanol precipitation and resuspension in an appropriate aqueous medium. Cellular nucleic acids are then removed from this crude nucleic acid preparation by various means, such as incubation with deoxyribonuclease to remove DNA, removal of high-molecular-weight RNAs by precipitation in 2 M LiCl, and removal of polysaccharides by treatment with 2-methoxyethanol, followed by precipitation of the nucleic acids with cetyltrimethylammonium bromide. Invariably, the last steps of PSTV purification consist of two or more cycles of PAGE, preferably first under native, then under denaturing conditions. For detailed procedures, the reader is referred to the original literature.

XII. PHYSICAL–CHEMICAL PROPERTIES

PSTV, as the prototype of viroids, displays all of the features now recognized as typical for viroids (see Chapters 2 and 3, this volume). All isolates so far analyzed consist of 359 nucleotides.

The type strain of PSTV was the first viroid whose nucleotide sequence was determined (Gross *et al.*, 1978). Since then the sequence of a number of isolates that differ in the severity of symptoms in tomato have been determined (Schnölzer *et al.*, 1985; Chapter 5, this volume).

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CHAPTER 10

Citrus Exocortis

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I. FIELD SYMPTOMATOLOGY

When exocortis was first described by Fawcett and Klotz (1948), its cause was unknown, but soon after Benton *et al.* (1949, 1950) and Bitters (1954) showed that scalybutt (exocortis) was bud-transmissible. Trees on trifoliate orange [*Poncirus trifoliata* (L.) Raf.] rootstock are severely dwarfed from an early age, show little enlargement of the stock, and exhibit heavy bark scaling of the butt below the bud union (Fig. 1). Trees on citrange [*P. trifoliata* × *Citrus sinensis* (L.) Osbeck] stocks are also reduced in size and the rootstock may show scaling due to exocortis in some countries (Garnsey, 1983). Olson (1952) and Moreira (1955, 1959) described a rootstock bark disorder of Rangpur lime (*C. limonia* Osbeck) stocks that was attributed to exocortis (Brown and Schmitz, 1954; Olson and Shull, 1956; Reitz and Knorr, 1957). Exocortis has also been associated with dwarfing of trifoliate orange stock in the absence of bark scaling (Broadbent *et al.*, 1971; Schwinghamer and Broadbent, 1987a,b). Gummy pitting symptoms in trifoliate orange rootstocks may also be associated with a strain of exocortis (Fraser and Broadbent, 1979).

Symptom expression (bark scaling and dwarfing) varies on different citrus hosts, and there is considerable variability in time taken for symptoms to appear (Wallace, 1978) depending on strain variability (Fraser and Levitt, 1959; Calavan and Weathers, 1961; Weathers and Calavan, 1961), host nutrition (Weathers, 1960; Weathers *et al.*, 1965), and temperature (Weathers *et al.*, 1962).

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FIGURE 1. Bark scaling of trifoliate orange (*Poncirus trifoliata*) rootstock caused by citrus exocortis viroid.

II. GEOGRAPHIC DISTRIBUTION

Exocortis is present in most, if not all, citrus-growing countries. Its occurrence in countries such as the United States, Australia, South America, and the Mediterranean region has been high, but has diminished in countries with established pathogen-tested schemes. By contrast, in Japan and China, where the principal rootstock, trifoliate orange, is native, exocortis has been found only in citrus introduced from foreign countries and apparently has not become established in commercial planting (Yamada and Tanaka, 1968; Tanaka and Yamada, 1969; Zhao *et al.*, 1983).

III. HOST RANGE

CEV infects many citrus species and cultivars and some citrus relatives. Many are latent hosts, but infected seedlings of trifoliate orange, Rangpur lime, and some citrons and lemons show stem blotching and/or bark splitting. Sensitive citrons also show leaf symptoms. Bark scaling occurs on the rootstock portion of trees grafted to sensitive rootstocks.

IV. IMPORTANCE OF EXOCORTIS

Exocortis can be a destructive disease of citrus trees on susceptible rootstocks. Size and yields of trees on trifoliate orange and Rangpur lime rootstocks are strongly suppressed by exocortis (Calavan *et al.*, 1968). A bark-scaling CEV strain inoculated into healthy nursery trees of "Bellamy" nucellar navel on citrange and trifoliate orange rootstocks reduced canopy surface area by 55 and 68% respectively and yield by 49 and 65.3% after 11 years (Bevington and Bacon, 1977). Inoculation with nonscaling CEV strains reduced canopy surface area of trifoliate orange, Troyer and Carrizo citranges, and Rangpur lime after 9 years by 51, 25, and 19%, respectively. There was no effect of inoculation on trees of Cleopatra mandarin, sweet orange, and rough lemon (Bevington and Bacon, 1977). By contrast, Sinclair and Brown (1960) reported a significant reduction in growth of CEV-infected Washington navel orange trees on sweet orange and Cleopatra mandarin rootstocks compared with healthy controls, even though the infected trees exhibited no bark scaling.

The destructive effects of exocortis have been greatest where changes to an exocortis-susceptible rootstock have been necessitated by other diseases. In Australia, trifoliate orange came into prominence in the 1940s after *Phytophthora* root and collar rots (*P. citrophthora* Sm. & Sm.) had caused heavy losses of trees on rough lemon and sweet orange stocks (Fraser and Broadbent, 1979). Rangpur lime was extensively used in Brazil as a replacement for sour orange when tristeza destroyed all existing groves (Wallace, 1978). In both areas, exocortis damage was widespread because its presence as a latent infection in sweet orange scions was not recognized.

V. STRAINS

The severity of bark scaling and degree of dwarfing (stunting) on various rootstock/scion combinations have been attributed to the existence of strains (Fraser and Levitt, 1959; Calavan and Weathers, 1961; Weathers and Calavan, 1961; Vogel *et al.*, 1965). Mild isolates of exocortis viroid were separated from severe isolates by knife transmission to Etrog citron (Roistacher *et al.*, 1969).

Strain variation among exocortis isolates has been mainly on symptomatology and it is possible that some variants may be distinct viroids (Schlemmer *et al.*, 1985; Duran-Vila and Semancik, 1985; Schwinghamer and Broadbent, 1987a,b). Two additional viroidlike RNAs (I and III), which are smaller than CEV and by dot-blot hybridization show no significant homology to CEV, infect citrus and produce a mild reaction in citron characteristic of the exocortis disease reaction (Duran-Vila *et al.*, 1986).

Cross-protection has not been demonstrated between mild and severe isolates of CEV (Kapur *et al.*, 1974; Semancik, 1980; Garnsey and Randles,

1987; Garnsey, unpublished observations), even though interference among viroids has been reported (Niblett *et al.*, 1978). The nucleotide sequences of several CEV isolates have been determined (see Section X).

VI. TRANSMISSION

A. Transmission by Grafting

Benton *et al.* (1949, 1950) and Bitters (1954) presented evidence that exocortis is bud-transmissible. Infection of trees adjacent to exocortis-infected trees in nurseries (Calavan *et al.*, 1959) and in orchards (Fraser and Levitt, 1959) suggested natural root grafting as a means of transmission.

B. Mechanical Transmission

CEV is mechanically transmissible from petunia to citron and from citron to citron (Weathers *et al.*, 1967) and on budding knives and pruning shears (Garnsey and Jones, 1967; Garnsey, 1968). The viroid is stable and survives 8 days on knife blades (Allen, 1968), and at least 4 months on contaminated razor blades (Roistacher and Calavan, 1974). Garnsey and Jones (1967) speculated that orchard operations, such as pruning and hedging, may transmit exocortis within an orchard and this has been verified by Broadbent *et al.* (in press) in a budwood multiplication block. Wutscher and Shull (1975) found that two of six uninoculated trees planted among other grapefruit and orange trees carrying CEV were found infected 2 years after the grove was machine-hedged.

The mechanical transmissibility of exocortis among citrus varieties varies: Garnsey and Weathers (1972) reported high rates of transmission to citron, Rangpur lime, trifoliate orange, and lemon and Morton citrange, but low rates to Duncan grapefruit and Orlando tangelo. Cartia (1978) found that, independent of the inoculum source, transmission was higher to citron than to other hosts tested. There was no transmission to grapefruit; lemon was more susceptible than orange. In Japan, Ushiyama (1978) mechanically transmitted exocortis from lemon to orange and mandarin with comparable success, but when the source of inoculum was satsuma mandarin leaves, the percentage of infection was lower. Transmission is generally higher between plants of the same species (Garnsey and Weathers, 1972; Cartia, 1978).

C. Transmission by Vectors

No evidence for transmission by a vector has been presented (Semancik, 1980). Several early reports suggesting natural spread were made

before spread by pruning tools was recognized (Garnsey and Randles, 1987).

D. Transmission through Seed

Seed transmission of exocortis in citrus apparently does not occur (Bitters, 1954; Frazer and Levitt, 1959). The viroid can be transmitted through the seed of *Lycopersicon esculentum* cv. *Rutgers* (Semancik, 1980).

E. Transmission by Dodder

CEV was successfully transmitted from citrus to Etrog citron and to petunia by *Cuscuta subinclusa* Dur. & Hilg. (Weathers, 1965).

VII. DETECTION

A. Indicator Plants

Initially, symptoms of rootstock bark scaling and dwarfing in trees on susceptible rootstocks (trifoliate orange and Rangpur lime) were used to detect clones carrying exocortis viroid (Fraser and Levitt, 1959; Moreira, 1959). Now, CEV can be detected by tissue-graft indexing on citron clones USDCS 60-13 (Calavan *et al.*, 1964), Arizona 861 (Allen and Oden, 1964), or 861-S-1 (Roistacher *et al.*, 1977). Symptom expression for severe isolates may occur in 1 to 3 months (Calavan *et al.*, 1964; Calavan, 1968), but some mild strains from dwarfed, nonscaling trees may take up to 27–29 months to express symptoms in citron indicators (Schwinghamer and Broadbent, 1987a). Several noncitrus hosts of CEV are known, including petunia (Weathers, 1965) and 12 species of plants in the family Solanaceae (Weathers *et al.*, 1967). The discovery of *Gynura aurantiaca* (Weathers and Greer, 1972) and chrysanthemum (Niblett *et al.*, 1980), as indicators of CEV, facilitated studies on its purification and classification (Runia and Peters, 1980; Visvader *et al.*, 1982; Visvader and Symons, 1983). *G. aurantiaca* is a good indicator of moderate or severe isolates of CEV, but may not react to mild isolates (Kapur *et al.*, 1974). Nine horticultural crop species (Runia and Peters, 1980) including tomato and potato (Singh *et al.*, 1972) are susceptible to CEV and seven are sensitive.

B. Biochemical Methods of Detection

There have been attempts to detect CEV by phloroglucinol staining of necrotic phloem tissue (Childs *et al.*, 1958, 1959) and chromatographic

methods (Schwarz, 1968; Feldman *et al.*, 1971; Fudl-Allah *et al.*, 1974), but these have not been used widely because of unreliability or technical difficulty (Baksh *et al.*, 1984).

PAGE has been used to isolate and purify CEV (Semancik and Weathers, 1972; Singh and Sänger, 1976) and as a means of routine detection (Baksh *et al.*, 1984; Boccardo *et al.*, 1984). Mild strains of CEV (associated with dwarfing) may not be detected by PAGE (Schwinghamer and Broadbent, 1987a), because the amount of CEV extracted from plants infected with mild isolates is consistently smaller than that extracted from plants with severe isolates (Baksh *et al.*, 1984). Detection of CEV by PAGE is enhanced after selective divalent cation fractionation (Semancik and Szychowski, 1983).

In vitro ^{32}P labeling of viroid RNA for hybridization studies was first described by Negruk *et al.* (1980). The potential applicability of dot-blot assays (Symons, 1984; Barber *et al.*, 1985; Schwinghamer and Broadbent, 1987a) or PAGE analysis (Baksh *et al.*, 1984), to CEV indexing programs has been discussed. However, reliability of these techniques, which are conducted at one point in time, depends on choice of tissue, sampling time in different citrus cultivars, as well as the severity of the CEV strain (Baksh *et al.*, 1984; Schwinghamer and Broadbent, 1987a).

VIII. CYTOPATHIC EFFECTS IN HOST

CEV can be isolated from all plant parts including roots and fruit (Semancik *et al.*, 1978). Studies of the viroid–host interaction have shown cell wall aberrations (Marton *et al.*,1982; Kojima *et al.*, 1983) and aberrations of the plasma membrane (Semancik and Vanderwoude, 1976), including alterations of plasmalemmasomes (Wahn *et al.*, 1980a,b). Analysis of viroid-infected tissues has indicated a decrease in the phytohormone levels of endogenous gibberellins (Rodriguez *et al.*, 1978) and a reduced ability of the tissues to respond to exogenously supplied auxins (Duran-Vila and Semancik, 1982). Increased accumulation of low-molecular-weight proteins accompanying CEV infection (Conejero and Semancik, 1977; Flores *et al.*, 1978) has been attributed to a stimulation of a host-specified maturation process (Conejero *et al.*, 1979).

Viroid-containing tomato cells demonstrate a higher temperature tolerance for growth than uninfected cells (Marton *et al.*, 1982), while increased freeze damage was associated with exocortis infection in navel oranges on Carrizo citrange rootstock (Garnsey, 1983). CEV-inoculated plants have been found tolerant to root rots under conditions in which viroid-free young trees declined shortly after planting (Rossetti *et al.*, 1962; Ashkenazi and Oren, 1977).

IX. CONTROL

Control of CEV is achieved either by use of tolerant rootstocks (Cohen, 1974), by eliminating CEV from budwood sources by nucellar propagation

(Weathers and Calavan, 1959), or by shoot tip grafting (Navarro *et al.*, 1975; Roistacher and Kitto, 1977), maintenance of indexed mother tree blocks, and prevention of root grafting and mechanical transmission by pruning and hedging tools.

A mixture of 2% NaOH plus 2 or 5% formaldehyde inactivates CEV (Garnsey and Whidden, 1972). Brief exposure to 70% ethyl alcohol (Garnsey and Jones, 1967) or flaming blades dipped in 95% alcohol did not completely inactivate the viroid (Roistacher *et al.*, 1969) and treatments with other chemicals and detergents gave variable results. A 20% household bleach solution (containing about 1% hypochlorite) was recommended as a disinfectant for pruning and grafting tools by Roistacher *et al.* (1969) and remains the most effective and simple procedure available.

Thermotherapy does not inactivate CEV (Calavan *et al.*, 1972).

X. PHYSICAL–CHEMICAL PROPERTIES

CEV has properties typical of viroids (Semancik and Weathers, 1972; Semancik *et al.*, 1975; Sänger *et al.*, 1976). All isolates sequenced contain 371 nucleotides (Gross *et al.*, 1982; Visvader *et al.*, 1982; Chapter 2, this volume). More recently, a range of 370–375 total nucleotide residues in CEV variants has been reported (Visvader and Symons, 1985).

Visvader and Symons (1983) compared the nucleotide sequences of two reference CEV isolates (Californian and Australian) with two CEV isolates from trees on trifoliate orange exhibiting dwarfing, but no scaling, symptoms. The two reference isolates and one dwarfing isolate differed in only four residues, while the second dwarfing isolate differed extensively from the reference scaling isolate and the 27 nucleotide changes included substitutions, insertions, and deletions. Inoculation of orange trees on trifoliate orange rootstock with purified viroid and observation of symptoms may eventually allow different pathogenic effects to be correlated with nucleotide changes in a defined region of the viroid (Visvader and Symons, 1983).

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CHAPTER 11

Chrysanthemum Stunt

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I. INTRODUCTION

Prior to the 1930s and early 1940s, florists' chrysanthemums were produced only in the fall and early winter. Plants were grown on a natural flowering cycle that extended from September through January with a succession of flowering determined by different genotypes selected as early, intermediate, and late varieties. The discovery of photoperiodic control of flowering led to precise timing of the crop and chrysanthemum production at all seasons of the year. Production expanded rapidly and chrysanthemums became one of the five leading florist crops.

Chrysanthemum stunt disease was first recognized in the cultivated chrysanthemum, *Chrysanthemum morifolium*, in 1945 when the industry was rapidly expanding. The disease was first described by Dimock (1947) who observed a reduced size of affected plants, leaves, and flowers. In 1946 and 1947, the disease was widespread throughout the United States and Canada. In some greenhouses, 50 to 100% of the plants were infected (Brierley, 1953a). Diseased plants of some varieties were so short that they were unsalable. In other varieties, stunting of vegetative growth was less pronounced; however, a few healthy, taller-growing plants could be distinguished from the majority of affected plants, which were uniformly reduced in stature. The disease soon became familiar to most producers of florists' chrysanthemums. Prentice (1951) described stunt in Australia and the disease was subsequently discovered in the Netherlands (Noordam, 1952) and the United Kingdom (Hollings, 1960). Stunt was probably rapidly transmitted to all areas of the world where the florist

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chrysanthemum is grown, due to the international transport of cuttings. In Britain the disease remained uncommon until the early 1960s when many stunt-affected chrysanthemums were imported from the United States (Hollings and Stone, 1973). Although it was speculated many years ago that the disease is endemic in one of the many species of Compositae that may be without disease symptoms, and perhaps spread to chrysanthemums (Brierley, 1953a), there is no evidence to support this assumption. Chrysanthemum stunt was identified as a viroid disease by Diener and Lawson (1973) and the unusual properties of the disease agent were also described by Hollings and Stone (1973).

II. SYMPTOMATOLOGY

A. CSV in Chrysanthemum

Symptoms on *C. morifolium* infected with CSV include stunting with a reduction of one-half to two-thirds the normal plant size. All portions of the plant are often proportionately reduced in size without malformation. Flowers are smaller and paler than normal and in some cultivars the flower color appears bleached. Flowers also open prematurely. The combination of reduced plant size and early opening of the flowers, in comparison to stunt-free plants, is a reaction that is typical in many infected cultivars.

In Britain, the incubation period for symptom development in chrysanthemums is usually longer than in the United States. In cultivars that flower year-round, only vegetative dwarfing may be obvious in cuttings derived from infected mother stock. Flower symptoms may not be recognizable until the second flowering in cuttings taken from infected plants. In addition to vegetative dwarfing, small flowers are produced that open 7–10 days earlier than normal (Hollings and Stone, 1973). Root development is reduced in infected plants. In two cultivars, Good News and Fred Shoesmith, the length of the leaf is reduced by 38%, stem internodes by 47%, and stem diameter by 37%.

Infection by CSV resulted in fresh weight reduction of 24, 30, and 65% on pompom chrysanthemums that flowered in spring, winter, and summer, respectively (Horst *et al.*, 1977). The effect on flowering was generally more severe on crops flowered in winter than those flowered in spring. CSV caused a great reduction in fresh weight, flower diameter, and stem length of cultivars flowered in summer with reductions of 30, 14, and 40%, respectively, for standard cultivars. When a comparison was made between pompom and standard chrysanthemum flower types, CSV caused the greatest reductions in total yield on the pompom type (Horst *et al.*, 1977).

B. Other Viroids in Chrysanthemum

Mistletoe chrysanthemum showed the same reaction when infected with CPFV or CSV but CEV-infected plants showed completely different symptoms consisting of severe growth reduction and leaf deformation in the top leaves, which were flecked with yellow spots and crinkled. The spots were smaller, and less clearly recognizable, than those caused by CSV or CPFV (Runia and Peters, 1980).

Symptoms of the severe (PS) and mild (PM) strains of PSTV, as well as CEV, CCMV, and CSV are distinguishable on "Bonnie Jean" chrysanthemum plants (Niblett *et al.*, 1978).

III. TRANSMISSION AND SPREAD

Spread of CSV by foliage contact, handling during cultivation, and by cutting knives has been known for many years (Brierley and Smith, 1951; Keller, 1953). Hollings and Stone (1973) tested formaldehyde and trisodium orthophosphate (TSP) as disinfectants of implements or hands. Although these additives are commonly used to disinfect tools and hands from viruses such as tobacco mosaic and potato virus X, the chemicals did not reduce CSV infectivity. Dipping of cutting knives for 2–3 sec in a solution of 2% TSP or in 2% formaldehyde or dipping of hands in 2% TSP before taking cuttings failed to prevent transmission (Hollings and Stone, 1973).

The extent of spread from CSV-infected to adjacent healthy plants depends on the cultivar (Hollings and Stone, 1973). Nineteen percent of the Mistletoe plants and 28% of American Beauty became infected by foliage contact when one infected plant was surrounded by eight healthy plants with pot-to-pot contact (Hollings and Stone, 1973).

Insect transmission of CSV has not been found and reports of seed transmission are contradictory. Whereas Monsion *et al.* (1973) obtained seed transmission in chrysanthemum, Hollings and Stone (1973) concluded that stunt is not seed transmitted.

