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Viroids: From Thermodynamics to Cellular Structure and Function

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Viroids are an extraordinary biological phenomenon in the sense that a small piece of RNA acts like a complete virus (for reviews, see Diener 1979; Sänger 1982; Riesner and Gross 1985; Diener 1987). This was evident from the days of the discovery and became even more obvious when their extraordinary structural features were elucidated. When viroids became accessible to structural studies in the mid-1970s, the picture of RNA structure was dominated by that of transfer RNA (tRNA), which was known in detail from many biochemical and biophysical studies and particularly from X-ray analysis. tRNA forms not only a secondary structure, which is described best as the well-known two-dimensional base-pairing scheme in the form of a clover leaf, but in addition the branches of the clover leaf are incorporated into a compact three-dimensional arrangement, the so-called L-shape. In fact, other RNAs, such as ribosomal RNA (rRNA), small nuclear RNA (snRNA), and messenger RNA (mRNA), seem to fit into the picture described.

The structure of viroids is clearly different from the prototype RNA structure described above. Viroids were the first circular RNAs to be discovered in nature (Sänger *et al.* 1976; McClements and Kaesberg 1977), and this feature evidently differentiates them from other RNAs. Because of this novelty and because of their extraordinary biological potency, viroids appeared to be very attractive objects for structural studies. Furthermore, their size of a few hundred nucleotides was readily accessible to physicochemical studies, and the nucleotide sequences became available for quite a few viroid species. Thus, several requirements for attractive and successful physicochemical studies on viroid structure were fulfilled.

Since viroids can have only a very limited coding capacity and since there is no experimental evidence for a viroid-coded translation product, one has to assume that viroid replication and pathogenesis depend completely on the enzyme systems of the host (for reviews, see Diener 1979; Sänger 1982; Riesner and Gross 1985; Diener 1987). Thus, their genetic information is the RNA structure, the ability to undergo structural transitions, and the capability to interact with host cell factors. Most results reported here were obtained from studies on the potato spindle tuber viroid (PSTVd).

Stable and metastable structures of PSTVd: Native structure and thermal denaturation. Under physiological conditions viroids form a rodlike structure that may be described as an unbranched arrangement of short helices and small internal loops (cf. Fig. 1A). It was shown by a whole set of experiments that the viroid molecule is present in solution in the rodlike structure, that is it does not fold back and form a more globular structure. During thermal denaturation viroids undergo several structural transitions from the rodlike structure to the single-stranded circle without intramolecular base pairing (for review, see Riesner and Steger 1990; Riesner 1987; cf. Fig. 1B). In a highly cooperative main transition, all base pairs of the rodlike structure are disrupted, and particularly stable hairpins are newly formed. In other words, an extended structure switches over into a branched structure with a concomitant loss of base pairing. Due to thermodynamic competition, the branched structure tends to destabilize the rodlike structure. At higher temperatures the stable hairpins dissociate independently from each other in the order of their individual thermal stabilities. Hairpin I is formed in a region of the molecule the sequence of which is highly homologous among all viroids (except avocado sunblotch viroid) and which is, therefore, called the central conserved region. Hairpin II (with slight sequence variation) is present in all viroids of the PSTVd class. Viroids of this group exhibit high sequence homology, and all can replicate in tomato and other host plants. Hairpin III was found only in PSTVd.

As outlined above, under physiological conditions the thermodynamically favored secondary structure contains none of the stable hairpins. Thus, any functional role for these hairpins has to be attributed to metastable structures that contain the hairpins even under physiological conditions. One may consider two situations that favor the formation of stable hairpins. First, there is the transition from the extended native structure to the branched, that is a hairpin-containing structure may be facilitated by protein binding. At present, no experimental data are available to support this possibility. Second, the stable

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This article is based on a lecture presented at a symposium marking the appointment of T. O. Diener as University of Maryland Distinguished Professor. The symposium, entitled "Subviral Pathogens: Their Impact on Molecular Biology, Plant and Human Medicine, and Evolutionary Thought," was held on March 5, 1990, at the University of Maryland. It was cosponsored by the Center for Agricultural Biotechnology, Maryland Biotechnology Institute, and the Department of Botany, University of Maryland, College Park.

hairpins may be formed during replication of the RNA strand as an intermediate conformation before the synthesis of the whole strand is completed. Since my colleagues and I regard the formation of a thermodynamically metastable structure as a fundamental feature of viroids, and probably of other RNAs, I will outline briefly the experimental evidence. The details have been published earlier (Hecker *et al.* 1988; Riesner *et al.* 1988).