IV. HOST RANGE

In early reports, only plants in the family Compositae were considered as hosts of CSV. Of 76 species inoculated, 39 were susceptible and 37 were nonsusceptible (Brierley, 1953b). Only 7 of the susceptible species developed recognizable symptoms. They include five species or cultivars of the genus *Chrysanthemum* and two species of *Senecio*. *C. parthenium* showed dwarfing, flowered prematurely with smaller flowers, and infected plants showed a high level of susceptibility to spider mites (Hol-

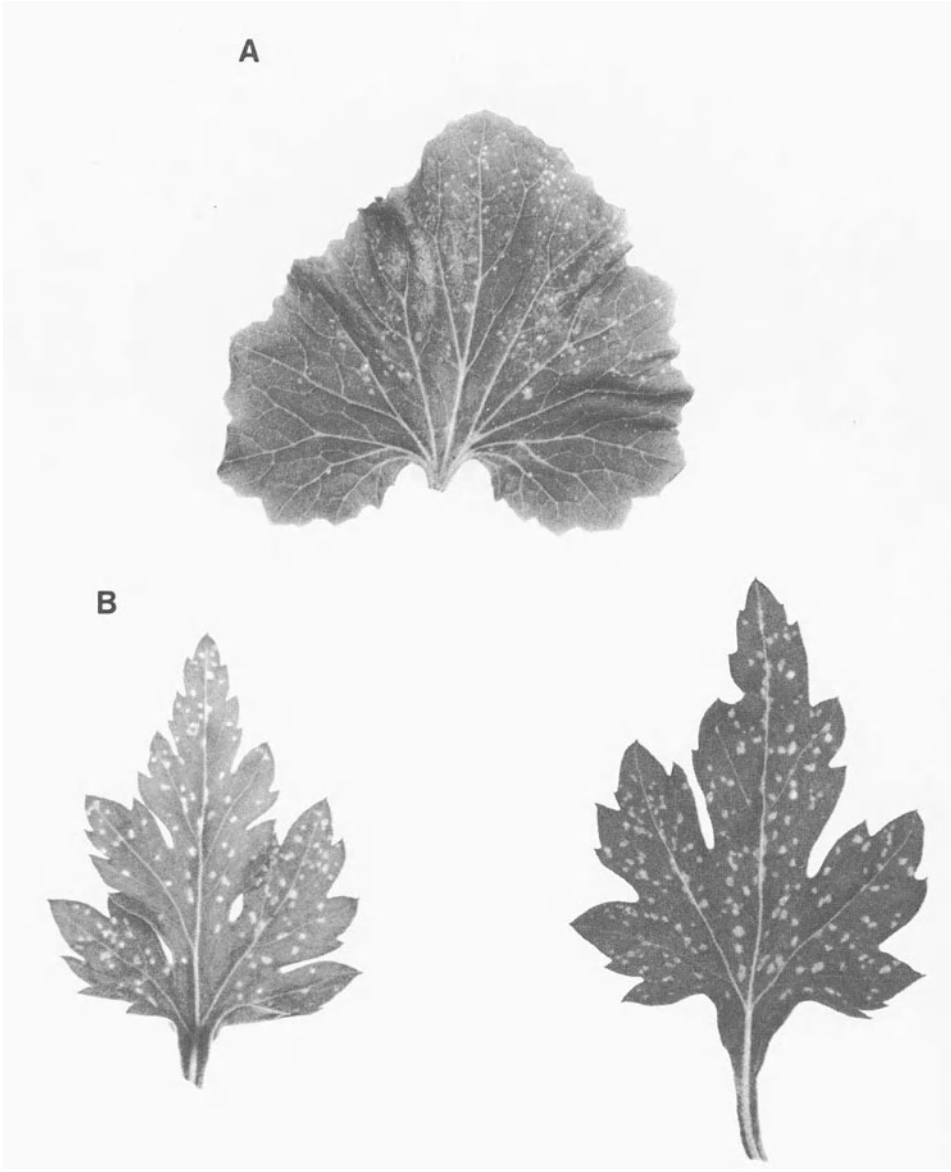

FIGURE 1. (A) Necrotic and chlorotic spots on *Senecio cruentus* leaf 30 days after inoculation. (B) Stunt-infected Mistletoe chrysanthemum showing chlorotic spotting.

lings and Stone, 1973). Florists' cineraria, *Senecio cruentus*, produces local necrotic and chlorotic lesions from 12 to 40 days after inoculation (Fig. 1a). Sites of starch accumulation were detected on inoculated leaves 12 to 15 days after inoculation before symptoms appeared on the unstained leaf. An 18-hr light period with 500 fc fluorescent illumination and a constant 21°C favored starch lesion formation (Lawson, 1968a). Variation in the number of lesions among cineraria plants, and between leaves on a single plant, precluded the use of this assay for detection of small differences in CSV concentration (Lawson, 1968a). *Verbesina encelioides* showed diffuse chlorotic local lesions after about 4 weeks, but the reaction was not consistent (Hollings and Stone, 1973). *C. carinatum, C. coccineum, Sonchus asper,* and *Zinnia elegans* were infected systemically without symptoms. Eight of twenty-nine species in the family Compositae were infected (Hollings and Stone, 1973).

More recent research has resulted in an expanded host range. CSV-infected *Gynura aurantiaca* shows slight stunting and mild rugosity (Niblett *et al.*, 1980), as well as mild leaf distortion and epinasty (Bachelier *et al.*, 1976). In the greenhouse, these symptoms occurred only 50 days after inoculation and were mild (Bachelier *et al.*, 1976). In contrast, in a controlled environment, severe symptoms developed after 18–20 days at 25–28°C and 2000 fc in plants inoculated by juice rubbing (Bachelier *et al.*, 1976). Tomato (*Lycopersicon esculentum* cv. Rutgers) is susceptible to CSV infection but, depending on the strain used, the infection remains symptomless (Niblett *et al.*, 1980) or results in symptoms similar to those induced by PSTV (Runia and Peters, 1980). Runia and Peters (1980) have studied the response of different plant species to viroid infection. They found that CPFV, CSV, and CEV cause identical symptoms on tomato and potato. All three viroids produce stunting on the tomato cultivar Rutgers. The symptoms were similar to those caused by PSTV. The potato cultivar Katahdin did not show symptoms when infected with these viroids but healthy plants produced more foliage 4 years after infection. Symptoms of CSV on tomato in the Netherlands contrast with previous reports and may represent infection by a different strain of the viroid (Runia and Peters, 1980).

V. DIAGNOSIS

A. Bioassay

An intensive search was made for plants that would express well-defined symptoms following sap inoculation (Brierley, 1953b). Although many plants in the family Compositae are susceptible, aside from chrysanthemum only two species of *Senecio* produce symptoms. *S. cruentus* produces recognizable symptoms and shows dwarfing and rosetting 2 or 3 months after inoculation. Although a seed-produced indicator plant was

sought, the discovery of chrysanthemum cultivars that clearly express stunt symptoms reduced the need for a seed-produced bioassay host plant.

1. Assay on Blanche Chrysanthemum

In the United States, a previously unreported virus (Keller, 1951) in the chrysanthemum cultivar Blanche was discovered. The virus, designated chrysanthemum virus Q, is masked in Blanche. In combination with chrysanthemum stunt, Blanche develops severe leaf distortion known as "crinkle." Leaves with crinkle also show streaks and small white flecks. Keller (1951) concluded that Blanche infected with Q virus is a valuable indicator plant for CSV diagnosis in graft inoculation tests. Q virus is synonymous with Noordam's B virus (Noordam, 1952).

The grafting procedure on Blanche has been used commercially in the United States for many years, because the reaction of CSV can be observed at any time of year, 4 to 6 weeks after graft inoculation. Hollings and Stone (1973) have compared the CSV reaction on symptomless Blanche infected with chrysanthemum virus B with the reaction of virus-free Blanche clones obtained by meristem-tip culture following heat therapy. Responses to CSV infection were similar but less pronounced in virus-free plants.

2. Assay on Mistletoe Chrysanthemum

A group of florists' "standards" or large-flowering types, known as Mistletoe chrysanthemums, all react to CSV infection with distinctive yellow leaf spotting called "measles." Following sap inoculation, stunt symptoms are expressed on some plants in 5 weeks (Brierley, 1953b), but plants have to be held for 6 months before symptoms are completely expressed. Symptoms on mechanically inoculated Mistletoe are often erratic, with less than 100% transmission occurring (Hollings and Stone, 1973). Without supplementary light, sap inoculation of Mistletoe often fails during winter months. High light intensities seem to improve the establishment of infection, as well as the development of symptoms.

Grafting is much more reliable than sap inoculation for indexing and the latent period is much shorter. Hollings and Stone (1973) reported that, for grafts made in spring, the mean incubation period was 8.1 weeks and for those made in fall, 24 weeks. With sap inoculations, the mean incubation period was 13.4 weeks for inoculations made in March–June and 25 weeks for those made in September–October (Hollings and Stone, 1973).

Lawson and Hearon (1971) described differences among cultivar Mistletoe selections in their reactions to CSV. In summer, CSV-free plants of the American yellow-flowered selection used for CSV diagnosis develop chlorotic spots on the fully expanded leaves in the greenhouse (Fig. 1b). The spots disappear from expanding leaves in winter, while chlorotic spots are present on leaves of CSV-infected plants of this Mistletoe se-

lection in both summer and winter. The nonspecific spotting on this Mistletoe selection limits its usefulness for CSV diagnosis during the summer months (Lawson and Hearon, 1971).

CSV-free Mistletoe from Britain produces white flowers and a few chlorotic leaf spots on unexpanded and fully expanded leaves in summer, but no symptoms in winter. Chlorotic spots up to 4 mm in diameter are produced on the leaves of CSV-infected American and British Mistletoe plants in summer with smaller spots (from 0.5 to 2 mm in diameter) in winter. In summer, British Mistletoe is a more useful indicator than American Mistletoe for CSV diagnosis (Lawson and Hearon, 1971).

3. Effects of Temperature and Light

Hollings and Stone (1973) held CSV-infected Mistletoe plants under different light and temperature regimens for 12 months, from September to August, and recorded the symptoms on new leaves. Symptoms were most prominent at 21°C with high light intensity (3000 to 13,000-lux natural light plus mercury vapor lamps of 400 W, giving 3800 lux with 12 hr of supplemental light); but at temperatures above 30°C, symptoms were masked even with the same high light intensity. Symptoms were also masked at 12°C with low light intensity. Symptoms were continuously masked from October to May under natural daylength.

Chip-budding of 10-cm-high Mistletoe plants results in symptoms in the greenhouse about 30 days after inoculation in the summer and 35 days in the winter. In the greenhouse at 20°C, with the addition of 700 fc artificial light, many small chlorotic spots 1–2 mm in diameter develop. At 28°C and a 16-hr light period with 3000 fc and 25°C in the dark period, fewer but larger (diameter 5–7 mm) chlorotic spots appear in 20 days (Bachelier *et al.*, 1976).

4. Symptom Masking in Mistletoe Chrysanthemum

Keller (1953) suggested shortening of the latent period in Mistletoe by repeated cutting-back of the stems, shading the shoot tips, and defoliation. After graft inoculation to Mistletoe in February–March and in September–October, these treatments result in a larger proportion of infections and a shorter latent period than in the untreated control groups; however, there is considerable plant-to-plant variation (Hollings and Stone, 1973). The latent period could be shortened by grafting very small Mistletoe scions to infected stocks (Teyssier and Dunez, 1971).

B. Polyacrylamide Gel Electrophoresis

Following the discovery that the chrysanthemum stunt agent is a viroid, more rapid methods for routine detection in commercial chrysanthemum culture have been developed. Horst and Kawamoto (1980) de-

scribed a PAGE procedure that is rapid, efficient, and reliable for routine assay of CSV. In this procedure the stunt viroid was detected in as little as 50 mg of infected *C. morifolium* tissue. Viroid nucleic acid was extracted by phenol with buffer containing SDS and LiCl. The preparation was then treated with chloroform and the separated aqueous phase was extensively dialyzed to remove the colored pigments. The ethanol-precipitated nucleic acid was separated by electrophoresis on a 5% slab gel. A more than threefold increase in recovery of CSV RNA was obtained (Horst and Kawamoto, 1980) as compared to a procedure described previously (Morris and Smith, 1977).

Monsion *et al.* (1980) modified the Morris and Smith (1977) procedure by substituting sodium diethyldithiocarbamate for β-mercaptoethanol. Dialysis time was reduced to 4 hr and the analysis could be completed in 2 days. The samples were electrophoresed in 5% slab gels followed by staining with ethidium bromide (Monsion *et al.*, 1980). Under these conditions, the viroid could be detected in as little as 6 mg of sample from infected chrysanthemum. A band could be visualized with 0.01 μg of purified viroid RNA.

CSV was detected from samples taken from stem tips, young and old leaves, laminae, veins, bark, and roots (Monsion *et al.*, 1980). The viroid could be detected in samples of less than 15 mg of young leaves or tips; but because of lower CSV concentration in older leaves, veins, and bark, 25-mg samples were required for detection from these tissues. In all cultivars tested, CSV could be detected in advance of the appearance of symptoms.

In addition to the requirement for a sensitive assay that can be reliably applied to small samples, such as individual mother plants, procedures for sampling large quantities of tissue are required where bulking samples are necessary for increased efficiency in large-scale indexing. Healthy chrysanthemum plants contain low concentrations of two small RNAs different from the 9 S cellular RNA that are not detected in small samples but become evident in the analysis of large samples. Although the 9 S RNA can be clearly distinguished from the viroid RNA on a 5% polyacrylamide gel, other small host RNAs migrate to a similar position as the viroid and may be mistaken for viroid RNA (Monsion *et al.*, 1981).

Small cellular nucleic acids can be eliminated after LiCl treatment by absorbing the viroid RNA to cellulose in the presence of 30% ethanol. Under these conditions the DNA and the single-stranded RNAs are not absorbed and can be eliminated (Monsion *et al.*, 1981). After elution of the viroid with ethanol-free buffer, the viroid RNA is precipitated with ethanol and separated by PAGE. Although this method has been successfully used on 5 to 50-g samples, care was required in interpretation because of the cellular RNAs migrating to the viroid position. This modified test can be completed in 3 days (Monsion *et al.*, 1981).

In the Netherlands (Mosch *et al.*, 1978), a modification of the Morris and Smith (1977) procedure was adopted for detection of CSV by PAGE.

Five grams of young shoots was sampled instead of 1 g and 2-mercaptoethanol was not used. The amount of buffer used in the extraction was doubled. The reliability of the PAGE method proved to be similar to that of the biological test on Mistletoe. CSV was detected by the PAGE method in one diseased chrysanthemum (1 g) in a total of ten plants (10 g) sampled from the new top growth (Mosch *et al.*, 1978). The number of bands in the gel varied and a host nucleic acid band may occur just below the viroid band. Because the front is not always in the same location in the gel, the host nucleic acid may make it difficult to identify the specific viroid band. To solve this problem, the presence of the viroid was determined by its relative mobility compared to the 5 S RNA band, which was always present (Mosch *et al.*, 1978).

C. Nucleic Acid Hybridization

A cDNA probe has been prepared to CSV that is specific for CSV sequences (Palukaitis and Symons, 1978). The ^{32}P cDNA to CSV hybridized only to low-molecular-weight RNA extracted from CSV-infected chrysanthemums of *Gynura aurantiaca*, but not to RNA from healthy chrysanthemums or gynuras or CEV-infected gynuras. A hybridization method was developed for the quantification of viroid RNA sequences in extracts of infected plants (Palukaitis and Symons, 1979). The rate of hybridization was influenced by the variation in residual secondary structure of the circular form, S_1-treated CSV, and linear form of the molecule. Optimal hybridization occurred at 50°C in the presence of formamide. It was calculated that CSV sequences were present in 1.65 and 0.095 mg/kg plant material from chrysanthemums and *G. aurantiaca*, respectively (Palukaitis and Symons, 1979).

Spot hybridization for viroid detection utilizing a ^{32}P-labeled cDNA probe to PSTV could be used to detect 100–250 pg of purified viroid RNA. The viroid could be identified in an extract from 0.1 mg of infected leaves (Macquaire *et al.*, 1984). The ^{32}P-labeled probe hybridized only with CSV in purified solutions under nonstringent conditions and not with CSV in crude sap. The reaction occurred only with 50 ng or more of CSV. Spot hybridization appeared to be more sensitive than the electrophoretic assay for diagnosis of PSTV. Utilizing a PSTV cDNA probe, only a limited degree of sequence homology to CSV was reported (Owens *et al.*, 1978).

VI. PURIFICATION

In early attempts to purify CSV, highly infectious supernatants were obtained following extraction in chloroform–butanol and infectivity was present in the interface pellets of reextracted pulp that was homogenized (Hollings and Stone, 1969). Infectivity was distributed about equally in

the first low-speed centrifugation supernatant and resuspended low-speed centrifugation pellet in extracts from stunt-infected cineraria (Lawson, 1968b). Infectivity was also present in supernatants following high-speed centrifugation. Phenol treatment of resuspended ethanol precipitates yielded samples with infectivity comparable to that of the untreated ethanol precipitates (Lawson, 1968b).

CSV was shown to have properties similar to PSTV (Diener and Lawson, 1973). In contrast to PSTV extraction from tomato, however, extraction from chrysanthemum leaves resulted in darkly pigmented preparations apparently due to the presence of phenolic compounds. Following the second ethanol precipitation, CSV preparations did not have UV absorption spectra typical of a nucleic acid. To separate the impurities, preparations were treated with hydroxyapatite (Diener and Lawson, 1973). Further purification involves extraction with ethylene glycol monomethyl ether to remove polysaccharides, DNA digestion and 2 M LiCl extraction to remove high-molecular-weight RNA, and gel filtration of total cellular low-molecular-weight RNA and DNA fragments. Final purification includes PAGE (Diener *et al.*, 1977; Diener, 1979).

A new extraction procedure has been developed for large-scale purification of CSV (Palukaitis and Symons, 1980). CSV was purified to homogeneity by one cycle of nondenaturing polyacrylamide slab gel electrophoresis followed by one cycle of denaturing slab gel electrophoresis. The covalently closed-circular form and the linear form of the viroid comigrated in the first gel but were completely separated in the second. Yield of the circular and linear forms were 200 and 35 μg/kg infected chrysanthemum shoots, respectively.

VII. PHYSICAL–CHEMICAL PROPERTIES

CSV is very heat tolerant and will survive boiling for 10 min in a tissue extract (Brierley, 1952; Hollings and Stone, 1973). The exceptional heat tolerance of CSV is responsible for the fact that procedures used for eradication of many plant viruses *in vivo* have not been successful in producing CSV-free material (Diener, 1979).

CSV tolerates freezing and thawing both in extracted juice and in leaf tissue and can be stored for several weeks in a frozen state without reduction in infectivity (Keller, 1953). In air-dried leaf tissue, the infectivity of CSV is retained at room temperature for 2 to 4 weeks in some trials and at least 8 weeks in others (Brierley, 1952). In other trails, infectivity was retained for 100 weeks (Keller, 1953). In buffer extracts, CSV is tolerant to sonication for 5 or 15 min (Hollings and Stone, 1973). Only partial loss of infectivity occurred after this treatment.

CSV displays properties characteristic of viroids, such as the highly base-paired secondary structure and the central conserved region. Two isolates have been sequenced. The isolate analyzed by Haseloff and Sy-

mons (1981) contains 356 nucleotides, whereas the one sequenced by Gross *et al.* (1982) contains 354 nucleotides (see Chapter 2, this volume).

VIII. CONTROL MEASURES

No natural resistance or tolerance to the disease has been identified. Control measures primarily consist in efforts to prevent transmission by hands, tools, and plant-to-plant contact (see Section III).

A. Elimination by Heat Treatment and Meristem-Tip Culture

A low percentage of CSV-free chrysanthemum plants have been produced by meristem-tip culture. CSV was not eliminated by thermotherapy or by meristem-tip culture alone and only with great difficulty by meristem-tip culture after prolonged heat treatment (Hollings and Stone, 1970). Ten plants, derived from 160 meristem tips from stunt-infected Mistletoe, remained healthy after 3 years (Bachelier *et al.*, 1976). More CSV-free Mistletoe plants were obtained when meristem-tips were cultured from terminal buds than from axillary buds (Paludan, 1980). More CSV-free plants were also produced with meristem tips 0.2 mm in length than with tips 0.25 or 0.5 mm long. The method was not very efficient, however, even when combined with heat treatment. Incorporation of the chemical amantadine into a tissue culture medium was reportedly effective in obtaining chrysanthemums free of CSV (Horst and Cohen, 1980). Culture of the shoot apices (0.3 to 1.0 mm) on an amantadine-containing growth medium resulted in about 10% of the plants free of CSV while none of the plants grown in the absence of the chemical was free of the viroid.

B. Cross-Protection

Inoculation with CSV protects chrysanthemum from expressing symptoms of CEV (Niblett *et al.*, 1978). CCMV, however, did not protect against the symptoms of CSV. More recently (Horst, 1980), CSV was found to protect against CCMV, but in reciprocal inoculations cross-protection did not occur. These results show that cross-protection is exhibited in a variety of combinations of PM, PS, CEV, and CSV, but not CCMV, indicating that PM, PS, CEV, and CSV affect a common biological process (Niblett *et al.*, 1978). These viroids differ significantly from one another in their primary structure (Dickson, 1979). So far, cross-protection has not been utilized as a potential means of control of CSV.

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CHAPTER 12

Cucumber Pale Fruit

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I. HISTORICAL

A disease in cucumber characterized particularly by a light green color of the fruits, but also with affected flowers and young leaves, was observed in 1963 in two glasshouses in the western part of the Netherlands. Since that time, the disease, now called *pale fruit disease,* has been observed in different places throughout that country (Van Dorst and Peters, 1974).

II. GEOGRAPHIC DISTRIBUTION

The disease has been reported to occur naturally only in the Netherlands (Van Dorst and Peters, 1974).

III. SYMPTOMATOLOGY

In its only reported natural host, cucumber (*Cucumis sativus* L.), the most distinctive symptom is found on the fruits. They are pale green, retarded in growth, and most are slightly pear-shaped (Van Dorst and Peters, 1974). Both male and female flowers often are stunted and crumpled. The edge of the petals is slightly notched. Developing leaves may be smaller, blue-green, and rugose. The leaf blades are undulated, their edges turned downward, and the tips bent downward or even turned backward. On aging, the leaf symptoms fade and a chlorosis appears.

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Because the internodes of the younger parts of affected plants are shorter than those of healthy plants, the former often are somewhat stunted. Symptoms develop earlier and are more intense when plants are grown at 30°C than at lower temperatures (Van Dorst and Peters, 1974).

IV. FIELD TRANSMISSION AND SPREAD

Pale fruit disease has been reported to occur naturally only in glasshouses. The disease agent can be transmitted during pruning operations, as was demonstrated experimentally (Van Dorst and Peters, 1974). Because the first diseased plants were near the sides and then often near fissures and near the main walk of glasshouses, Van Dorst and Peters (1974) suggested that the agent might be introduced by a vector, supposedly an insect. Results of tests to determine whether a common aphid species (*Myzus persicae*) was able to transmit the agent were, however, negative. Neither was there any evidence that the disease was soil borne or seed transmitted (Van Dorst and Peters, 1974).

V. ECONOMIC LOSSES

Although the pale fruit disease has been detected in many glasshouses in various parts of the Netherlands, the number of infected plants is mostly small, usually less than 20 plants in a crop of 2500 to 25,000 plants. Some higher incidences, however, have been found, perhaps due to spreading by pruning and other cultivation measures (Van Dorst and Peters, 1974). Nevertheless, losses to date seem to have been relatively small.

VI. CONTROL

Apparently, no special measures have been taken to control the disease (Van Dorst and Peters, 1974), but in light of existing knowledge, these would be the same as those used with other viroid diseases.

VII. PURIFICATION

Purification of CPFV and demonstration of its viroid nature was first reported by Sänger *et al.* (1976). Methods were the same as used with other viroids.

VIII. HOST RANGE

Originally, CPFV was reported to infect only several cultivars of cucumber (*C. sativus* L.) (Van Dorst and Peters, 1974). Now that the viroid is known to be closely related to HSV (Sano *et al.*, 1984a), its host range, presumably, extends to all species susceptible to HSV.

IX. STRAINS

CPFV causes symptoms in cucumber identical with those of HSV (Sano *et al.*, 1984a); it has been shown by nucleotide sequence analysis to be a strain of HSV, composed of 303 nucleotides and differing from the latter at 16 positions, resulting in about 95% sequence homology (Sano *et al.*, 1984b; Chapter 14, this volume).

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CHAPTER 13

Coconut Cadang-Cadang

JOHN W. RANDLES

I. INTRODUCTION

"Cadang-cadang" is derived from a word in the Bicol dialect, "gadan-gadan," which means dead or dying (Rillo and Rillo, 1981). It now refers to a premature decline and death of coconut palms in the Philippines that is associated with viroid infection (Fig. 1). The synonym "yellow mottle decline" is not commonly used, and "tinangaja" is the name used for a similar disease with the same etiology in Guam (Boccardo *et al.*, 1981; Boccardo, 1985).

Some terms and abbreviations used here are: CCCV, coconut cadang-cadang viroid, without reference to the molecular form; CC1S, monomeric form of the viroid in its small (S) form (246 or 247 nucleotides); CC2S, dimer of CC1S; CC1L, CC1S with reiteration of a sequence of between 41 and 55 nucleotides at the right-hand end of the molecule to produce a large (L) form; CC2L, dimer of CC1L.

Circular and linear forms are annotated with "c" or "l." Molecular forms with a single or a double cytosine residue adjacent to the central conserved region (e.g., at position 197–198 of CC1S) have the suffix "(c)" or "(cc)."

II. HISTORY

The earliest unsubstantiated report of cadang-cadang was in Camarines Sur province of the Philippines in 1914 (De Leon and Bigornia, 1953;

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FIGURE 1. A coconut plantation affected by cadang-cadang. Late-stage nonbearing palms are shown together with numerous dead palms.

Kent, 1953; Velasco, 1961). A disease with symptoms now known to be typical of cadang-cadang was first described on San Miguel Island in 1931 (Ocfemia, 1937) with an incidence of 25% in some areas. A sequence of reports from sites at increasing distances from the Bicol peninsula then followed (Price, 1971).

International support for research on cadang-cadang commenced after 1950 (Reinking, 1950), leading to a wider recognition of the importance of the disease and a detailed description of symptomatology (Price, 1958; Nagaraj *et al.*, 1965). The etiology of the disease was the subject of intense speculation (Rillo and Rillo, 1981; Velasco, 1982) until the discovery that a viroidlike RNA was associated with the disease (Randles, 1975; Randles *et al.*, 1976; Randles and Hatta, 1979). Proof that this RNA was infectious (Randles *et al.*, 1977; Mohamed and Imperial, 1983) confirmed the viroid etiology and led to the development of diagnostic tests (Randles and Palukaitis, 1979; Mohamed and Imperial, 1984; Imperial *et al.*, 1985), elucidation of primary and secondary structure (Haseloff *et al.*, 1982; Randles *et al.*, 1982; Riesner *et al.*, 1982), and comparisons with other viroids.

Tinangaja was first reported on Guam in 1917 (Weston, 1918), and although somewhat different in symptomatology from cadang-cadang, the causal viroid is homologous with CCCV (Boccardo *et al.*, 1981). Its incidence on Guam is high but of no commercial importance.

No vector has been found for cadang-cadang despite evidence for natural spread (Anonymous, 1982; Zelazny and Pacumbaba, 1982a). The

search for a vector, and a source of resistance to the disease, continues with the ultimate objective of developing control measures.

III. DISTRIBUTION

Cadang-cadang disease has been recognized only in the central Philippines (Zelazny, 1979) (Fig. 2). Tinangaja has been found on Guam only (Boccardo *et al.*, 1981). CCCV has not been detected in diseased coconut

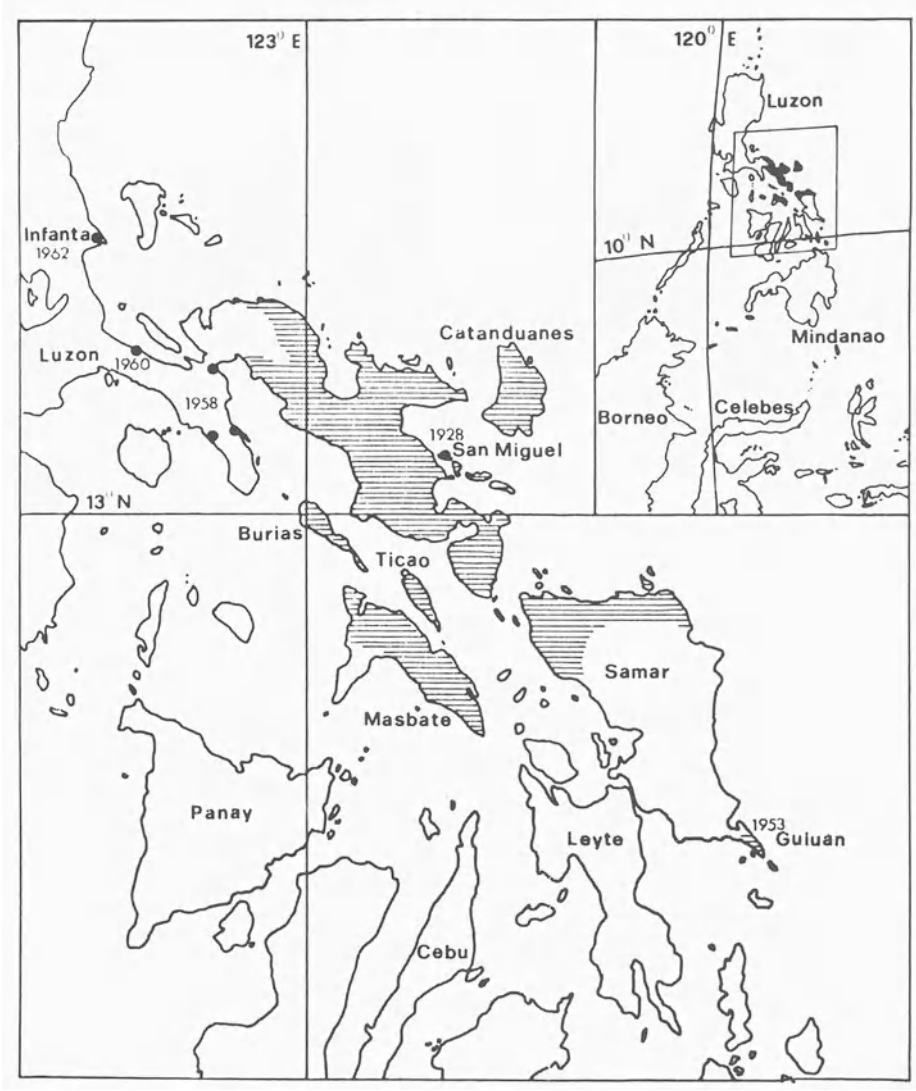


FIGURE 2. Distribution of cadang-cadang disease in the Philippines, showing the year of first recognition at several sites.

palms in India, Vanuatu (Randles, 1985), other parts of the Philippines, or Micronesia (J. W. Randles and B. Zelazny, unpublished results).

In the Philippines the disease now has an apparently stable distribution with only slow outward movement of the boundaries. Isolated pockets of disease exist, jumps of several hundred meters have been observed, and neither rivers nor sea seem to have interrupted the spread (Zelazny, 1979).

Within the areas of disease occurrence, the incidence of cadang-cadang is highly variable. In small areas, diseased palms are not clustered, but over large areas zones of high and low incidence are seen (Zelazny, 1980), which can shift with time (Zelazny *et al.*, 1982).

IV. SYMPTOMS AND HOST RANGE

Infected bearing coconut palms progress through a well-defined series of changes culminating in death (Randles *et al.*, 1977; Mohamed *et al.*, 1982; Randles, 1985). The first symptom is the rounding and equatorial scarification of new developing nuts, 1–2 years after CCCV is first detected in the youngest fronds. Chlorotic leaf spots then appear on young expanded fronds and inflorescences become stunted. Leaf spots enlarge, nut production declines, spathes and newly emerging inflorescences become more stunted, and then become sterile. This "early" stage occupies an average of 2 years in 19- to 30-year-old palms and 3.75 years in older palms (Zelazny and Niven, 1980). Spathe, inflorescence, and nut production then decline and cease. This is the "mid" stage and has an average duration of about 2 years. Leaf spots become more numerous, coalescing to give a general chlorosis, fronds decline in size and number, leaflets become brittle, crown size is reduced, and the palm dies. This "late" stage averages about 5 years.

The estimated duration from initial disease appearance to death is 7.5 and 15.9 years respectively for palms 22 and 44 years old (Zelazny and Niven, 1980). Palms naturally or artificially infected before bearing are stunted and generally fail to bear nuts.

Root deterioration (Calica and Bigornia, 1962) and leaf hypoplasia (Rillo *et al.*, 1972) have been reported. In the chlorotic leaf spots, chloroplasts appear vesiculate, they accumulate starch, and lamellae become disorganized (Randles, 1985).

Other palm species also show mild or severe leaf spotting, and general stunting, following inoculation with CCCV (Imperial *et al.*, 1985). Naturally infected African oil palm (*Elaies guineensis*) (Randles *et al.*, 1980) becomes sterile, and dies.

Tinangaja differs in that nuts are characteristically small and elongated and lacking a kernel (i.e., "mummified"; Boccardo *et al.*, 1981).

The host range of CCCV comprises members of the *Palmae* only. The following species have been infected by inoculation: *Areca catechu*

(betel nut palm), *Corypha elata* (buri palm), *Adonidia merrillii* (manila palm), *E. guineensis*, *Chrysalidocarpus lutescens* (palmera), *Oreodoxa regia* (royal palm) (Imperial *et al.*, 1985) and *Phoenix dacrylitera* (date palm; J. S. Imperial and M. Rodriguez, unpublished results).

V. EPIDEMIOLOGY

Natural spread of cadang-cadang occurs through an unknown agent or vector and the rate of seed transmission is so low (below 0.3%; Anonymous, 1982; Randles and Imperial, 1984) as to be unimportant except perhaps for primary introduction of CCCV to new planting sites.

Coconut plantations less than 10 years old have negligible incidence of disease, then cadang-cadang shows an almost linear increase with age until palms are about 40 years old (Zelazny and Pacumbaba, 1982b). The source of infection is not known. Removal of diseased palms does not appear to prevent disease spread (Anonymous, 1982; Zelazny *et al.*, 1982) and so if palms are the source of infection, it is probable that those at the very early stage of infection are the most important source (see Section XIII).

Outward spread of the disease from margins of distribution now appears to be less than 500 m per year, and isolated groups of infected palms detected around 1960 appear not to have expanded markedly (Zelazny, 1979). The present distribution of the disease and the slow outward rate of spread suggest that cadang-cadang is an endemic disease rather than a newly introduced disease originating from a single source 70–90 years ago (a hypothesis arising from the sequential reports of disease at increasing radii from San Miguel Island).

Epidemics have risen and declined at different times in different places. An epidemic in Albay province in 1951–1957 is now declining, while disease incidence in parts of the neighboring Camarines Sur province has risen from below 3% in 1956 to 50–70% at present (Zelazny *et al.*, 1982).

In the Albay and Camarines Sur provinces, no correlation was found between incidence and site, abundance of a range of plant species, palm density, rainfall, or soil conditions. However, incidence was correlated with palm age, and negatively correlated with altitude (Zelazny, 1980). Three beetle species were more abundant in areas of high incidence (Zelazny and Pacumbaba, 1982a) but so far no insects have been shown to transmit cadang-cadang.

VI. ECONOMIC LOSSES

During the period of high incidence in the 1950s, roadside surveys in the Bicol region gave the following estimates of disease incidence: 1.8×10^6 diseased palms in 1951; 4.6×10^6 in 1952; 5.5×10^6 in 1953;

7.9×10^6 in 1957 (Kent, 1953; Price, 1958). Incidence ranged between 9.1 and 61% in different provinces. Zelazny and Pacumbaba (1982b) estimated that there were 391,000 new cases in 1978 and 209,000 in 1980, indicating a decline in incidence since 1960. Zelazny *et al.* (1982) estimate that up to 30×10^6 palms have been killed by cadang-cadang since it was first recognized.

Infected palms cease production an average of 5 years before death, and replacement palms do not reach full bearing for 5–8 years after planting. Thus, 10–13 years' production may be lost for each planting site occupied by an infected palm. Assuming an average nut production of 80 per year per palm, and a copra yield of 250 g per nut, the total loss per site would be 200–260 kg of copra. At a price of $0.40 per kg, $80–$104 would be lost per site. The 209,000 new cases in 1980 could therefore have resulted in a maximum total loss of ca. $\$20 \times 10^6$. A minimal loss would occur if palms were removed when disease first appeared, and assuming an average loss of half the bearing life of palms from infection, a cost of $\$3.5 \times 10^6$ can be calculated (Zelazny and Pacumbaba, 1982b).

VII. DIAGNOSIS

Symptomatology is unsatisfactory for disease diagnosis because of the delay of 2–4 years between inoculation and symptom appearance, and its unreliability in inoculated seedlings or hosts other than coconut palm. CCCV is detected on the basis of size and structure by gel electrophoretic methods, and on the basis of sequence by molecular hybridization. CCCV in preparations precipitated with polyethylene glycol (PEG) appears to be nonimmunogenic (J. W. Randles and O. W. Barnett, unpublished results) and serological techniques are therefore not applicable.

CCCV is concentrated from sap extracts with PEG (Randles, 1975; Imperial *et al.*, 1985), extracted with phenol–sodium dodecyl sulfate–chloroform, and precipitated with ethanol.

CCCV is detected by single-dimension electrophoresis on 3.3%–20% polyacrylamide gels, the high-concentration gels allowing separation and identification of all the molecular forms present. Toluidine blue and ethidium bromide detect CCCV in leaf sample equivalents of 1–2.5 g (Randles, 1985) while silver staining is at least 100-fold more sensitive. Two-dimensional and bidirectional electrophoresis, which has been developed for general use in the detection of viroidlike RNAs (Schumacher *et al.*, 1983), is useful for identifying CCCV in mixtures when linear RNAs of similar mobility are present.

Synthesis of a radioactive cDNA probe using viroid as a template for reverse transcription (Randles and Palukaitis, 1979), or cloning of the viroid sequence in an M13 phage vector (Barker *et al.*, 1985) allows CCCV to be detected by solution hybridization or dot-blot hybridization analysis (Mohamed and Imperial, 1983; Imperial *et al.*, 1985). The use of cloned

probe labeled with biotin, and enzymatic detection of the probe (Forster *et al.*, 1985) should provide a stable reagent and avoid the use of radioactive labeling for detection of CCCV.

Both electrophoresis and dot-blot hybridization are in regular use for the assay of samples, the former method having been shortened to a minimum of 1 day for an assay, the latter to 2 or 3 days. This has resulted from selecting plant tissue with relatively high viroid concentration, and deletion of the PEG precipitation step (J. S. Imperial and J. W. Randles, unpublished results).

VIII. STRAINS

Variation in symptomatology of cadang-cadang within the Philippines is apparently attributable to nonuniformity of palms, rather than to variation in the viroid. Haseloff *et al.* (1982) showed that a range of isolates had the same minimal nucleotide sequence and any modification of the structure of the viroid was related to the stage of disease development (Imperial *et al.*, 1981) and not to strain differences.

Tinangaja viroid has not been sequenced but it may be considered a strain of CCCV because of its geographical separation and different symptomatology. No direct comparisons of the biological properties of cadang-cadang and tinangaja viroids have been made.

IX. EXPERIMENTAL TRANSMISSION

Artificial transmission of CCCV is relatively inefficient, incubation times to detection of viroid are long, and early symptoms of infection are unreliable for diagnosis. The procedure used for experimental transmission is as follows:

Inoculum: nucleic acids are extracted from the PEG-concentrated sap of palms at the early stage of infection, and adjusted to a concentration of ca. 1 mg/ml in 0.15 M NaCl, 0.015 M Na citrate; fractionated RNA is used at 20–40 μg/ml (Randles, 1985; Imperial *et al.*, 1985).

Test plants: use coconut seedlings with newly emerged shoots, or seedlings of other species at the two- to three-leaf stage (Imperial *et al.*, 1985); preinoculation darkening of 72 hr is generally practiced.

Inoculation: by high-pressure injection using a hand-held "Panjet" injector, delivering 0.05–0.1 ml of inoculum in each of five injections at sites on folded fronds and frond bases (Randles *et al.*, 1977; Imperial *et al.*, 1985); razor slashing or pricking is combined with injection by cutting the leaf surface through droplets of inoculum left on the leaf surface.

Assay: by detection of viroid 0.5–2 years after inoculation using gel electrophoresis or molecular hybridization.

Sap is not infectious by this method, and inoculation by rubbing

inoculum in the presence of abrasive, pseudografting, or insect transmission have been unsuccessful (Price, 1971).

X. PURIFICATION

Fronds in positions four or more below the spear leaf are harvested. Leaflets without mid ribs are chopped and blended with 3 volumes of 0.1 M Na_2SO_3. The extract is filtered through fine nylon mesh, clarified by centrifugation at 10,000 *g* for 10 min, and PEG 6000 is added to 5% (w/v). After 1–2 hr at 4°C, the precipitate is collected by low-speed centrifugation and nucleic acids extracted by one of a range of procedures (Randles, 1975; Randles *et al.*, 1976; Boccardo *et al.*, 1981). CCCV may be recovered directly from the PEG precipitate by resuspending it in 1 mM EDTA and precipitating it with ethanol (Randles and Imperial, 1984).

Extracts are subjected to electrophoresis in 5% polyacrylamide gels, the bands are detected by brief staining with aqueous toluidine blue, and gel pieces containing the bands are laid over or under a denaturing gel containing 8 M urea (Randles *et al.*, 1982). A second electrophoresis separates circular and linear forms of the CC1 and CC2. These bands are again excised, and RNA extracted either by (1) maceration and extraction of the gel followed by sucrose density gradient centrifugation (Randles and Palukaitis, 1979), (2) preparative gel electrophoresis, or (3) electrophoresis into agarose gel (Randles *et al.*, 1982) followed by gel maceration and extraction. RNA is precipitated with ethanol, dried, resuspended in 0.2 M NaCl, and reprecipitated with 1% cetyl trimethyl ammonium bromide.

XI. PHYSICAL PROPERTIES

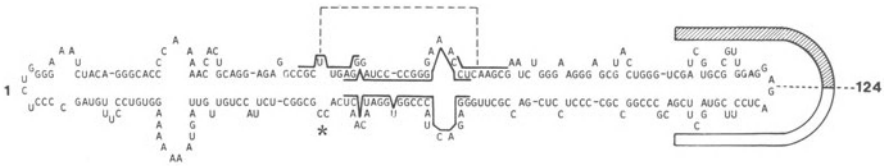
Four circular forms of CCCV can be extracted from an infected palm during the course of the disease: CC1S, CC1L, CC2S, CC2L. In their native state they are partially base-paired rods (Randles and Hatta, 1979) with a flexibility (determined by sedimentation analysis) the same as that of PSTV, greater than that of dsRNA and dsDNA, and less than that of the nonviroid circular RNAs of VTMoV and SNMV (Riesner *et al.*, 1982). Although a cruciform native structure of CC2 is possible (Haseloff *et al.*, 1982; Riesner *et al.*, 1982), this has not been detected by sedimentation analysis.