Experimental analysis of stable and metastable structures of circular viroids. The structural transitions of both the circular PSTVd and of its oligomeric replication intermediates were studied with the recently developed method of temperature gradient gel electrophoresis (TGGE) (Rosenbaum and Riesner 1987; Riesner *et al.* 1989). This method is explained most easily with the example of circular PSTVd (Fig. 2A). A linear temperature gradient is established in a horizontal slab gel. The sample is applied in the broad sample slot that extends over nearly the entire width of the slab gel. The direction of electrophoresis is from top to bottom, that is perpendicular to the temperature gradient. Thus, every individual molecule runs at constant temperature, those at the left side at low temperature, at the right side at high temperature, and in between at all intermediate temperatures. Since the instrument is currently industrially manufactured (Diagen-TGGE system, Diagen, Düsseldorf, Federal Republic of Germany), the method may be used as a routine procedure.

As seen in Figure 2A, PSTVd migrates fastest in its native conformation and is drastically retarded within a narrow temperature range that corresponds to the main transition (cf. Fig. 1B). A further decrease in mobility is observed at higher temperatures. Some portion of the PSTVd sample was in a linear form due to single nicks in the circle. Nicks at different sites lead to different stabilities of the native structure; correspondingly different transition curves are visible as faint bands, all of which merge at high temperatures into the curve of the completely denatured PSTVd RNA. The advantages of TGGE over the conventional optical melting curves are obvious from Figure 2A. A mixture of different molecules, for example circular and linear viroids, may be analyzed in the form of individual transition curves, whereas in optical melting curves only the superimposition of all curves is measurable (cf. Riesner 1987). The amount of material needed for one TGGE is as little as 100 ng or less if silver staining is used for detection. Radioactively labeled nucleic acids may be recorded as well, and if the detection is conducted by molecular hybridization with a specific probe, a single nucleic acid species may be analyzed even if it is present as a minor portion of a nucleic acid crude extract. The