The native CC1 is partially sensitive to the single-strand-specific S1 nuclease (Randles *et al.*, 1976; Randles and Palukaitis, 1979) as expected for a partially base-paired structure. Thermodynamic studies indicate that the native molecules do not adopt significant tertiary folding, but they exhibit highly cooperative melting of base-paired regions at a T_m of 49°C in 10 mM NaCl and a half-width of melting ($\Delta T_{1/2}$) of 1.2–1.4°C (Table I)

TABLE I. Some Physical Properties of the Coconut Cadang-Cadang Viroid

Form of viroid		Number of nucleotides	$s_{20,w}$	Thermodynamic properties					
				T_{m_1}	$\Delta T_{\frac{1}{2}}$	Hy	GC	T_{m_2}	Hy
CC1S	circular	246/7	5.91	49.1°C	1.4°C	15%	70%	57.5°C	2.3%
	linear	"		45.6	2.9	10.5	73.5	56.5	NA
CC1L	circular	287–301	6.34	49.4	1.2	16.5	72.5	57.5	2.0
	linear	"		45.2	2.8	15.0	73.0	55.0	1.5
CC2S	circular	492/4	7.57	46.7	2.4	12.0	73.0	57.0	1.9
	linear	"		45.7	2.7	8.5	72.5	56.0	1.5
CC2L	circular	574–602	8.16	47.1	1.6	14.5	74.0	56.0	<1.8
	linear	"	NA	NA	NA	NA	NA	NA	NA

[a] T_m values refer to 1 mM Na-cacodylate, 10 mM NaCl, 0.1 mM EDTA, pH 6.8. $\Delta T_{\frac{1}{2}}$ is the half-width of the thermal transition. Hypochromicity (Hy) is the percentage change in A_{260} associated with the first (T_{m_1}) or the second (T_{m_2}) thermal transition. GC is the percentage of GC base pairs dissociating (values from Randles *et al.*, 1982; Riesner *et al.*, 1982, 1983).



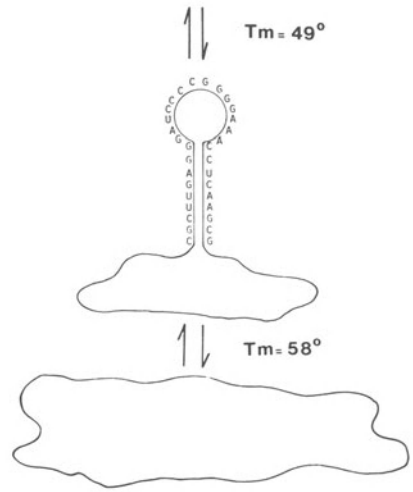


FIGURE 3. Primary sequence of the 247-nucleotide form of CCCV (after Haseloff *et al.*, 1982). The minimal 246-nucleotide form has one cytosine deleted from position 198 (asterisk). The inner bold line (52–71, 171–184) shows the conserved region. The outer bracketed line marks the complementary sequences on either side of the conserved region which can pair to produce an intermediate form following the cooperative melt at 49°C of the native molecule. The loop at the right enclosed by the hatched and clear blocks is duplicated to give the large forms of CCCV.

(Randles *et al.*, 1982; Riesner *et al.*, 1983). Less cooperativity is shown by the native linear forms that arise through a single cleavage of the circular form. Although rodlike, their secondary structure differs from that of the circular form as shown by a lower T_m, a wider thermal transition, lower hypochromicity, and hence lower cooperativity of melting (Table I). CC2 also has lower cooperativity than CC1, and its linear form has even lower cooperativity (Table I). The small and large circular forms of CC1 (CC1S and CC1L) exhibit the same cooperativity.

An intermediate structure forms at temperatures just above the T_m, apparently resulting from the base pairing of complementary sequences previously separated from each other in the native form. A hairpin comprising a single-stranded loop of 14 bases and a stem of 9 base pairs results (Fig. 3). The stem melts at about 58°C (Table I) to produce a completely denatured circle. The short sequence that is exposed as a single-stranded loop occurs in the central conserved region common to most of the other viroids, and where homology with introns has been proposed.

XII. NUCLEOTIDE SEQUENCE

Of the four basic molecular forms of CCCV, the 246-nucleotide form of CCCV (Haseloff *et al.*, 1982) is the smallest known viroid. CCCV has the conserved sequence common to PSTV, CEV, CSV, HSV, TASV, and TPMV, which is shown in the center of the molecule in Fig. 3. A variable region occurs at the right-hand end of the molecule as a result of 41, 50, or 55 nucleotides in the bracketed region being reiterated to produce the CC1L forms of CCCV, which are between 287 and 301 nucleotides long (Haseloff *et al.*, 1982). Another site of variation is at cytosine residue 197 where a second cytosine may be inserted at site 198 to give a molecule of 247 nucleotides. The dimeric forms detected concurrently with their respective monomer are covalently linked forms of the monomer, incorporating the same sequence variations.

XIII. RELATIONSHIP BETWEEN VIROID STRUCTURE AND DISEASE PROGRESS

CC1S is replaced by CC1L during disease development (Fig. 4) (Imperial *et al.*, 1981; Mohamed *et al.*, 1982). CC1S is first detected in young fronds before symptoms appear in the palm, and is the only form detected in all subsequently developing fronds for about 2 years, during the period when nut symptoms develop but before leaf symptoms appear. Fronds developing after this time, during the period when leaf symptoms appear, contain progressively increasing amounts of CC1L and decreasing amounts of CC1S. This phase occupies approximately 1 year. As these fronds mature, move down through the canopy, and senesce, they are replaced by fronds containing CC1L only, and this form persists throughout the late

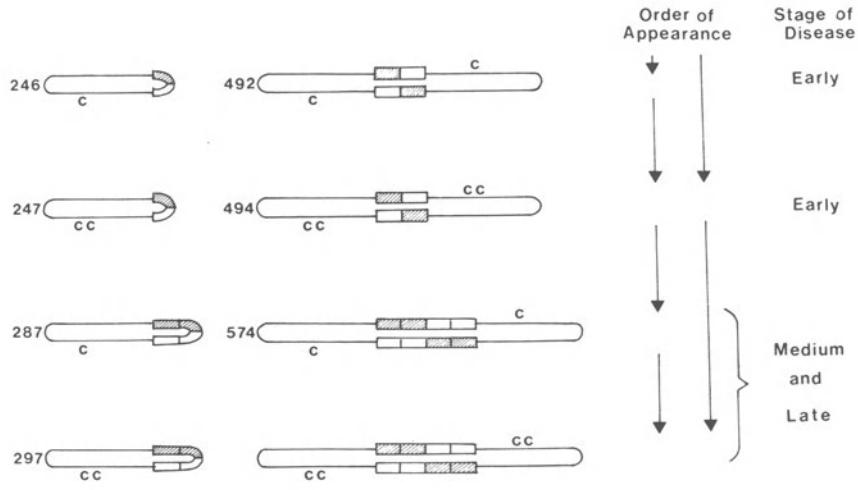


FIGURE 4. The four forms of monomeric (left) and dimeric (right) circular CCCV shown in relation to the stage of disease development. The small form (246 or 247 nucleotides) is modified to the large (287 or 297 nucleotides) by duplication of the right end shown in Fig. 3, as palms progress from the early to the medium stage of the disease. Note also the progression from the single cytosine (c) to the double cytosine (cc) form. The arrows indicate alternate modes of progression through the forms, i.e., 246, 247, 287 to 297, or 247 to 297 nucleotides. The progression of the dimer is parallel to that of the monomer.

stage of disease. The corresponding forms of CC2 appear simultaneously with CC1.

Some evidence has been obtained for a similar transition in other infected palm species (Imperial *et al.*, 1985).

The presence or absence of a cytosine residue at nucleotide position 198 (Fig. 4) represents another variation that may be related to disease development. Imperial and Rodriguez (1983) found that in palms in which CC1S(c) was first detected, the sequence of development was CC1S(cc), CC1L(c), CC1L(cc). When CC1S(cc) was the form first detected, it was replaced by CC1L(cc).

The systematic modification of viroid primary structure with time and disease development appears to be unique to CCCV. It is interesting in that it may be an indication that primary structural changes are associated with changes in function of the viroid, as has also been suggested above for a change in secondary structure. This view must remain speculative until the nature of viroid replication and pathogenesis is understood.

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CHAPTER 14

Hop Stunt

EISHIRO SHIKATA

I. DISEASE OCCURRENCE

Abnormal growth involving severe stunting of Japanese commercial hop plants (*Humulus lupulus* L.) has been observed occasionally in the fields of Fukushima prefecture, Tohoku district of Honshu (northeast of the main island of Japan) since 1952. At that time, the disease was suspected of being a physiological disorder. In the early 1960s, a disease called "dwarfed hop" became prevalent at Fukushima prefecture (Yamamoto *et al.*, 1970, 1973).

A similar disease, locally called "Tozama disease," was first recognized in the Tozama area in Iiyama city and also in Nagano prefecture, the central mountainous area of Honshu (Mori, 1965, 1966, 1967).

After thorough investigations, started in 1965 by Yamamoto *et al.* (1970), it was concluded that the disease was of viral origin and was named "hop stunt disease," although no causal virus particles were detected. Yamamoto *et al.* found that the disease had spread over Tohoku district, including 17% of the total acreage of hop yards in Fukushima prefecture. The authors suggested that the "Tozama disease" in Nagano prefecture was almost certainly the same as hop stunt disease. There was no report of the disease occurring in Hokkaido.

Because of the increase of the disease and of damages in the hop-growing areas, intensive investigations on the etiology of hop stunt disease have been conducted at the Department of Botany, Faculty of Agriculture, Hokkaido University, Sapporo, since 1975. Following the find-

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ing of cucumber plants as a susceptible host plant, the disease was shown to be of viroid causation (Sasaki and Shikata, 1977a,b).

The Japanese commercial hop was first introduced in 1875–1876 from the United States and Germany. Since then, most of the hop varieties grown in Japan were obtained from the United States or European countries. Hop stunt disease, however, has not been reported to occur in these countries, despite the presence of the pale fruit disease on cucumber in Holland (Van Dorst and Peters, 1974). Thus, the disease is specific to Japan.

The outbreak of HSV in Japan is, therefore, very interesting with regard to cucumber pale fruit disease, which is now known to be caused by an HSV strain designated as cucumber isolate (HSV-c) of HSV (Sano *et al.*, 1984b).

A detailed retrospective investigation on the source of HSV infection in the field was made in Fukushima prefecture by Yamamoto *et al.* (1970, 1973). It was found that all cuttings of the infected gardens in Fukushima prefecture had been obtained, directly or indirectly, from a source garden, into which the cuttings were suspected to have been introduced from Yamanashi prefecture in 1941. On the other hand, Mori (1965, 1966, 1967) has reported that the hop cuttings originally cultivated in Yamanashi prefecture had been transferred from Nagano prefecture about 47 years ago, where "Tozama disease" has been found since 1965.

Because Yamamoto *et al.* (1973) failed to detect "Tozama disease" in Yamanashi prefecture at that time, it is not clear whether the origin of HSV was at Yamanashi or Nagano prefecture.

II. SYMPTOMS AND DAMAGE TO HOP PLANTS

Typically, infected hop plants are stunted, reaching only approximately 5 m high, instead of 8 m as do healthy plants. Thus, the total height of diseased plants is 60–66% that of healthy ones (Fig. 1). Typical symptoms of HSV-infected hop plants consist of shortening of the internodes of the main and lateral bines and curling of the upper leaves. The upper lateral bines of diseased plants grow poorly and the shape of the entire plants resembles that of Japanese cedar trees. The main bines grow thinner and hooked hairs and ridges on the underpart of the bines are poorly developed. Leaves are smaller and more yellowish green than those of HSV-free plants. One of the upper leaves curls outwards in June. These symptoms vary according to growing conditions and it is therefore sometimes difficult to diagnose the disease in the field.

The total weight of green bines and leaves decreases about 30% as compared with healthy plants. Cone weight is reduced about 50%. In addition, serious damage is caused by changes in the resin content of cones. The alpha acid content in the bitter substances of cones from HSV-

FIGURE 1. Hop infected with HSV (right) and healthy hop (left). Note that the apical bines of the healthy hops (three plants at left) reach the upper wire, while the diseased ones (three plants at right) do not.

infected hop plants is only one-third of that from HSV-free cones, whereas beta acid content is almost the same in HSV-infected and healthy cones (Yamamoto *et al.*, 1970, 1973). One of the serious effects of HSV infection of hop is a 60% decrease in the amount of lupulin glands on bracteoles. A scanning electron microscopic study revealed that most of the lupulin glands on bracteoles from the infected cones were severely shriveled (Momma and Takahashi, 1984).

III. HOST RANGE AND SYMPTOMATOLOGY

Yamamoto *et al.* (1970) showed that wild hop (*Humulus japonica* Sieb. et Zucc.) could be infected only by grafting. In 1977, Sasaki and Shikata (1977a) reported that some cucurbitaceous plant species are susceptible to HSV (Table I).

By mechanical inoculation, cucumber (*Cucumis sativus* L.) is the most susceptible species and shows severe symptoms on leaves and flowers, as well as stunting (Fig. 2), pale fruits, and sterile seeds. Symptoms described by Sasaki and Shikata (1977a, 1978a) are quite similar to those of cucumber plants infected by CPFV, as reported by Van Dorst and Peters

TABLE I. Host Range of HSV

Susceptible	*Benincasa hispida*	*Cucumis melo*	*C. melo* var. *conomon*
	C. sativus	*Lagenaria siceraria* var. *clavata*	
	L. siceraria var. *gourda*	*L. siceraria* var. *microcarpa*	
	Luffa cylindrica	*Humulus japonicus*	*H. lupulus*
	Lycopersicon esculentum		
Nonsusceptible	*Tetragonia expansa*	*Gomphrena globosa*	*Vinca rosea*
	Beta vulgaris var. *sacchariferra*		*Chenopodium album*
	C. amaranticolor	*C. murale*	*C. quinoa*
	Cucurbita moschata	*C. pepo*	*C. pepo* var. *medullosa*
	Citrullus vulgaris	*Glycine max*	*Phaseolus angularis*
	P. radiatus	*P. vulgaris*	*Pisum sativum*
	Vicia faba	*Vigna sesquipedalis*	*Morus bombycis*
	Sesamum indicum	*Datura stramonium*	*Nicotiana clevelandii*
	N. debneyi	*N. glutinosa*	*N. sylvestris*
	N. tabacum	*Petunia hybrida*	*Solanum melongena*[a]
	S. tuberosum	*Pharbitais nil*	*Juglans* sp.

[a] But is susceptible (without symptoms) when inoculated with highly concentrated inoculum (Shikata, unpublished results).

(1974). The only difference is that *Cucurbita moschata* is susceptible to CPFV but not to HSV.

Characteristic cytopathic changes of leaf tissues of hop, cucumber, and tomato plants infected with HSV were shown in the form of aberrations of cell walls, chloroplast disintegration, and disappearance of tonoplasts (Kojima and Murai, 1982; Momma and Takahashi, 1982; Kojima *et al.*, 1983). Undulated cell walls were also noted in third primordium cell walls, shoot, and apical meristems of infected hop plants (Momma and Takahashi, 1983). It is unlikely that paramural bodies are the primary cytopathic effects in infected plants because these bodies are also present in healthy plants. It is interesting to note that disintegration of chloroplasts sometimes occurs also in latently infected tomato plants.

Scanning electron microscopy revealed that infected hop plants lack foldlike structures of cuticular wax deposits over the epidermis (Momma and Takahashi, 1982). In transverse sections examined by light microscopy, irregular cell arrangements, such as severely distorted epidermal and mesophyll cells, were noted in infected hop plants. On the other hand, HSV infection of cucumber plants caused effects on cell arrangement, mostly in the upper and lower epidermis (Momma and Takahashi, 1982). However, Kojima *et al.* (1983) indicated that the disintegrated cells were vertically distributed from the palisade to the spongy parenchyma.

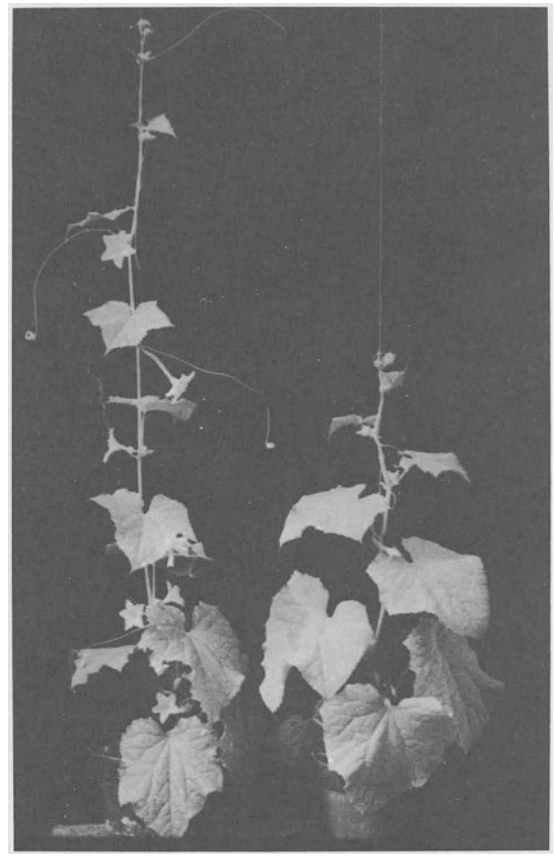

FIGURE 2. Healthy (left) and HSV-infected (right) cucumber plants.

IV. TRANSMISSION AND DISTRIBUTION

HSV is transmissible by grafting and mechanical inoculation (Yamamoto *et al.*, 1970, 1973; Sasaki and Shikata, 1977a, 1978a). No transmission occurred by aphids, through soil, by nematodes, or through seeds (Yamamoto *et al.*, 1970, 1973). No transmission through pollen ovule could be demonstrated (Yaguchi and Takahashi, 1984a). In hop gardens, it was shown that the viroid is transmitted by hands, sickles, scissors, and contact with bines during handling of the plants, such as dressing, pulling, and picking (Yamamoto *et al.*, 1970, 1973; Sasaki and Shikata, 1978a,c). Originally the disease was spread over the fields by introduction of diseased cuttings. In the field, plants gradually become infected along the ridges next to an infected plant, as has been shown clearly by Yamamoto *et al.* (1970, 1973) and by Sasaki *et al.* (1982). This indicates that contact of healthy plants with bines and leaves, and perhaps with roots, of infected plants may cause spread of the disease in the field. The viroid

was shown to survive in roots for more than a year (Yaguchi and Takahashi, 1984b).

V. ETIOLOGY AND PURIFICATION

The first evidence of the viroid etiology of hop stunt disease was shown by Sasaki and Shikata (1977b, 1978b,d) on the basis of the physicochemical properties of the disease agent. The agent was not inactivated at 84°C for 10 min, or after storage for 3 days at 4°C. Infectivity was lost when sap was diluted 10^4 to 10^5 or kept for 1 day at 20°C. Air-dried infected cones maintained their infectivity for more than 1 year (Sasaki and Shikata, 1980).

HSV was more readily extracted in 0.5 M K_2HPO_2 buffer than in 0.005 M K_2HPO_4 buffer. Infectivity remained in the supernatant after high-speed centrifugation at 45,000 rpm for 2 hr, as well as after ultracentrifugation of chloroform–butanol extractions. The agent could be extracted with phenol and precipitated from the aqueous phase with ethanol. It was inactivated by RNase, but not DNase. The sedimentation coefficient of HSV was estimated to range between 6 and 9 S by sucrose density gradient centrifugation. The infectious low-molecular-weight nucleic acid extracted from diseased cucumber by the Morris and Smith (1977) procedure was detected in the fraction of 7 S RNA by 5% polyacrylamide gel electrophoresis, indicating that the molecular weight of HSV is lower than that of PSTV (Sasaki and Shikata, 1978b,d).

Because no viruslike particles were found in extracts or cells of ultrathin sections, and based on the physicochemical properties of the agent, Sasaki and Shikata (1977b, 1978b,d) concluded that the disease was caused by a viroid. The viroid etiology was later confirmed by similar experiments (Takahashi, 1981).

Purification of HSV was achieved by several workers (Ohno *et al.*, 1982; Yoshikawa and Takahashi, 1982), and that of HSV-c by Uyeda *et al.* (1983, 1984). Procedures employed by Uyeda *et al.* (1984) are as follows:

Nucleic acids were extracted from the frozen tissues of infected cucumber plants by phenol–$CHCl_3$–SDS, and precipitated with ethanol. Polysaccharides were removed by ethylene glycol monomethyl ether treatment and phenolic substances by cetyltrimethylammonium bromide. After treatment with 2 M LiCl, the soluble fraction was treated with DNase I, the viroid separated from other low-molecular-weight RNAs by CF-11 cellulose column chromatography and 15% PAGE. Yield of the purified viroid was about 3–6 μg/200 g tissue. Uyeda *et al.* (1984) indicated that the RNA bands were sharper in 15% PAGE than in 7.5% PAGE as described by Yoshikawa and Takahashi (1982), or in 10% PAGE as described by Ohno *et al.* (1982).

VI. STRUCTURE AND FUNCTION

Both circular and linear forms of HSV and HSV-c have been found to be infectious (Ohno *et al.*, 1982; Sano *et al.*, 1984a). Electron micrographs of denatured HSV showed a covalently closed circular form, 77–100 nm long, suggesting 296 nucleotides (Ohno *et al.*, 1982). However, Sano *et al.* (1984a) measured the length of HSV and HSV-c as 83 ± 2.4 and 82 ± 4.2 nm, respectively. Under nondenaturing conditions, rodlike molecules of HSV-c, about 50 nm long, were also observed. Therefore, biologically and morphologically, HSV and HSV-c are indistinguishable. Ohno *et al.* (1983a) established the nucleotide sequence of HSV by cloning of HSV cDNA. The molecule is a covalently closed circular ssRNA consisting of 297 nucleotides. The most probable secondary structure of HSV has 67% of its residues base-paired. The central region of native HSV has a similar conserved region to other viroids. The sequence homology to the 5′-end of U1a RNA is also found in the HSV sequence (Fig. 3).

RNA molecules synthesized *in vitro* from HSV cDNA are highly infectious if they consist of two or four units of tandemly repeated plus strand (Ohno *et al.*, 1983b). The plus and minus monomeric units, and the minus two- and four-unit RNAs are not infectious.

Based on the results with HSV-related RNAs, a possible modified rolling-circle model for viroid replication has been presented by Ishikawa *et al.* (1984). Plus-strand RNA synthesis would start at a specific site of a circular double-stranded region according to this model.

Double-stranded cDNA fragments corresponding to 1, 2, or 3 units of HSV sequences were prepared and inoculated into cucumber plants. The results indicated that HSV ds cDNA with 1, 2, or 3 monomeric units is infectious (Meshi *et al.*, 1984). Excised fragments containing 2 or 3 units of HSV sequences were highly infectious at 0.3–0.4 μg/ml. A monomeric fragment was less infectious at 2 μg/ml. Northern blot analysis revealed that, independent of the repeating of HSV sequences in the inoculum cDNA, only RNA molecules corresponding to authentic HSV were detected.

VII. CUCUMBER AND GRAPEVINE ISOLATES

During the investigation of HSV, it was suspected that CPFV, reported in Holland (Van Dorst and Peters, 1974), might be related to HSV, owing to the similarity of its host range and symptomatology. Comparative host range studies indicated that the two viroids are biologically indistinguishable (Sano *et al.*, 1981). Further experiments revealed that both viroids comigrate to almost the same position in 15% polyacrylamide gels under nondenaturing conditions. Under denaturing conditions (7 M urea), however, they migrate at slightly different rates in 5 or 7.5%

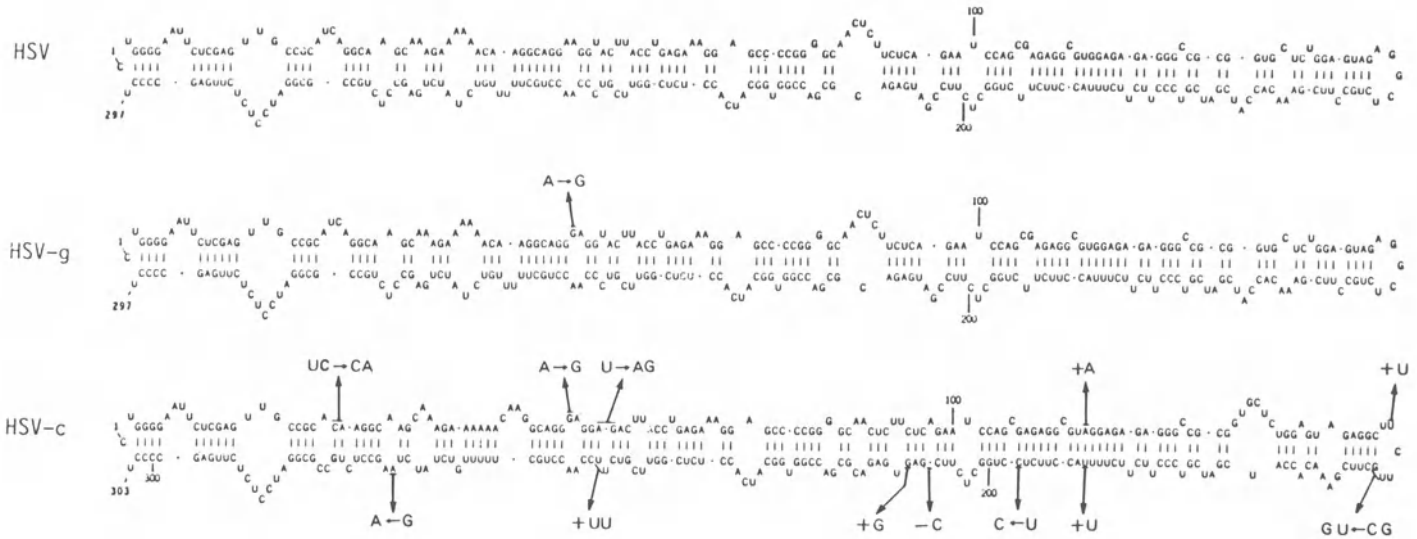

FIGURE 3. Nucleotide sequences and predicted secondary structures of HSV, HSV-g, and HSV-c. Nucleotides differing from those of HSV are indicated by arrows.

gels. The circular form of HSV apparently migrates faster than that of CPFV, suggesting a difference of five nucleotides (Sano *et al.*, 1984a).

Double-stranded cDNA of CPFV has been cloned and the complete nucleotide sequence established. The covalently closed circular molecules of ssCPFV consist of 303 nucleotides (Fig. 3). The nucleotide sequence of CPFV differs from HSV at 16 positions, which includes 8 exchanges, 7 insertions, and 1 deletion. Because both viroids exhibit about 95% sequence homology, and similar pathogenic properties, it is concluded that CPFV is a cucumber isolate of HSV (HSV-c) (Sano *et al.*, 1984b).

An infectious low-molecular-weight RNA was detected in grapevines by molecular hybridization with HSV-c cDNA (Shikata *et al.*, 1984). The RNA produced symptoms on cucumber plants indistinguishable from those infected with HSV. At present, it is not known if this agent is responsible for any specific disease symptoms of grapevines. However, such viroidlike RNA was detected in almost all grapevines collected in Japan, including stocks imported from the United States and Europe (Sano *et al.*, 1985a). As hops and grapevines share almost the same cultivated areas in Japan, such as Nagano, Yamanashi, and Tohoku districts, it will be interesting to see whether the grapevine isolate is the same or similar to HSV, or to HSV-c in its nucleotide sequence.

The complete nucleotide sequence of grapevine viroid has been established (Sano *et al.*, 1985b). The sequence of grapevine viroid consists of 297 nucleotides, and differs from that of HSV only in one nucleotide (99% sequence homology) and from that of HSV-c in being 6 nucleotides smaller and 15 nucleotides different (95% sequence homology) (Fig. 3). The result indicates that grapevine viroid is a grapevine isolate of HSV (HSV-c).

VIII. DIAGNOSIS AND CONTROL

In field-grown hops, diagnosis of the hop stunt disease can be achieved by observation of the growth conditions and other characteristic symptoms of leaves and bines. This is usually possible in June and July in Japan, but it is difficult even for experts to identify practically all infected plants showing mild symptoms.

Analysis of alpha- and beta-acid contents in cones represents a rapid and reliable diagnosis of the disease (Yamamoto *et al.*, 1970, 1973). Since Sasaki and Shikata (1977a) had established the cucumber bioassay, it was widely employed for the indexing of hop plants. Comparison of these three diagnostic methods for hop stunt disease indicates that the biological assay in cucumber is more sensitive than the other two methods (Sasaki and Shikata, 1980; Takahashi *et al.*, 1983). Symptoms on cucumber appear earlier when plants are held after inoculation at about 33°C

than when held at 21°C (Sasaki and Shikata, 1978a, 1980; Takahashi and Takusari, 1979b). HSV can be easily extracted in high-ionic-strength buffers, for example in 0.5 M K_2HPO_4–TGA (Sasaki and Shikata, 1977b, 1978b,d). HSV can be detected by the cucumber bioassay in a mixture of 1 HSV-infected leaf disk of 10-mm diameter and 200 HSV-free disks (Sasaki and Shikata, 1980). Practically, the viroid can be detected in a 1:50 leaf disk mixture. In the cucumber bioassay, inoculation at the first true leaf-stage was more consistent and the length of incubation period was shorter than inoculation at the cotyledon stage (Takahashi and Takusari, 1979a,b). However, a detailed investigation by Sasaki *et al.* (1982) indicated that the most favorable method is simultaneous inoculation of cotyledons and hypocotyl. The infectivity indexes obtained were 474 for both cotyledon–hypocotyl inoculation, 419 for hypocotyl only, 400 for cotyledon–hypocotyl–first leaf, 320 for cotyledon only, and 178 for first leaf only. In the cucumber bioassay, HSV could be detected from hop plants that appeared to be HSV-free by either symptom diagnosis or alpha-acid analysis (Sasaki and Shikata, 1980).

A simplified analysis by PAGE may also be used for the rapid indexing of hop plants for the presence of HSV (Takahashi *et al.*, 1983). The procedure can be completed in 9 hr and allows detection in as little as 0.25 g of leaf.

Molecular hybridization by cDNA of HSV successfully detected the viroidlike RNA in grapevines (Shikata *et al.*, 1984). These biochemical techniques may assume an important role in the future for the indexing of HSV.

The most successful method for eradication of HSV in Japan was to remove the diseased plants and replant healthy ones. This was achieved by overall indexing of field-grown hops by the cucumber bioassay and alpha-acid analysis. In this regard, careful treatment of rootstocks and deep roots with urea or chloropicrine after removing the hop plants is required (Sasaki, 1981).

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CHAPTER 15

Chrysanthemum Chlorotic Mottle

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I. INTRODUCTION

The chrysanthemum chlorotic mottle disease was first described by Dimock and Geissinger (1969), who noted that the disease was initially seen in 1967 in a number of commercial greenhouses. Dimock *et al.* (1971) concluded that the disease had a viral etiology since graft transmission was successful and since no pathogenic bacterium or fungus was found associated with the disease. The viroid nature of chrysanthemum chlorotic mottle was first reported by Romaine and Horst (1975). CCMV was determined to have physical characteristics similar to other viroids but differed by having a very narrow host range and by being less contagious than other viroids. The only hosts reported to be susceptible to CCMV are cultivars of *Chrysanthemum morifolium, C. zawadskii* var. *latilobum*, and *C. zawadskii* var. *latilobum* cv. Clara Curtiss. Fifty-one species and cultivars were tested, including 9 chrysanthemum species and 35 genera, to determine whether CCMV replication was supported. CCMV was infectious to 2 species of chrysanthemum and noninfectious to 11 species of plants known to support RNA replication of other viroids. CCMV has been reported to occur in infected chrysanthemums in Denmark (Paludan, 1980), France (Monsion *et al.*, 1980), and India (Singh *et al.*, 1978).

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II. SYMPTOMATOLOGY

Affected plants exhibit a striking mottling on leaves with acute symptoms followed by complete chlorosis on leaves with chronic symptoms (Fig. 1). Additional symptoms include dwarfing of leaves, flowers, and the entire plant and delay in flower development (Dimock *et al.*, 1971; Horst *et al.*, 1977). Under natural conditions, complete foliar chlorosis symptoms associated with CCMV can be easily confused with nutrient problems; however, vegetative production stock plants may or may not show symptoms under natural conditions depending on the average temperature.

Symptoms are best expressed with minimal light intensities of 10,760 lx, photoperiods of 12 hr, and temperatures of 24 to 27°C (Horst and Kryczynski, 1971; Horst, 1975). No symptoms develop at constant temperatures of less than 21°C and light intensities less than 5380 lx. Chrysanthemum cultivars Bonnie Jean and Deep Ridge exhibit symptoms within 14 days under optimal environmental conditions and may be used for bioassays.

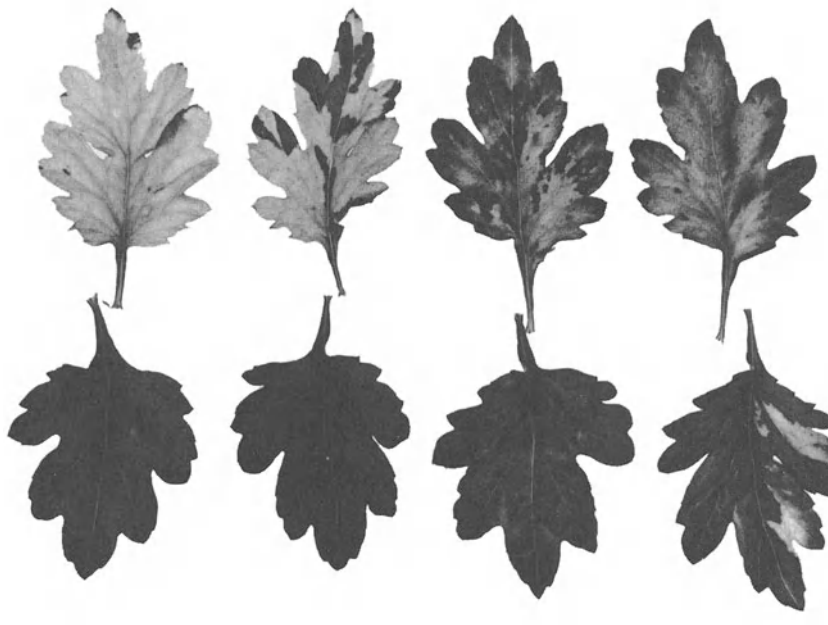

FIGURE 1. Progression of symptoms in chrysanthemum following inoculation with CCMV. First new leaf to expand after inoculation is at lower left; subsequently expanding leaves are in counterclockwise order with first acute (mottle) symptoms and finally chronic symptoms (complete chlorosis, upper left); From Dimock *et al.* (1971).

III. ETIOLOGY

CCMV has a dilution end point of less than 10^{-3} and a thermal inactivation point of about 95°C. The infectivity of CCMV in crude leaf extracts is lost within 30 min at room temperature and within 2 to 3 hr at 4°C. Infectivity is retained for at least 1 year in freeze-dried leaf tissue.

No insect vector is known. CCMV is transmitted by juice inoculation, grafts, and tissue implantation. Spread of the pathogen under natural conditions occurs through leaves and stems as they are handled during cultural procedures, the pathogen being on the hands of workmen or on knives and tools used. Long-distance spread is primarily by shipment of infected cuttings or plants. The principal means of carryover of CCMV from season to season is in infected stock plants as with CSV.

In experiments where grafts are made between CCMV-infected plants and healthy test plants or where tissue implants are made from infected plants to healthy test plants, symptoms are detected within 10–15 days. Symptom expression in nonreactors is encouraged by removing the terminal growth after 21 days; symptoms develop 7 to 14 days later on the new growth.

IV. PHYSICAL CHARACTERISTICS

Romaine and Horst (1975) reported the viroid nature of CCMV and suggested that the narrow host range and the less contagious characteristics as compared with other viroids were due to instability of the agent in crude extracts. Other viroids have been separated from host nucleic acids by PAGE (Morris and Wright, 1975; Semancik *et al.*, 1975; Horst and Kawamoto, 1980). CCMV reportedly has been visualized through PAGE (Monsion *et al.*, 1980) and purified (Langowski *et al.*, 1978). Kawamoto *et al.* (1985) were unable to confirm these results, either with cultures from Europe or with their own cultures of CCMV.

Kawamoto *et al.* (1985) showed CCMV to differ from other viroids by being more infectious in the 2 M LiCl-insoluble RNA fraction than in the soluble fraction (Table I). The RNAs of CSV, PSTV, CEV, and CLV are primarily soluble in 2 M LiCl. This phenomenon raises interesting questions about the structure of CCMV. Double-stranded RNA, hairpin-shaped RNA, and circular RNA with a high degree of base pairing are soluble in 2 M LiCl. Linear ssRNA with little base pairing is insoluble in 2 M LiCl, as is dsRNA with enough single-stranded component (Barlow *et al.*, 1963; Baltimore and Girard, 1966; Sänger *et al.*, 1979).

The insolubility of CCMV in 2 M LiCl is not unique to CCMV. Utermohlen and Semancik (1982) reported a strain of CEV that was more insoluble than soluble in LiCl.

Horst (1975) isolated a strain of CCMV that expresses no symptoms

TABLE I. Relative Infectivity (RI) of the 2 M LiCl-Soluble and -Insoluble Fractions from Plants Infected with Different Viroids

Viroid	RI[a]		Ratio
	2 M LiCl supernatant	2 M LiCl pellet	
CSV	717	22	33 : 1
PSTV	18,117	1002	18 : 1
CEV	704	138	5 : 1
CLV	462	13	36 : 1
CCMV-s	833	1877	1 : 2
CCMV-1[b]	47	81	1 : 2

[a] RI = Σ[No. of infected plants × No. of days infected × dilution].
[b] CCMV-1 RI based on number of symptomless plants after challenge by CCMV-s times dilution.

in chrysanthemum even under optimal environmental conditions. The latent strain of CCMV (CCMV-1) is detected by its protection against infection by CCMV in double inoculations. CCMV-1, like CCMV, is insoluble in 2 M LiCl (Kawamoto *et al.*, 1985).

CCMV and CCMV-1 migrate at the same rate as 7 S RNA in 5% polyacrylamide gels (Kawamoto *et al.*, 1985). No RNA band unique to CCMV-infected tissue was found in 5, 10, or 15% nondenaturing gels. Attempts to recover infectious CCMV RNA from gels under fully denaturing conditions were unsuccessful.

V. CONTROL

The disease caused by CCMV poses a potentially serious threat to the chrysanthemum industry. Control of CCMV chiefly consists of using indexing procedures for obtaining a nucleus of disease-free propagating stock (Horst *et al.*, 1977). Vigorous control measures that include indexing are carried out by specialist propagators and losses from this disease have been generally avoided.

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CHAPTER 16

Columnea Latent

T. O. DIENER

The geographical distribution of this viroid is unknown. The viroid was discovered by transfer of nucleic acid preparations made from normal-appearing *Columnea erytrophae* leaves obtained from a commercial garden shop in Beltsville, Maryland, to Rutgers tomato plants, in which the viroid causes symptoms similar to those produced by PSTV (Owens *et al.*, 1978). *C. erytrophae* (lipstick vine), which is frequently used as an ornamental house plant, is an epiphyte from Central America. No viroids could be isolated, however, from about 120 *Columnea* specimens collected in Costa Rica (T. O. Diener and R. Gamez, unpublished observations).

In Saco potato, the viroid causes symptoms similar to those of the type strain of PSTV, but hybridization of the viroid with PSTV cDNA revealed that its primary structure is distinct from that of PSTV (Owens *et al.*, 1978).

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CHAPTER 17

Avocado Sunblotch

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I. INTRODUCTION

The sunblotch disease of avocado has been known for over 50 years. It was first described as a physiological (Coit, 1928) or genetic (Horne, 1929) disorder. Shortly thereafter, Horne and Parker (1931) transmitted the causal agent from diseased scions to healthy rootstocks. By 1941, graft transmissibility of the agent was well established (Horne *et al.*, 1941). Hence, the causal agent was considered to be a virus, and like some of the other plant viroids was studied as such for many years (Whitsell, 1952; Wallace, 1958; Wallace and Drake, 1962; Desjardins *et al.*, 1980).

By 1974 three lines of evidence in our laboratory suggested that the causal agent might be a viroid rather than a virus. These were: the thermophilic nature of the agent; our failure to find virions in either ultrathin sections from infected tissue or in extracts from such tissues; and the failure of light and electron microscopy to demonstrate the presence of any microorganisms or virus particles in association with the disease. By 1979–1980 evidence from several laboratories had demonstrated that the causal agent is indeed a viroid (da Graca, 1979; Dale and Allen, 1979; Thomas and Mohamed, 1979; Desjardins *et al.*, 1980) and by 1981 that highly purified ASBV eluted from polyacrylamide gels can cause the disease in avocado seedlings (Allen *et al.*, 1981; Utermohlen *et al.*, 1981).

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II. GEOGRAPHIC DISTRIBUTION AND ECONOMIC IMPORTANCE

Avocado sunblotch disease has been reported in Australia, Israel, Latin America, South Africa, and the United States (Whitsell, 1952; Zentmyer, 1959; Dale *et al.*, 1982).

Few studies have been made to assess the economic importance of the disease. Infected trees, however, are known to be less productive than healthy ones (Wallace, 1958). da Graca *et al.* (1983) found a 27.3% reduction in fruit yield from viroid-infected Fuerte variety trees. More than 50% of such fruit were undergrade while none of the fruit from healthy trees were undergrade. da Graca (1985) reported that the reduction in yield over a 3-year period for this variety was 14%—with 50% of the fruit undergrade. He stated that the reduction in yield from symptomless carrier trees of the Edranol variety was as high as 82%. With trees of certain varieties (e.g., Caliente and Reed) in our symptomless carrier plot in Riverside, we have found even more dramatic yield reductions of up to 95% and higher (Desjardins and Sasaki, unpublished observations).

In another study, da Graca and van Vuuren (1977) found that fruit produced on symptomless carrier branches of the Edranol cultivar had a higher oil content than fruit from healthy trees of the same variety, but this higher oil content may shorten their storage life and thus cannot be considered a good feature. Undoubtedly, a comprehensive study of the economic losses caused by ASBV is needed.

III. HOST RANGE

ASBV is found naturally only in avocado (*Persea americana* Mill.). da Graca and van Vuuren (1980, 1981a) have experimentally transmitted ASBV by grafting bark patches into other species in the family Lauraceae (of which avocado is a member). They successfully transmitted the viroid to *Persea schiedeana* Nees, *Cinnamomum zeylanicum* Blume, *C. camphora* (Li) Nees and Eberm, and *Ocotea bullata* (Burch) Benth. They were unable to transmit the viroid to *C. liebertiana* and *P. indica* Spreng.

In our laboratory we attempted graft transmission of the viroid to *P. indica,* and mechanical transmission to tomato (*Lycopersicon esculentum* Mill.) and *Gynura aurantiaca* (Bl.) DC, but were unable to infect any of these species (Desjardins and Drake, unpublished observations).