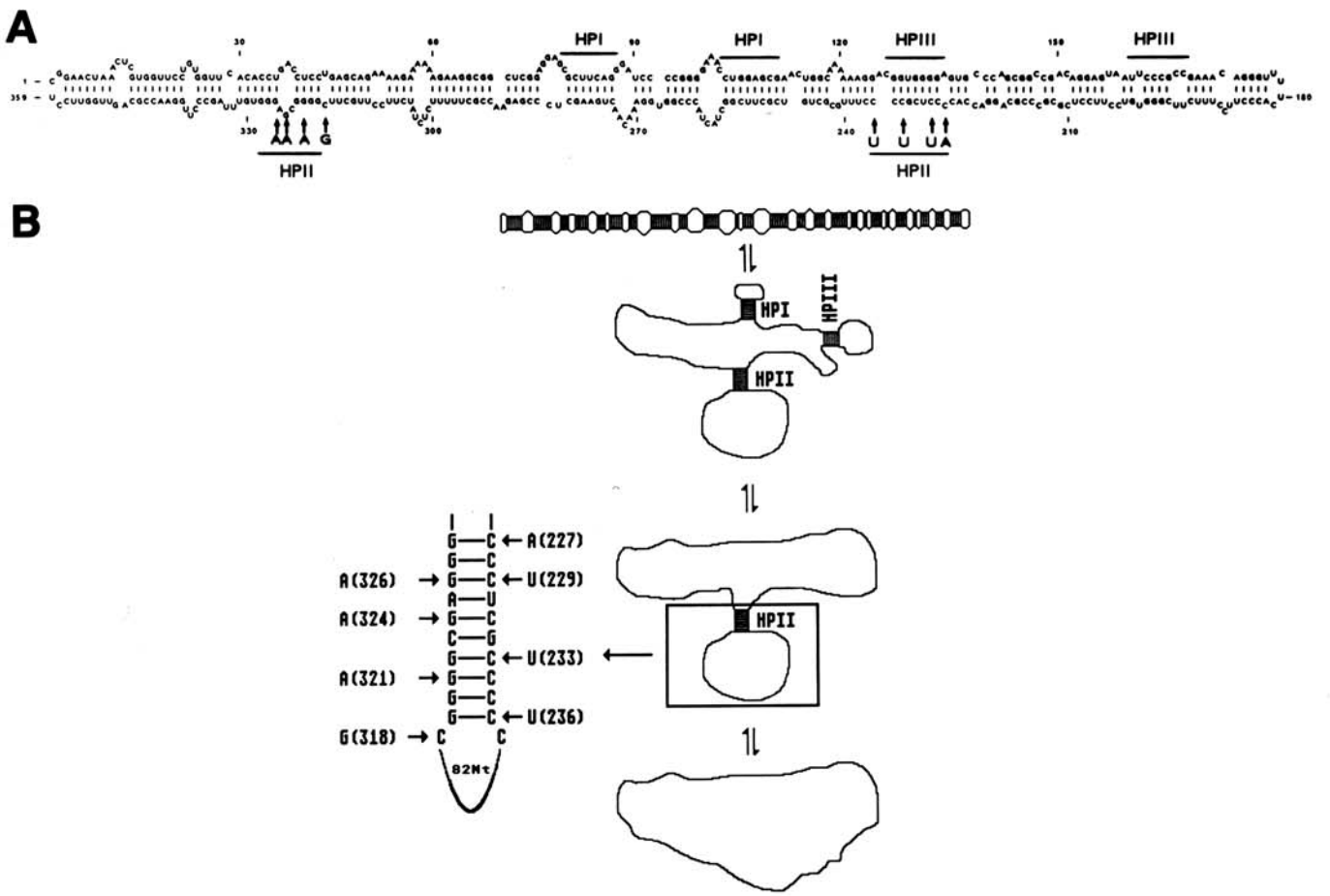

Fig. 1. Secondary structure (**A**) and various structural forms produced during thermal denaturation (**B**) of the potato spindle tuber viroid. The arrows indicate mutations that are described in the text. HPI, HPII, and HPIII indicate hairpins I, II, and III, respectively. This figure is reproduced from Loss *et al.* (in press). (Reprinted by permission of Oxford University Press.)

method of TGGE was originally developed for studies of viroids, but has been applied in the meantime to a large series of other problems of nucleic acids and proteins (cf. review in Riesner *et al.* 1989).

Replication intermediates. The mechanism of viroid replication is known to a certain extent (cf. Riesner and Gross 1985; Branch and Robertson 1984). The circular (by definition) (+) strand viroid is transcribed into an oligomeric (−) strand RNA. The (−) strand acts as a template for synthesis of an oligomeric (+) strand RNA. Both transcription steps are catalyzed by a host enzyme, the DNA-dependent RNA polymerase II (Mühlbach and Sänger 1979; Rackwitz *et al.* 1981; J.-M. Schindler and H. P. Mühlbach. *In vitro* studies on PSTVd synthesis in nuclei and nuclear extracts with α-amanitin and actinomycin C1, International Congress of Virology, 7th, Berlin, August 26-31, 1990, p55-002). This enzyme, which normally transcribes mRNA from double-stranded DNA, does accept viroid RNA as a template. The (+) strand oligomeric RNA is cleaved enzymatically to unit-length molecules that are then ligated to the mature viroid circles. Autocatalysis of cleavage and ligation was not found in viroids; a definite exception is, however, the avocado sunblotch viroid, which undergoes autocatalytic cleavage and ligation and is also different in respect to other details of the replication cycle.