IV. SYMPTOMATOLOGY

A. Symptomatic Trees

Probably the most consistent symptoms exhibited by infected avocado are yellow, white, or pink streaks on stems (Fig. 1A). Occasionally,

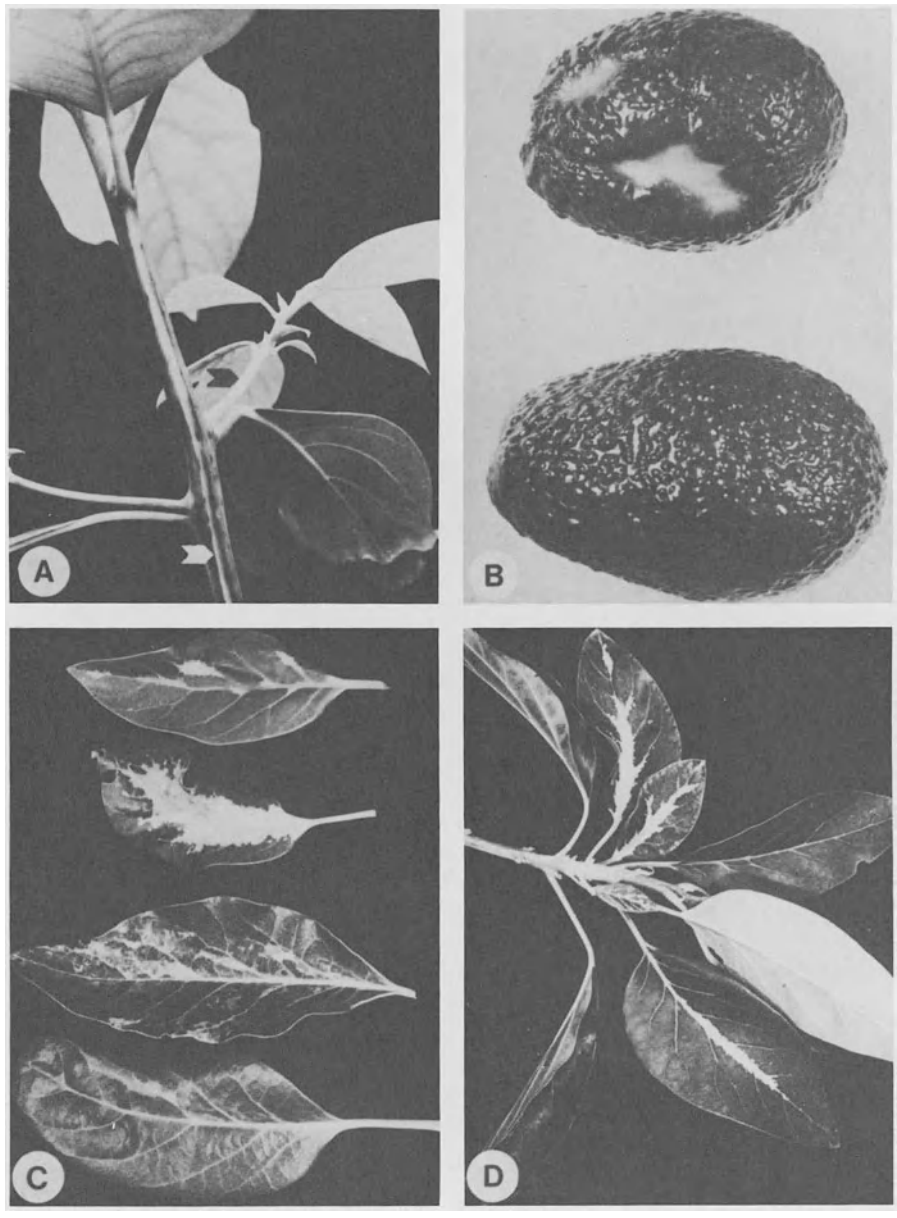

FIGURE 1. (A) Characteristic yellow stem streak (white arrow) on avocado shoot. Also small completely chlorotic side shoot (black arrow) with chlorotic leaves. (B) Upper: viroid-infected Hass variety fruit. Lower: healthy fruit of the same variety. (C) Four leaves showing variation of chlorotic splotches associated with vascular tissue. (D) Arrangement of symptomatic leaves originating from a single growth flush.

small side shoots or growth flushes that are completely chlorotic, including any leaves, can be found (Fig. 1A). The stem streaks are sometimes depressed and this is also thought by many to be characteristic (Whitsell, 1952). Fruit symptoms, when found, consist of depressed craters on the fruit surface, which may be yellow, greenish yellow, or deep pink (Fig. 1B). Leaf symptoms seem to appear somewhat sporadically and vary somewhat in character. The most striking symptom is a very chlorotic area of the leaf generally associated with the midrib or other vascular tissue. Occasionally, an infected leaf may exhibit a variegated pattern not unlike that of genetic leaf variegations sometimes found on viroid-free tree leaves (Fig. 1C). Commonly, leaves exhibit varying degrees of distortion. Frequently, leaf symptoms are absent for several growth flushes and then appear suddenly in a cluster as shown in Fig. 1D.

Symptomatic field trees exhibit a sprawling, somewhat stunted type of growth, which enables one to distinguish readily an infected tree from healthy trees in the field. Recovered, symptomless carrier trees do not have this appearance (see next section).

A bark symptom characterized as a browning and cracking of the surface has been described for ASBV-infected trees (Whitsell, 1952), but this is not recommended as a diagnostic symptom because it varies from cultivar to cultivar. Also, other agents can sometimes produce somewhat similar symptoms.

B. Symptomless Carrier Trees

Viroid-infected trees sometimes appear to recover with the disappearance of the typical symptoms described above. These trees are still infected but appear normal in all respects except that some varieties have a greatly reduced fruit yield, which must be considered as a symptom. A normal-appearing tree that produces very few fruit compared to healthy trees of the same variety and age should be suspected of being a symptomless carrier and be tested for the presence of the viroid. Occasionally, a recovered symptomless carrier tree will yield abundant fruit.

C. Effect of Light and Temperature

da Graca and van Vuuren (1981b) reported that the rate of symptom development can be accelerated by keeping Collinson variety indicator seedlings at high temperature (30/28°C, day/night). They indicated that cutting back the inoculated seedlings and the inoculum source also contributed to this acceleration. However, in tests run in a heat chamber under similar temperature conditions, we did not obtain a significant acceleration of symptoms with several inoculated avocado varieties un-

less we subjected our test seedlings to both elevated temperatures and continuous light (Desjardins, unpublished findings). These growth conditions are similar to those used by Yang and Hooker (1977) to enhance symptom development in PSTV-infected Rutgers tomato and resulted in greatly reduced incubation periods of ASBV in two cultivars.

V. STRAINS

It is likely that several strains of ASBV exist. The inherent requirement of cross-pollination for embryo development and fruit set in avocado cultivars results in considerable genetic variation among seedlings derived from a single seed source. This seedling variation makes it somewhat difficult to differentiate strains on the basis of pathogenicity or even on the basis of viroid yield.

A herbaceous host for ASBV would undoubtedly facilitate differentiation of viroid strains. Palukaitis *et al.* (1981) showed that two ASBV isolates had minor but significant differences in RNase digest fingerprint patterns and hence in nucleotide sequences.

VI. RECOVERY PHENOMENON

A. Development of Symptomless Condition

Several examples of partial recovery of a host plant from an acute phase of a viral disease are known with varying degrees of cross-protection against reinfection and recurrence of severe symptoms. Normally, however, the host plant remains in a mild chronic stage of the disease, often with specific, though mild, symptoms of the disease. Symptomatic, ASBV-infected trees occasionally recover by sending up a shoot that is normal. Except for the greatly reduced fruit production in many varieties, this recovery is so complete that the tree appears completely normal. Recovery appears to be complete and permanent (Wallace, 1967; Wallace and Drake, 1962). Because the recovered trees still carry the viroid, such trees are called symptomless carrier trees. Wallace and Drake (1962) demonstrated that a symptomless carrier tree may arise as the result of the recovery of a symptomatic tree or because a seedling is derived from a symptomless carrier tree that earlier went through the recovery stage. Occasionally, symptomatic, infected seedlings will recover while quite young and still in greenhouse containers by sending up a shoot that is normal in appearance and that becomes dominant (Desjardins and Drake, unpublished observations). The first examples of recovered, symptomless carrier trees were field trees initially thought to be healthy (Wallace, 1967; Wallace and Drake, 1962).

B. Consequences of the Symptomless Carrier Condition

Upon recovery, dramatic changes in the interaction of the viroid with the avocado host occur: (1) symptoms cannot be induced in symptomless carrier trees; (2) there is a dramatic increase in the rate of seed transmission of the viroid (from less than 5% to 90–100%); (3) progeny seedlings from symptomless carrier trees are themselves symptomless carriers and cannot be induced to exhibit symptoms; (4) there is a high rate of seed transmission by the symptomless carrier progeny seedlings; (5) symptomless carrier trees can serve as a reservoir for mechanical and pollen transmission of the viroid; and (6) there is a great reduction in fruit yield with many varieties. The dramatic change in the rate of seed transmission upon development of the symptomless carrier condition, and the fact that progeny seedlings themselves are symptomless carrier trees are unique among recovery phenomena.

VII. TRANSMISSION

A. Graft Transmission

ASBV can be graft transmitted by buds, scions, bark patches, or leaf patches (Whitsell, 1952; Wallace, 1958). Transmission by natural root grafts can occur (Whitsell, 1952).

The incubation period for the disease following graft transmission is from 6 months to 2 years depending on the source of the tissue used for grafting. Drake and Wallace (1974) described an embryo graftage technique that reduces the incubation period to as little as 45 days in some cases.

B. Mechanical Transmission

ASBV can be mechanically transmitted by the razor-slash technique described by Semancik and Weathers (1968), but apparently not by the standard rubbing technique usually employed in plant virus transmission. The razor-slash technique has permitted transmission of the viroid directly from avocado to avocado and also from extracts from infected tissues, as well as of highly purified ASBV eluted from gels (Desjardins *et al.*, 1980; Utermohlen *et al.*, 1981; Desjardins and Drake, 1983). Direct transmission from avocado to avocado results in an incubation period of 10–22 months whereas with concentrated extracts or purified viroid the incubation period may be as short as 4 months (Desjardins *et al.*, 1980; Utermohlen *et al.*, 1981; Desjardins and Drake, 1983).

C. Seed Transmission

Although Whitsell (1952) had suggested that the sunblotch agent was seed transmitted, experimental demonstration was first presented by Wallace and Drake (1953). Wallace and Drake (1962) elucidated and described a dramatic increase in the rate of seed transmission when infected trees recover and become symptomless carriers.

D. Pollen Transmission

Pollen transmission of the viroid was first suspected when an occasional seedling was found to be infected, while the seed-mother tree from which it came was known to be viroid-free (Desjardins *et al.*, 1979; Desjardins and Drake, 1983). A 5-year study using bee pollination provided evidence that the viroid is pollen transmitted in field trees at a rate of 1 to 4% and that both symptomatic and symptomless carrier trees can serve as pollen donors (Desjardins *et al.*, 1984). In addition, the study demonstrated that the pollen-recipient tree does not become infected during the course of pollen transmission. Most of the infected progeny seedlings exhibited symptoms on germination.

VIII. MIXED INFECTIONS WITH VIRUSES

A. Viruses Involved

For many years the causal agent of avocado sunblotch was thought to be the only virus infecting avocado. In 1978, Alper *et al.* described a strain of tobacco mosaic virus (TMV) infecting avocado that differs slightly from common TMV.

By dsRNA analysis, Jordan *et al.* (1983a) have demonstrated the presence of three latent viruses in individual plants of several cultivars of avocado. These workers designated the viruses as 1, 2, and 3, and suggested that virus 2 either alone or in combination with virus 3 may be involved in the black streak disease of avocado. They found the viruses widely distributed in field trees of several avocado cultivars.

B. Problems for ASBV Research

Because of the more or less latent nature of these viruses and because the so-called avocado viruses 1, 2, and 3 are so widespread, mixed infections with ASBV are likely to occur frequently. This in turn makes more difficult certain types of studies on the viroid and viroid-infected tissues.

The problem is further complicated by the fact that it has been found recently that these viruses are not only seed transmitted from the female parent, but they are frequently pollen transmitted as well (H. D. Ohr, personal communication). Because of this pollen transmission, one must test seedlings from virus-free seed sources to ensure that they are virus-free before using them in many types of viroid studies.

IX. ISOLATION AND CHARACTERIZATION

A. Methods of Isolation and Purification

ASBV isolation and purification protocols do not differ significantly from those described for other viroids and can be found in several publications (Semancik and Weathers, 1972; Semancik *et al.*, 1973; Diener, 1979; Palukaitis *et al.*, 1979; Semancik and Desjardins, 1980; Mohamed and Thomas, 1980). Only a brief description of various procedures will be given.

1. Viroid Extraction

One can utilize high-salt buffer containing sodium dodecyl sulfate (SDS) and various antioxidants as an extraction medium (Palukaitis *et al.*, 1979; Palukaitis and Symons, 1980) or media containing phenol, SDS, and bentonite (Semancik and Weathers, 1972; Semancik and Desjardins, 1980). Chloroform and butanol has also been used to help release infectious viroid (Desjardins *et al.*, 1980).

2. General Fractionation Methods

Viroid RNA, once extracted, can be fractionated by ethanol precipitation at reduced temperatures, by 2 M LiCl partitioning, and by PAGE (Palukaitis *et al.*, 1979; Semancik and Desjardins, 1980; Mohamed and Thomas, 1980). Certain avocado tissues, especially fruit tissue, contain rather high concentrations of polysaccharide. The latter can be removed by processing the extract with methoxyethanol (Semancik and Desjardins, 1980).

3. Further Purification

The most common method for the final preparation of highly purified viroid RNA is by recycling of gel electrophoresis fractions on new gels with subsequent elution from the gel (Palukaitis *et al.*, 1979; Semancik and Desjardins, 1980). Another method that has proved to be useful in combination with gel electrophoresis is chromatographic fractionation on CF-11 cellulose columns (Semancik, 1986).

B. Multiple RNA Species

Thomas and Mohamed (1979) and Dale and Allen (1979) first demonstrated the presence of a low-molecular-weight RNA band in gels of extracts from sunblotch-diseased avocado which was absent from similar extracts from healthy avocado. Palukaitis *et al.* (1979) also found a single band in their initial studies and demonstrated that there are both circular and linear forms of the RNA. Infectivity of the RNA was not demonstrated in these early reports.

Semancik and Desjardins (1980) first described the presence of five different RNA species in PAGE-fractionated preparations from infected avocado. They utilized ethidium bromide for RNA staining, which is more sensitive for RNA detection than is the toluidine blue used by the earlier workers. This may have precluded them from finding the higher-molecular-weight RNA species. The presence of five disease-associated RNA species was later confirmed by da Graca (1981). In symptomatic chlorotic tissue, monomeric ASBV reaches a concentration almost equal to that of 5 S RNA. In extracts of symptomless leaves of otherwise symptomatic plants, no ASBV can generally be detected by PAGE, whereas in extracts from ASBV-infected, symptomless carrier trees (Topa Topa variety), only monomeric ASBV could be detected (Semancik and Desjardins, 1980). In the latter case the concentration of the monomeric ASBV is much lower than that found in symptomatic, chlorotic tissue.

Bruening *et al.* (1982) found by Northern blot assay an oligomeric series of ASBV-specific RNAs in avocado extracts which are integral multiples of unit-length ASBV and have the same (+) polarity. They also reported the presence of complementary (−) RNAs but in much lower concentrations. The lowest-molecular-weight ASBV-specific RNA molecule is infectious (Utermohlen *et al.*, 1981; Allen *et al.*, 1981). Palukaitis *et al.* (1979) have presented electron micrographs of circular and linear ASBV.

C. Nucleotide Sequence

Symons (1981) has determined the primary and most likely secondary structures of ASBV (see Chapter 2, this volume). ASBV consists of 247 nucleotides.

X. ULTRASTRUCTURAL CYTOLOGY

In his first report on ultrastructural studies of ASBV-infected avocado leaves, da Graca (1979) described the presence of plasmalemmasomes and gross changes in chloroplast structure. Later, da Graca and Martin (1981) reported paramural bodies and grossly disorganized chloroplasts in the yellow areas of the infected leaf, whereas the chloroplasts in the green areas of the leaf had a normal appearance.

Desjardins and Drake (1981) also found plasmalemmasomes (Fig. 2A), paramural bodies, and disorganized chloroplasts in symptomatic areas of young leaves, but in addition, they found bodies composed of large whorls of membrane similar to those found by Hari (1980) in PSTV-infected tomato leaf tissue. In about 5% of the cells in the symptomatic areas of young leaves, Desjardins and Drake (1981) found vesicles present in the nuclei (Fig. 2A). It has not been established whether these are unequivocally related to viroid infection or directly related to one of the latent avocado viruses. In cells from symptomatic areas of the leaf that had been infected for considerable time, these workers found such gross changes in cellular structure that recognition of some organelles became difficult. More recently, in studies of leaves that were completely chlorotic upon emergence, Desjardins *et al.* (unpublished observations) found gross alterations of structure in some chloroplasts while others had an appearance similar to proplastids (Fig. 2B). In all instances, the chloroplast membrane remained intact. This differs from the findings of Kojima *et al.* (1983) who, in studies of various viroid-infected hosts, reported breakdown of the chloroplast envelope and release of the inner contents into the cytoplasm.

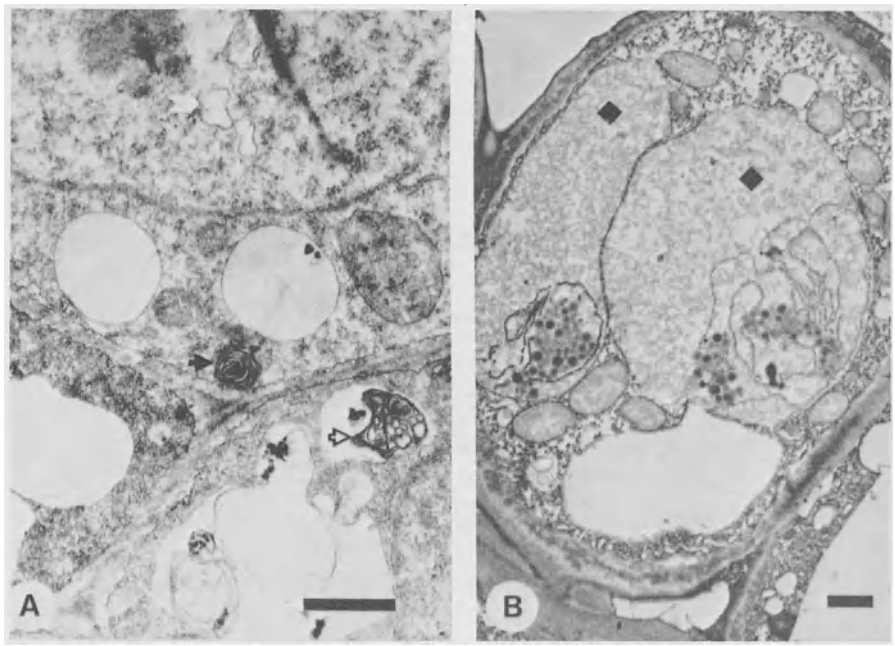

FIGURE 2. (A) Section of a cell in the symptomatic area of an infected leaf but near the boundary between symptomatic and asymptomatic tissues. Solid black arrow: plasmalemmasome in contact with the plasmalemma; open black arrow: plasmalemmasome partitioned into a cytoplasmic vesicle; solid white arrow: intranuclear vesicle. (B) Cell in a leaf that emerged in a totally chlorotic state. Black squares designate two chloroplasts with abnormal structure; stacks of grana are absent. Bars = 1 μm.

XI. DETECTION AND CONTROL

A. Diagnosis

1. Biological Indexing

For many years, the only method of establishing that either seed (rootstock) or scion sources of avocado were sunblotch disease-free was the use of biological indexing. This is basically a method of tissue implantation using a seedling as rootstock or test seedling and implanting a scion, bud, or leaf or bark patch. The method is still generally used and has resulted in the establishment of quite successful indexing programs for the production of viroid-free propagation materials (Wallace and Drake, 1971). The disadvantage of the method is the long incubation period required before one can evaluate the results (Wallace, 1958). This might be overcome somewhat by using special techniques such as embryo grafting (Drake and Wallace, 1974), or high-temperature regimes (da Graca and van Vuuren, 1981b). It is even conceivable that one might utilize mechanical inoculations of concentrated tissue extracts from the sources under test to decrease the incubation time (Desjardins and Drake, 1983; Desjardins *et al.*, 1980). If one were to do this, however, it would be prudent to inoculate several test seedlings because of the characteristic genetic variation among seedlings.

The biological indexing method of detection is still mainly used in many control programs (Spiegel *et al.*, 1984; da Graca and Trench, 1985).

2. Biophysical and Biochemical Techniques

Biophysical and biochemical techniques of detection fall into three general categories: (1) standard PAGE (Utermohlen and Ohr, 1981; da Graca, 1981; da Graca and Goodman, 1982; Allen and Dale, 1981; Jordan *et al.*, 1983b); (2) hybridization analysis with a ^{32}P-labeled cDNA probe (Palukaitis *et al.*, 1981; Allen and Dale, 1981); (3) dot-spot self-hybridization with ^{32}P-labeled ASBV (Rosner *et al.*, 1983).

Although standard PAGE has been widely advocated, it apparently does not in all instances provide 100% accuracy of indexing (Spiegel *et al.*, 1984). A number of factors may be involved; e.g., variation in the application of a specific protocol by different laboratories, variation of viroid concentration in different tissues at different times of the year and under different growing conditions. da Graca and Goodman (1982) indicated that flower bud tissue was the ideal tissue source for testing by gel electrophoresis. Later, however, da Graca (1984) and da Graca and Trench (1985) found variability in viroid concentration of flower tissues.

The cDNA probe is considered to be 1000-fold more sensitive than gel electrophoresis (Palukaitis *et al.*, 1981), yet it was found not to be 100% accurate when applied to field trees in Israel (Spiegel *et al.*, 1984).

The self-hybridization technique was found to be less sensitive than the cDNA probe (Spiegel *et al.*, 1984). As a consequence, the Israeli control program continues to utilize biological indexing along with biochemical methods. This is presently also the policy in the California program.

B. Viroid Inactivation on Tools

Because 5% commercial bleach (sodium hypochlorite) had been shown to inactive other viroids, Desjardins and colleagues (Desjardins and Drake, 1983; Desjardins *et al.*, 1980) recommended it for use in disinfecting tools utilized in avocado culture. The results of a recent 3-year study have now experimentally confirmed inactivation of ASBV by this chemical (Desjardins *et al.*, unpublished results). The study also showed that a 1:1 mixture of 2% sodium hydroxide and 2% formaldehyde or a 6% solution of hydrogen peroxide are equally effective in inactivating the viroid on cutting surfaces.

C. Use of Symptomless Trees

Wallace (1967) earlier suggested that the use of symptomless avocado trees might be a means of avoiding the sunblotch disease. It is now known, however, that with most varieties there is a greatly reduced fruit yield (da Graca, 1985; Desjardins and Sasaki, unpublished observations). Because symptomless carriers can act as a reservoir for the viroid, they certainly complicate the problem of controlling the disease.

XII. DISCUSSION

Visvader *et al.* (1982) have suggested that ASBV be placed in a group by itself on the basis of nucleotide sequence homologies and lack of most of the central conserved region present in other viroids (see Chapter 2, this volume).

Other differences between ASBV and other viroids include: the presence of multiple RNA species or oligomers in extracts from infected trees (Semancik and Desjardins, 1980; Bruening *et al.*, 1982) and the unique change in the viroid–host relationship upon recovery of the host (establishment of the symptomless carrier condition).

Because of the limited host range of the sunblotch viroid, studies of its interaction with its host are made more difficult. Two problems are involved. The first has to do with the genetic variation of the primary host, namely avocado. For viroid strain studies it would be ideal to have a herbaceous host for the viroid or at least one that is less variable genetically than the avocado.

The second problem has to do with the mixed infections of ASBV with the three latent viruses frequently found in avocado. Even though as Diener (1983) has pointed out that no synergisms or antagonisms in plants doubly infected with a viroid and a plant virus have been noted, the frequency of mixed infections of ASBV with these viruses does present difficulties for certain types of research. These would include certain biochemical and cytological studies at the ultrastructural level.

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CHAPTER 18

Tomato Planta Macho

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I. HISTORICAL

This disease was first observed by tomato growers in the state of Morelos, Mexico. It was initially reported as a viral disease by Belalcazar and Galindo in 1974. Viral etiology was suggested by the appearance of a prominent light-scattering band in a density gradient tube in which sap from infected plants was subjected to high-speed centrifugation. No comparable band appeared in a sister tube, in which sap from healthy plants was centrifuged. Infectivity was associated with the light-scattering band and injection of rabbits with material from the band resulted in the production of a disease-specific antiserum (Belalcazar and Galindo, 1974). The antigen was later identified as a host protein whose concentration increases about 200-fold in infected plants (Galindo *et al.*, 1984). Some evidence for a viroid etiology of the disease was obtained by Galindo and Rodriguez (1978), and was fully demonstrated finally by Galindo *et al.* (1982). Since the viroid appeared to be different from those already described, it was designated by the local name of the disease, "planta macho" ("male plant"); i.e., tomato planta macho viroid.

II. GEOGRAPHIC DISTRIBUTION

The tomato "planta macho" (PM) disease has been recorded only in Mexico, but similar tomato diseases have been described in the Ivory Coast as apical stunt (Walter, 1981, and Chapter 19, this volume) and in

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South Africa as bunchy top (McClean, 1931). PM and apical stunt are now confirmed to be different diseases on the basis of the nucleotide sequences of their respective viroids, as determined by Kiefer *et al.* (1983).

The identity of the tomato bunchy-top disease, however, will remain uncertain until the primary structure of its viroid is determined.

The PM disease has been observed in the Mexican states of Morelos, Mexico, Guanajuato, and Michoacan. In Morelos the disease distribution is sharply defined by the 22°C isothermal. Tomato fields in districts with an annual mean temperature above 22°C (800- to 1400-m altitude) have a disease incidence ranging from 5 to 45%, whereas tomato fields in districts with an annual temperature below 22°C (1400- to 2000-m altitude) are free of the disease (G. Orozco and J. Galindo, unpublished observations).

III. SYMPTOMATOLOGY

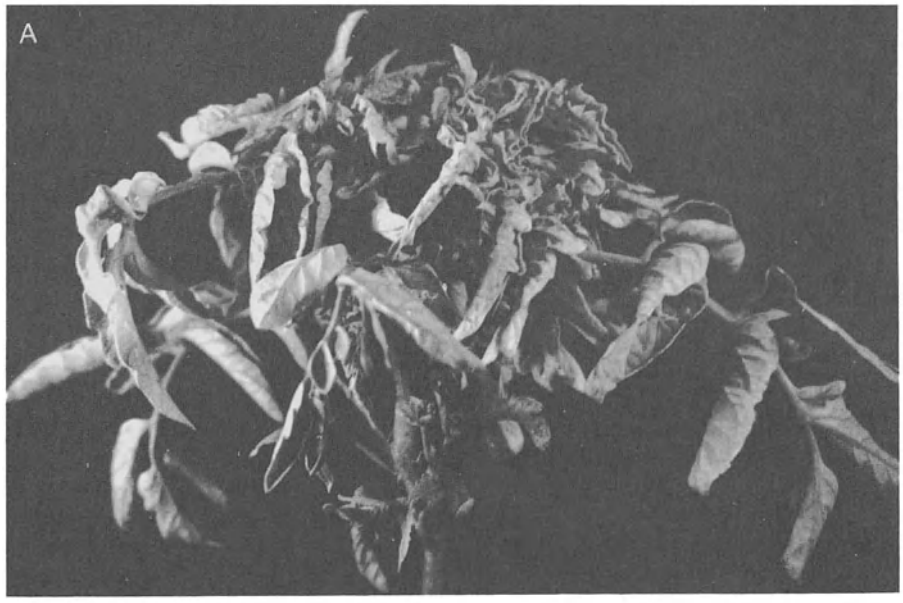
Tomato plants infected with TPMV show two consecutive and well-defined syndromes. The first syndrome starts 10 to 15 days after infection with the following symptoms: drastic arresting of growth, strong epinasty of leaves and leaflets (Fig. 1), crinkling and brittleness of leaflets, and veinal necrosis. After several weeks of the epinastic and static phase, the second syndrome begins. It is characterized by renewed slow growth of small, upright, yellow leaves; upward bending of leaflets along the rachis; assumption of a wavy leaflet margin; and very small fruits with few or no seed. The fact that infected plants do not produce marketable fruits has led to the name "planta macho," because in native Mexican cultures, fertility is attributed only to the female domain.

The main internal symptom observed with light microscopy is the collapse of phloem elements in those veins that have developed necrosis (Belalcazar and Galindo, 1974).

IV. EPIDEMIOLOGY

The primary source of inoculum appears not to be among the tomato populations, because negative evidence has been obtained for seed transmission of the viroid (Belalcazar and Galindo, 1974). Furthermore, the tomato seed for Morelos is produced in other regions where no PM disease is known to occur. Some of the seven solanaceous species found recently as natural hosts of TPMV (Orozco, 1983; Orozco and Galindo, 1986) could

FIGURE 1. (A) Symptoms of the first syndrome showing strong epinasty of leaves and leaflets and complete stoppage of growth. (B) Symptoms of the second syndrome characterized by a very slow renewal of growth of erected and yellowish thin leaves.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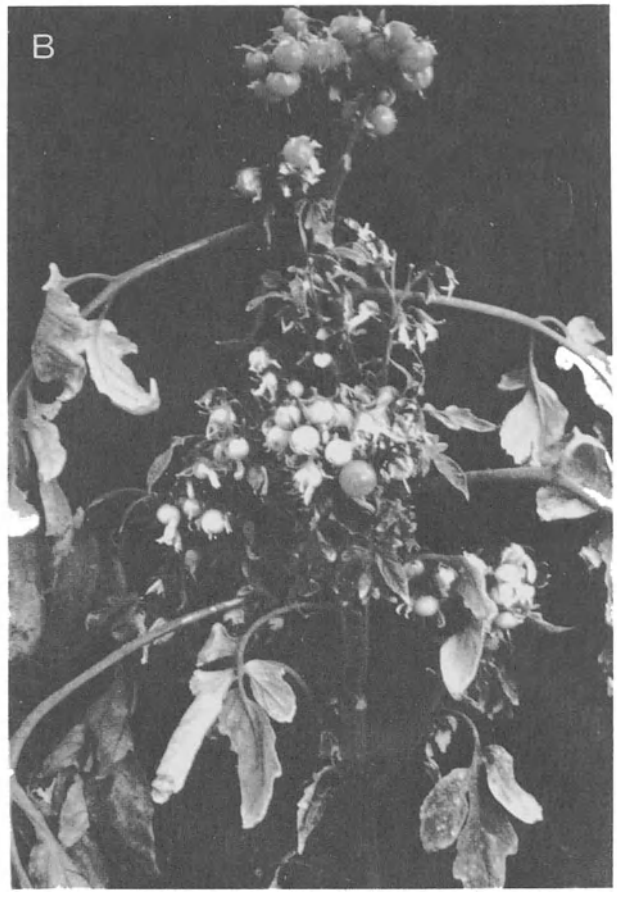
B

represent primary inoculum sources for the tomato plants, since seed transmission of TPMV has already been detected in some of the wild natural hosts (Galindo and Aguilar, unpublished observations). Transfer of the viroid from the wild natural hosts to tomato plants appears to be possible by a *Myzus persicae* biotype which is able to transmit the viroid (Galindo and Aguilar, 1985), and which colonizes and reproduces well in many of the TPMV natural host species (Galindo *et al.*, 1986).

V. ECONOMIC LOSSES

When tomato plants are infected before blooming, they fail to produce any marketable fruits. Consequently, the percentage of crop loss is approximately equal to the percentage of PM incidence found in the tomato field. The losses are negligible in the summer in Morelos, but they are important in autumn and winter, when a 16% average incidence occurs (Orozco, 1983).

The uneven PM incidence among the tomato fields has a special significance for farmers who cultivate tomato on small pieces of land, as is the case in Morelos, because the family economy is upset for those farmers whose complete crop is lost.

VI. DIAGNOSIS

The characteristic leaf and leaflet epinasty of PM is a good diagnostic symptom to distinguish this viroid disease from all known tomato virus diseases, even in those cases in which the virus causes distinct epinasty, as with tobacco etch virus and tomato bushy stunt virus, when the latter infects the tomato plant at the state of two to four leaves.

On the other hand, the epinasty symptom, or even the whole PM disease syndrome, has no diagnostic value in distinguishing TPMV infection from infections caused by other viroids in the tomato plant, such as TASV, TBTV, or the syndrome induced in the tomato plant by PSTV (Raymer and O'Brien, 1962). In these cases, comparison of the TPMV host range with those of the other viroids may be useful for a tentative diagnosis [host range studies helped to establish TPMV as a new viroid (Galindo *et al.*, 1982)]. However, for a more precise and definitive diagnosis, the primary molecular structure of the viroid has to be determined, either partially through molecular hybridization with appropriate probes or RNA fingerprinting or completely, by RNA sequencing.

VII. STRAINS

No information is available.

VIII. EXPERIMENTAL TRANSMISSION

Mechanical transmission is achieved by the usual method of rubbing the infectious sap onto carborundum-dusted foliage. Incubation of the inoculated plants in a growth chamber gives good results at a constant temperature of 28°C and a 14-hr photoperiod with 15,000-lx intensity. Under greenhouse conditions, the temperature should be around 28°C.

Myzus persicae aphids from a colony established from *Physalis aff. foetens* growing in the Morelos region where the highest PM incidence occurs, showed the capacity to transmit TPMV (Galindo and Aguilar, 1985). The aphids, after a 24-hr acquisition period, transmitted the viroid for 8 consecutive days, with daily transfers to tomato seedlings. When chile pepper, *S. nigrescens*, and *P. aff. foetens* were used as viroid sources for the aphid, 100% transmission was common in several experiments. On the other hand, the tomato plant was the less suitable viroid source, with only a 33% transmission average. Tomato seedlings developed earlier symptoms when inoculated by aphid transmission than by mechanical transmission (Galindo *et al.*, 1986).

IX. PURIFICATION

TPMV has been partially purified by using the methods of Morris and Wright (1975), Diener (1973), and Pfannenstiel *et al.* (1980).

X. PHYSICAL PROPERTIES

In 20% polyacrylamide gels, the electrophoretic mobility of TPMV is identical to that of PSTV (Galindo *et al.*, 1982). Thus, TPMV should have about the same molecular weight as PSTV, i.e., 1.27×10^5 (Sänger *et al.*, 1976).

XI. NUCLEOTIDE SEQUENCE

Kiefer *et al.* (1983) have determined the primary structure of TPMV. They found (1) that the viroid molecule has 360 nucleotides, in which the G-to-C, A-to-U, and purine-to-pyrimidine ratios are near one; (2) that there are almost twice as many G:C as A:U base pairs; (3) that 9% of the base pairs are G:U; and (4) that neither TPMV nor its putative complementary RNA contains AUG initiation codons. They also found that TPMV has an 83% sequence homology with PSTV; 75% with TASV; 74% with CEV; and 72% with CSV.

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CHAPTER 19

Tomato Apical Stunt

B. WALTER

I. HISTORICAL; GEOGRAPHIC DISTRIBUTION

The tomato apical stunt disease was first described in 1980 (Walter *et al.*); it has been found only occasionally in the northern part of the Ivory Coast, near Korhogo, where large market gardenings were put into place at that time.

The causal agent of the tomato apical stunt disease is the first characterized viroid on the African continent (Walter, 1981).

The symptoms of the disease are very similar to those of the tomato bunchy top disease in South Africa (McClean, 1931). Raymer and O'Brien (1962) noted the similarity of the symptoms induced in tomato by PSTV and those typical of the bunchy top disease of tomato. Based on other similarities—such as thermal inactivation points and longevity *in vitro*—PSTV and the bunchy top "virus" were considered closely related strains of the same "virus" (Benson *et al.*, 1965). Although there is no unequivocal proof that the causal agent of the bunchy top disease in South Africa is really a viroid (TBTV), this appears most likely.

In 1982, Declert and Thouvenel described symptoms resembling those of bunchy top on the tomato variety "Rianto," grown in cooled greenhouses near Arlit (Niger). They could transmit the syndrome to "Marmande" tomatoes by grafting and by mechanical inoculation. No virus particles were seen in juice from infected plants. The thermal inactivation point was over 90°C; no symptoms were visible after mechanical inoculation on *Nicotiana glutinosa, Physalis floridana,* and *Torenia fournieri,* but typical symptoms were observed on tomato after back inoculation from *T. fournieri* (Thouvenel, personal communication).

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Though probable, the presence of a viroid responsible for these symptoms in Niger remains to be demonstrated. A relationship with TASV is possible.

II. SYMPTOMATOLOGY, EPIDEMIOLOGY, AND STRAINS

The symptoms originally described on tomato infected by TASV are curling of the leaves and apical stunting. These symptoms are comparable to those caused by TPMV in Mexico (Galindo *et al.*, 1982).

After experimental inoculation of TASV, different tomato varieties ["Heinz 1350" (Fig. 1), "Marmande," "Claudia," "Moneymaker," "Rutgers," "Rentita," "Hilda"] show the following symptoms: 10 to 12 days after inoculation, leafs are crinkling and downwards rolling; necrotic spots appear and extend along the main and secondary leaf-veins. Later, the whole plant has a stunted and bushy appearance. The upper leaflets remain very small, are yellowish, and sometimes roll upwards like spoons.

There is no information about the epidemiology of TASV or the economic losses it induces, as the disease was identified only occasionally. No strains of TASV have been distinguished, but TASV, TBTV, and

FIGURE 1. Symptoms of TASV on *Lycopersicon esculentum* cv. Heinz 1350.

the pathogen inducing the tomato "bunchy top" in Niger may be strains of the same viroid.

III. EXPERIMENTAL TRANSMISSION

A. Mechanical Inoculation

TASV has been transmitted to several species, mainly of the family Solanaceae, by rubbing carborundum-dusted leaves with an extract obtained by grinding tomato leaflets in Hepes, phosphate, or Tris-HCl buffers. Presence or absence of the viroid in the test plants was determined by back inoculation to healthy tomato plants and observation of symptoms. The types of symptoms induced by TASV on the various host plants are described in Table I.

TABLE I. Suscepts of TASV and Symptoms Induced by Mechanical Inoculation

Chenopodiaceae	
Beta vulgaris (Detroit Dark Red)	NS[a]
Compositae	
Chrysanthemum carinatum	Stunting
Scrophulariaceae	
Nemesia floribunda	Mottling, crinkling
Penstemon hirsutus	Mottling, crinkling, top necrosis
Torenia fournieri	NS
Solanaceae	
Browallia demissa	Faint mottling
Datura inoxia	Faint mottling
D. metel	Faint mottling
Nicotiana alata	NS
N. benthamiana	Leaf narrowing, wilt
N. bigelovii	Mosaic
N. debneyi	NS
N. edwardsonii	Necrotic points
N. forgetiana	Mottling, stunting
N. glutinosa	Necrotic lesions
N. goodspeedii	NS
N. langsdorfii	NS
N. longiflora	Leaf crinkling
N. megalosiphon	Vein yellowing, leaf deformation
N. nudicaulis	Top deformation
N. plumbaginifolia	NS
N. quadrivalvis	NS
N. repanda	NS
N. rustica	NS
N. sylvestris	NS
N. tabacum (Maryland, Samsun, Samsun NN, Virginiana, Xanthi, Xanthi nc)	Mosaic, leaf deformation

(*continued*)

TABLE I. (*Continued*)

Petunia hybrida (Crown jewels, Nain conseiller, Nain mandarin)	Mosaic, deformation
Physalis alkekengi	NS
P. curassavica	Mosaic
P. floridana	Chlorotic lesions, vein yellowing, leaf curling
P. peruviana	Faint mottling
P. philadelphica	Mosaic
P. pubescens	Mosaic
Scopolia corniolica	Necrotic lesions
S. lurida	Necrotic lesions
S. tanguticus	Necrotic lesions
Solanum aviculare	Vein necrosis, leaf curling, stunting
S. berthaultii (Singh, 1984)	Necrotic spotting of petioles and stems, stunting
S carolinense	NS
S. dulcamara	Leaf deformation, stunt
S. indicum	Faint mottling
S. lycopersicum	Deformation
S. melongena (Burpee's Black Beauty)	NS
S. nigrum	NS
S. nigrum guinense	NS
S. quitoense	NS
S. sisymbrifolium	NS
S. topiro	Vein necrosis, leaf curling

[a] NS, no symptoms, presence of viroid ascertained by back inoculation into tomato.

TASV could not be transmitted to species from the families Aizoaceae, Amaranthaceae, Campanulaceae, Caryophyllaceae, Cruciferae, Cucurbitaceae, and Leguminosae, as well as to some Chenopodiaceae (*Chenopodium* spp.), Compositae (*Gynura crepidoides*), and Solanaceae (*Capsicum* spp., *Nicotiana clevelandii*).

There are some apparent discrepancies between the host ranges of TASV and TBTV (for review see Diener, 1979). For example, *Capsicum annuum* and *Datura stramonium* are hosts of TBTV, but not of TASV.

B. Aphid Transmission

Experiments were done to transmit TASV from tomato to tomato with *Aphis craccivora, A. spiraecola, Toxoptera citricidus, Acyrthosiphon pisum,* or *Myzus persicae.*

Aphis craccivora, after an acquisition feed of 2 hr and a transmission feed of 24 hr, transmitted TASV to 1 of 12 tomatoes in a first experiment, and 4 of 20 in a second (Walter, 1981). In comparable conditions, *Acyrthosiphon pisum* transmitted the viroid to 1 of 12 tomatoes (Walter,

unpublished results). *Myzus persicae,* with acquisition feed of 24 hr and transmission feed of 24 hr, transmitted TASV to 1 of 20 tomatoes (Walter, unpublished results).

There is no proof of a true aphid transmission. In our experiments, TASV could have been transmitted externally (e.g., by the legs of the insects).

IV. PURIFICATION

TASV was purified by using one of two methods (Walter, 1981): the first was derived from the polyethylene glycol method described by Randles (1975) for CCCV; the second is a rapid method similar to that of Morris and Smith (1977).

A. Polyethylene Glycol Method

Twenty grams of tomato leaves are crushed in a Waring Blender with 80 ml buffer (0.1 M Na_2HPO_4, 0.01 M sodium diethyldithiocarbamate, 0.1% 2-mercaptoethanol, pH 8.7). After filtration through cheesecloth and low-speed centrifugation, polyethylene glycol is added to the supernatant to a final concentration of 5%. Nucleic acids are extracted by three successive phenol treatments and are precipitated by adding 2 volumes of ethanol. The nucleic acids are fractionated by centrifugation on sucrose density gradients or electrophoresis on 2.5% polyacrylamide, 0.5% agarose gels.

B. Rapid Method

One gram of young tomato leaflets is crushed in a mortar in the presence of 0.5 ml buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, 1% sodium dodecyl sulfate, pH 9.5) and 2 ml of water-saturated phenol. After clarification with 2 ml of a chloroform–butanol mixture (1 : 1, v/v), lithium chloride is added to the aqueous phase to a final concentration of 2 M. After incubation for 2 hr at 4°C, low-speed centrifugation, and overnight dialysis against distilled water, nucleic acids are fractionated by 5% PAGE.

No disease-specific band was visible in the sucrose gradients, but fractions from the upper part of the gradients—when inoculated on tomato—induced typical symptoms of TASV.

In the gels, a band present only in extracts from TASV-infected plants was visible. Its measured R_f was 0.60 ± 0.02. Only the fraction of the gel containing this band was infective.

V. PHYSICAL–CHEMICAL PROPERTIES

Incubation of TASV preparations with ribonuclease (final concentration of 0.1 to 10 μg/ml) inactivates the viroid, whereas incubation with deoxyribonuclease has no effect on infectivity (Walter, 1981).

The infectivity dilution end point of 0.5 M phosphate buffer extracts (pH 7.6) of TASV-infected tomato leaves is between 10^{-3} and 10^{-4}; of 0.005 M phosphate buffer extracts (pH 7.1) it is only 1/5.

Infectivity of crude extracts from TASV-infected plants survived boiling for 10 min.

In vitro longevity of TASV in tomato sap at 24°C was between 24 and 36 hr in all buffers tested; at 4°C it was more than 38 days in 0.5 M phosphate buffer and 20 days in 0.005 M buffer. At −20°C, extracts were still infectious after 6 months (Walter, 1981).

VI. NUCLEOTIDE SEQUENCE

The nucleotide sequence of TASV has been determined (Kiefer *et al.*, 1983). TASV is composed of 360 nucleotides; its most probable secondary structure is similar to those of other viroids of the PSTV group, consisting of short base-paired regions, interrupted by small internal loops.

TASV displays 73, 77, and 78% homology with PSTV, CSV, and CEV, respectively. TASV and TPMV show 75% sequence homology. TASV contains the highly conserved central region found in many viroids.

TASV and its putative complementary RNA contain three AUG initiation codons and could encode polypeptides with approximate M_r of 800, 1900, and 5500 (Kiefer *et al.*, 1983).

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CHAPTER 20

Tomato Bunchy Top

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I. SYMPTOMATOLOGY

The tomato bunchy-top disease is known to occur naturally only in South Africa (McClean, 1931). Characteristic symptoms are severe stunting of the entire plant, in particular the production of small leaves, a necrosis of the leaves and stems, and various forms of leaflet distortion, such as curling and an abnormal unevenness of the surface (McClean, 1931). Chlorosis or mottling are not features of the disease.

The first indication of the disease is a sudden and almost total cessation of growth at the branch extremities, with the result that at these points the leaves become closely crowded, giving to the plant a bunched appearance, which is typical of the early stages of infection. Leaves fully developed at the time of infection do not undergo any change but remain normal and healthy in appearance. In the condensed region, however, there is a progressive decrease in leaf size, and closer crowding of the leaflets on the rachis. The leaflet margins become crowded toward the undersurface, the tips frequently are twisted downwards, and the surfaces show a puckered condition. Axillary buds, particularly those of the lower leaves, are forced into early activity (McClean, 1931).

The check on the upward growth of the axis is not permanent and is followed by a definite elongation of the internodes, with the production of a somewhat spindling type of growth. A plant at this stage shows a lower region that is apparently normal, a middle region with condensed axis, bunching of foliage, a progressive reduction in leaf size and various forms of leaf distortion, and an upper region in which the internodes have

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again lengthened, and characterized by a thin axis and small leaves, which show little or no distortion.

As the diseased plant matures, the older leaves, including apparently healthy ones and those in the bunched region on the main axis, die off and are shed. Thus, a plant in an advanced stage of the disease bears only the typical dwarfed leaves produced after infection.

II. HOST RANGE

In nature, the disease is known to occur only in tomato, but the viroid can be transmitted to a number of plant species in addition to tomato. It is not known whether any of these or other plant species harbor the viroid in nature (McClean, 1931) [see Diener (1979) for a list of susceptible species].

Evidently, there is much overlap among species susceptible to PSTV and TBTV. Some apparent discrepancies, however, exist. For example, *Zinnia elegans* has been reported as a susceptible species for TBTV, but as a resistant species for PSTV (O'Brien and Raymer, 1964).

III. STRAINS

No definitive evidence exists as to whether TBTV occurs in the form of different strains. Raymer and O'Brien (1962) noted the similarity of symptoms induced in tomato by PSTV and those typical of the bunchy top disease of tomato (McClean, 1931). Later, Raymer and O'Brien (see Benson *et al.*, 1965) reported that in comparative studies with the bunchy top "virus" from South Africa and PSTV from the United States the two "viruses" produced the same symptoms in both tomato and Saco potato and that both are very similar in their thermal inactivation "points" and longevity *in vitro*. On the basis of these similarities, the two agents were considered identical or closely related strains of the same "virus" (Benson *et al.*, 1965). Later results with PSTV and CEV (which also resemble one another closely and were thought by some to be identical) revealed, however, that despite close similarity of the biological properties of the two viroids (such as identical host range and type of symptoms produced in different hosts), they may be distinct entities chemically.

With the discovery of TASV in tropical Africa (Chapter 19, this volume) and of TPMV in Mexico (Chapter 18, this volume), the identity of the viroid causing tomato bunchy top in South Africa again has become of interest. Unfortunately, no source of infected material could be located in South Africa (von Wechmar, personal communication) for comparison. Thus, the identity of TBTV still is uncertain.

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CHAPTER 21

Burdock Stunt

TIEN PO AND CHEN WEI

I. HISTORICAL

After the discovery of PSTV (Diener, 1971), a survey of plant viroid diseases in China was initiated (Tien, 1973). In addition to well-described viroids such as PSTV, CEV, and CSV, other viroids have been discovered in China. One of these is BSV (Tien, 1985).

The burdocks, *Arctium tomentosum* and *A. lappa*, are grown as biennial or perennial medicinal plants in several areas of China. Many of these in Beijing have been affected with a stunt disease every year since the 1960s.

II. GEOGRAPHIC DISTRIBUTION

Burdock stunt disease (BSD) is confined to an area around Beijing (northern China). In different fields in Beijing, about 0–30% of burdock plants show stunting symptoms. These plants contain one of two viroid RNAs. However, burdocks from Wuhan (central China) and Kunming (southern China) have no stunt diseases. These plants also have no symptoms or viroid RNAs.

III. SYMPTOMATOLOGY

The most characteristic symptom of BSD is stunting of the plants (Fig. 1). Other symptoms, such as vein clearing and leaf mottling (Fig. 2), usually also develop. Seriously diseased plants do not elongate, flower,

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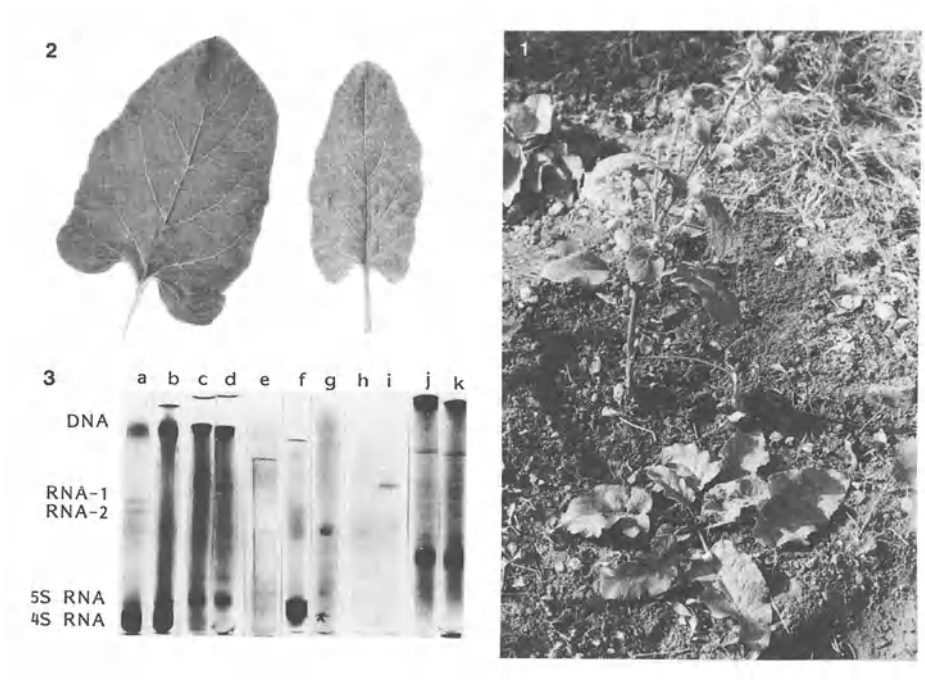


FIGURE 1. Burdock (*A. tomentosum*) stunt diseased plant (dwarfed plant in foreground) and healthy plant (background).
FIGURE 2. Leaf symptom of burdock (*A. tomentosum*) stunt disease (right) and healthy leaf (left).
FIGURE 3. Polyacrylamide tube gel electrophoresis of nucleic acids from (a–d, f–k) *A. tomentosum* and (e) *A. lappa*. (a) Diseased and (b) healthy *A. tomentosum* on 4% gels; (c) several diseased plants and (d) one diseased plant of *A. tomentosum* on 5% gels; (e) purified BSD RNA-2 from *A. lappa* on 4% gels; (f, g) purified BSD RNA-2 in TBE buffer and 8 M urea (f) electrophoresed at 40°C or (g) at 50 to 60°C; (h, i) BSD RNA-1 after incubation with RNase in (h) 0.01 × SSC and (i) 2.5 × SSC; (j) incubation without DNase I; (k) incubation with DNase I.

or bear fruit (Fig. 1). Thus they yield no seeds, which are the only parts of medicinal value. All of the symptoms are more pronounced the second year of growth. Unfortunately, there are no detailed records of the extent of damage and loss caused by BSD. There is no evidence for the existence of different strains of BSV.

IV. TRANSMISSION

Evidence accumulated thus far indicates that BSV cannot be transmitted through seeds. None of 100 plants grown from seeds from diseased plants showed stunting symptoms. Efforts to transmit BSV by mechanical inoculation or aphids also failed.

Healthy plants were inoculated either with crude extracts from infected leaves or with purified preparations of BSV RNA-1 and/or RNA-2, by rubbing and puncturing with needles. Some of these burdock seedlings

developed ambiguous symptoms of BSD. However, when nucleic acids were extracted form leaves developing symptoms, and subjected to electrophoresis on analytical gels, no BSV-specific bands could be detected. Similarly inoculated *Gynura aurantiaca* and Rutgers tomato plants developed no definite BSD symptoms.

In another set of experiments, the aphids *Myzus persicae* and *Macrosiphum gobonis* also failed to transmit BSV from diseased to healthy burdock plants.

V. VIROID ETIOLOGY

Two low-molecular-weight RNA species are found by nondenaturing PAGE in preparations from diseased leaf tissues of *A. tomentosum* or *A. lappa*, but not in preparations from healthy plants. The two bands, RNA-1 and RNA-2, have electrophoretic mobilities between those of high-molecular-weight host DNA and 5 S RNA (Fig. 3a,b). The ratio of RNA-1 to RNA-2 varies with different samples.

Both RNA-1 and RNA-2 are degraded by RNase A at low ionic strength (0.01 × SSC) (Fig. 3h), but they are not degraded by RNase A in 2.5 × SSC (Fig. 3i) or DNase I (Fig. 3j, k) (Chen *et al.*, 1983).

VI. PURIFICATION

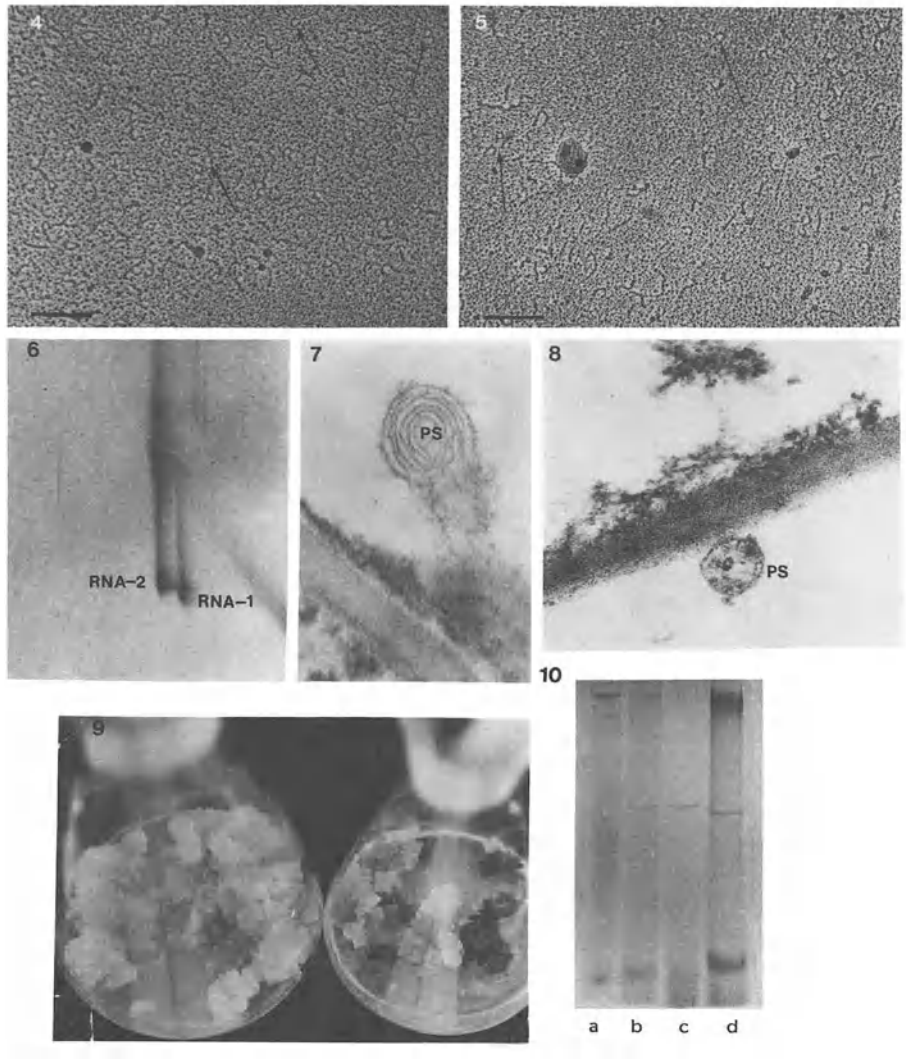
A crude nucleic acid extract form diseased leaves was applied to a Whatman cellulose CF-11 column, then eluted with an ethanol gradient. Nucleic acid in the fraction eluting in 14% ethanol was recovered by ethanol precipitation, resuspended, and subjected to preparative gel electrophoresis. The appropriate bands were cut out and the RNAs electroeluted from the gel slices. Further purification of RNA-1 and RNA-2 was achieved by absorption onto, and elution from, CF-11 or DEAE-32 cellulose columns. The eluted BSV RNAs were then precipitated with ethanol and vacuum-dried.

VII. SOME PHYSICAL PROPERTIES OF BSV

The molecular weights of BSV RNA-1 and RNA-2 were estimated by slab-gel electrophoresis and compared to molecular weight standards. RNA-1 and RNA-2 have approximate M_r values of 1.9×10^5 and 1.7×10^5, respectively (Chen *et al.*, 1983).

Denatured BSV RNA-1 and RNA-2 molecules were prepared for electron microscopy by the method of Randles and Hatta (1979). Both BSV RNA-1 (Fig. 4) and RNA-2 (Fig. 5) molecules are circular (Chen *et al.*, 1983).

Crude nucleic acid extracts containing BSV RNA-1 and RNA-2 molecules were analyzed by the two-dimensional electrophoresis technique



FIGURES 4 AND 5. Electron micrographs of BSV RNA-1 (Fig. 4) and BSV RNA-2 (Fig. 5), prepared as described by Randles and Hatta (1979). Bars = 500 nm.

FIGURE 6. Two-dimensional PAGE of crude extract containing BSV RNA-1 and RNA-2. In the first direction, electrophoresis was carried out under native conditions, in the second direction under denaturing conditions (Schumacher *et al.*, 1983). Two circular viroid bands are clearly visible below the diagonal front.

FIGURES 7 AND 8. Malformed plasmalemmasomes (PS) from diseased leaf.

FIGURE 9. Tissue culture of healthy (left) and diseased burdock containing BSV RNA-1 (right).

FIGURE 10. Polyacrylamide (5%) slab gel electrophoresis of nucleic acids from (a) healthy calli; (b) diseased calli developed from a leaf containing BSV RNA-1; (c) diseased leaves containing BSV RNA-1; (d) diseased leaves containing BSV RNA-2.

of Schumacher *et al.*, (1983). Two bands, not present in uninfected tissue, were well separated from cellular nucleic acids (and possibly polysaccharides and polyphenols) (Fig. 6). The extremely low electrophoretic mobility of these two bands under denaturing conditions indicates that the RNAs are circular. However, when the nucleic acids were separated in the second dimension at 50°C, BSV RNA-1 and RNA-2 molecules migrated faster than the diagonal front, indicating that they were not denatured. Under the same conditions, PSTV is completely denatured (Chen *et al.*, 1986). These results indicate that the secondary structures of BSV RNAs are more stable than that of PSTV (Chen *et al.*, 1986).

VIII. THE OCCURRENCE OF BSV RNA-1 AND RNA-2 IN SEPARATE PLANTS

To determine whether the viroid RNA type in individual plants changes over the course of infection, as with coconut cadang-cadang disease (Randles and Hatta, 1979), or if the viroid type remains constant, 11 plants were repeatedly analyzed by PAGE over 2–3 years. Individual diseased plants contained either RNA-1 or RNA-2, but not both (Table I). No

TABLE I. Occurrence of BSV RNA-1 and RNA-2 in Separate Plants[a]

		Detected in			
Plant number	BSV RNA	1980	1981	1982	1983
1	RNA-1	+	+	Died	
	RNA-2	−	−		
2	RNA-1	−	−	Died	
	RNA-2	+	+		
3	RNA-1	−	−	Died	
	RNA-2	+	+		
4	RNA-1		+	+	+
	RNA-2		−	−	−
5	RNA-1		+	+	Died
	RNA-2		−	−	
6	RNA-1		−	−	−
	RNA-2		+	+	+
7	RNA-1		−	−	Died
	RNA-2		+	+	
8	RNA-1		−	−	Died
	RNA-2		+	+	
9	RNA-1		+	+	Died
	RNA-2		−	−	
10	RNA-1	−	−	−	Died
	RNA-2	−	−	−	
11	RNA-1	−	−	−	Died
	RNA-2	−	−	−	

[a] From Chen *et al.* (1986).

change was observed in the electrophoretic mobility of the viroid in any plant, even over a period of 2 or 3 years (Table I). Diseased plants, which were derived from specific parental plants, contained the same BSV RNA species as did the original plant (Chen *et al.*, 1986).

IX. ULTRASTRUCTURE OF DISEASED LEAF TISSUES

A. tomentosum leaves from healthy and diseased plants (with either BSV RNA-1 or RNA-2) were fixed and processed for transmission electron microscopy. No viruslike particles were found in thin sections from diseased tissue. Also, no significant differences between the chloroplasts or nuclei of healthy and diseased cells were observed (Chen *et al.*, 1983). However, abnormal plasmalemmasomes were observed (Figs. 7 and 8). Some of the malformed plasmalemmasomes appeared similar to the multilayered membrane bodies described by Hari (1980).

X. PROPAGATION OF BSV RNA-1 IN TISSUE CULTURE

Tissue cultures were established from healthy and diseased burdock leaves (containing BSV RNA-1) (Chen *et al.*, 1986). Callus tissues developed from healthy leaves after 1 1/2 months, whereas after 2 months only a few of the diseased leaf pieces developed calli. The calli from diseased leaves were small dark brown and grew more slowly than those from healthy leaves, which were larger and white (Fig. 9). PAGE of nucleic acids from calli derived from healthy and diseased (containing BSV RNA-1) leaves showed the presence of BSV RNA-1 in diseased calli that had been continuously grown for 8 months. The mobility of the disease-specific RNA band from diseased calli was the same as that of BSV RNA-1 from diseased leaves. No band with similar mobility was detected in gels from healthy calli (Chen *et al.*, 1986) (Fig. 10).

XI. CONCLUSIONS

Two low-molecular-weight RNA species (RNA-1, RNA-2) have been purified from BSV-infected leaves, but not from healthy leaves. Both of these molecules are circular, as determined by electron microscopy and two-dimensional gel electrophoresis. We also have evidence for the continuous replication of BSV RNA-1 in permanent cell cultures of burdock over a period of at least 8 months. These properties are similar to those of other known viroids and strongly suggest that BSD is viroid caused.

Some properties of BSV RNAs, however, differ from those of other viroids. Individual diseased plants contain either RNA-1 or RNA-2, but not both. Also, the molecular weight of either RNA is higher than that

of other known viroids. The marked difference in the electrophoretic mobilities of RNA-1 and RNA-2 suggests that the primary structures of the two RNAs are sufficiently different to consider them as distinct viroids. Preliminary evidence indicates that both RNAs are thermodynamically more stable than other viroids investigated.

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Index