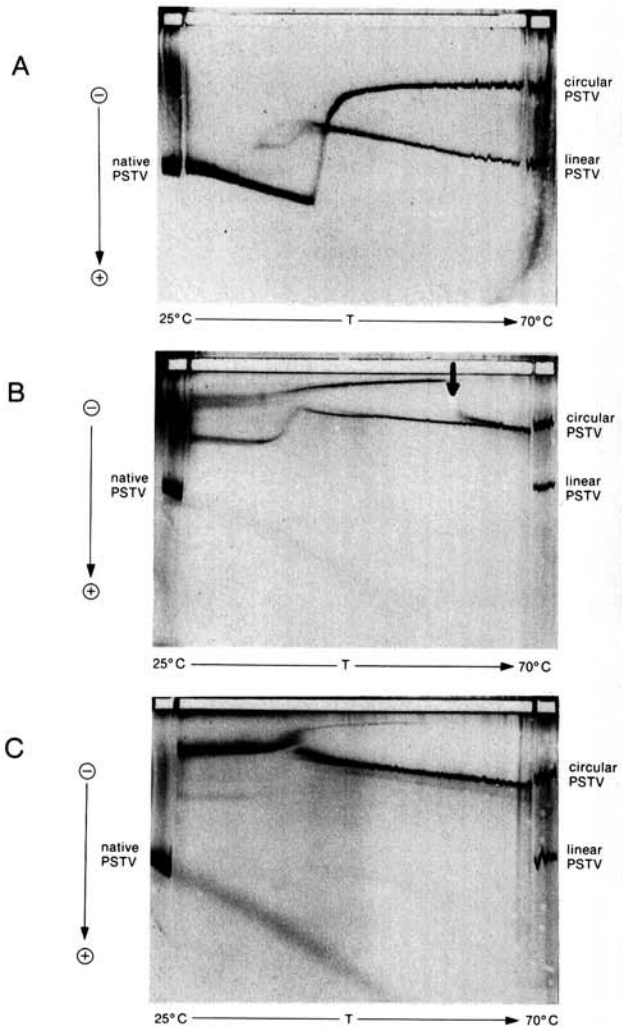

Fig. 2. Analysis of structural transitions of circular and linear potato spindle tuber viroid (PSTVd) (**A**) and dimeric (+) strand RNA transcripts (**B** and **C**) by temperature gradient gel electrophoresis in a 5% polyacrylamide gel. Buffer conditions: 17.8 mM Tris, 17.8 mM boric acid, 0.4 mM EDTA. The direction of the electrophoresis (top to bottom) and the linear temperature gradient (left to right) are indicated. The gel was silver-stained. Marker slots (left and right side of the broad sample slot) contain natural circular and linear PSTVd. **B**, Transcript isolated from the transcription mixture, incubated in a high salt/urea buffer (1 mM sodium-cacodylate, 500 mM NaCl, 4 M urea, 0.1 mM EDTA, pH 6.8) for 1 hr at 53° C, cooled, and analyzed. **C**, Transcript analyzed right after transcription without further treatment. This figure is adapted from Hecker *et al.* (1988). (Reprinted with permission of Elsevier Science Publishers, Amsterdam.)

Linear oligomeric RNA transcripts. Structure formation during replication was simulated by synthesizing linear oligomeric RNA transcripts from PSTVd cDNA clones with T7 polymerase (Hecker *et al.* 1988). An oligomeric RNA transcript is considered to represent a replication intermediate. Since the dimeric RNA (+) strand transcripts are the smallest model for oligomers, a series of studies were conducted on these molecules.

The secondary structures to which dimeric molecules may adapt are depicted in Figure 3.

Extended structure. An extended secondary structure without bifurcations (Fig. 3E) was calculated to be the most stable structure. It is described best as a doubling of the secondary structure of native circular viroids (cf. Fig. 3B).

Trihelical structure. Because the region that forms hairpin I in circular viroids is present twice in the dimeric transcripts, both of them together may form a particularly stable double-helical region. This region, shown in detail in Figure 3A, contains three consecutive helices with a total of 28 base pairs and a G+C content of 71%. The central helix is formed only in dimeric transcripts, because the sequence is located in the loop region of hairpin I. Once these helices form, the other parts of the dimeric transcript will assume the structure as depicted in Figure 3C. The structure is 34 kJ/mol or 7% thermodynamically less favorable than the extended structure. Consequently, the trihelical structure is metastable.

Transient structures during synthesis (multihairpin structures). During synthesis, structures other than the extended or the trihelical structure may be formed and may persist as metastable structures for some time. It was assumed that in a transient structure such as shown in Figure 3D, a hairpin is formed as soon as its sequence has been synthesized. In the particular model of Figure 3D, the well-known stable hairpins of PSTVd and the left- and right-ended hairpins of the extended structure (designated by small arrows in their loops) are taken into account. Alternative models of a multihairpin structure could also be designed.

TGGE of dimeric transcripts. These analyses were conducted after a pretreatment of the sample under different solution conditions (for details, see Hecker *et al.* 1988; Riesner *et al.* 1988): a) thermal equilibrium between the extended structure (Fig. 3E) and its denatured conformation (as shown for PSTVd in Fig. 1B), and b) the conditions of RNA synthesis, that is TGGE conducted immediately after T7 transcription without further

treatment.

In Figure 2B and C the results from TGGE after treating the dimers under solution conditions a and b are shown. Under the equilibrium conditions (Fig. 2B) two dominant bands and one faint band were visible, corresponding to three different structures. This is a graphic example of the potency of TGGE when analyzing coexisting structures. The fastest migrating band was clearly identified as the extended structure, because the transition was very similar to that of a circular PSTVd or a monomeric linear viroid (Fig. 2A). The band of slowest mobility has to represent the trihelical structure, because the three consecutive helices are the only structural elements that are stable enough to account for the transition at 58–60° C (designated in Fig. 2B by an arrow).

The faint band in Figure 2B between the bands of the extended structure and the trihelical structure was identical to that obtained under the conditions of synthesis b (Fig. 2C). Therefore, it has to be the transient structure (cf. Fig. 3D). One should note in Figure 2C that to a minor extent, the extended structure and the trihelical structure are also present in the transcription mixture. Thus, one has to conclude that the transient structure is able to switch over into the other structures under conditions which are close to physiological.

In summary, the experiments and calculations have shown that viroids assume under physiological conditions a rodlike structure which switches over in a hairpin-containing structure at elevated temperatures. The same hairpins were found, however, under physiological conditions when viroid replication intermediates were analyzed right after their synthesis.

Involvement of hairpin structures in replication: Hairpin I and processing. The formation of hairpin I, together with its location in a region of highly conserved sequences, suggested several hypotheses on its functional relevance. Hypotheses about the origin, processing, and pathogenicity of viroids have been discussed. In the present context, only the involvement of the stable hairpin I in viroid processing will be considered, because experimental support for this hypothesis has been accumulating during the past several years.

R. Owens and co-workers (Cress *et al.* 1983) and subsequently several other research groups have shown that

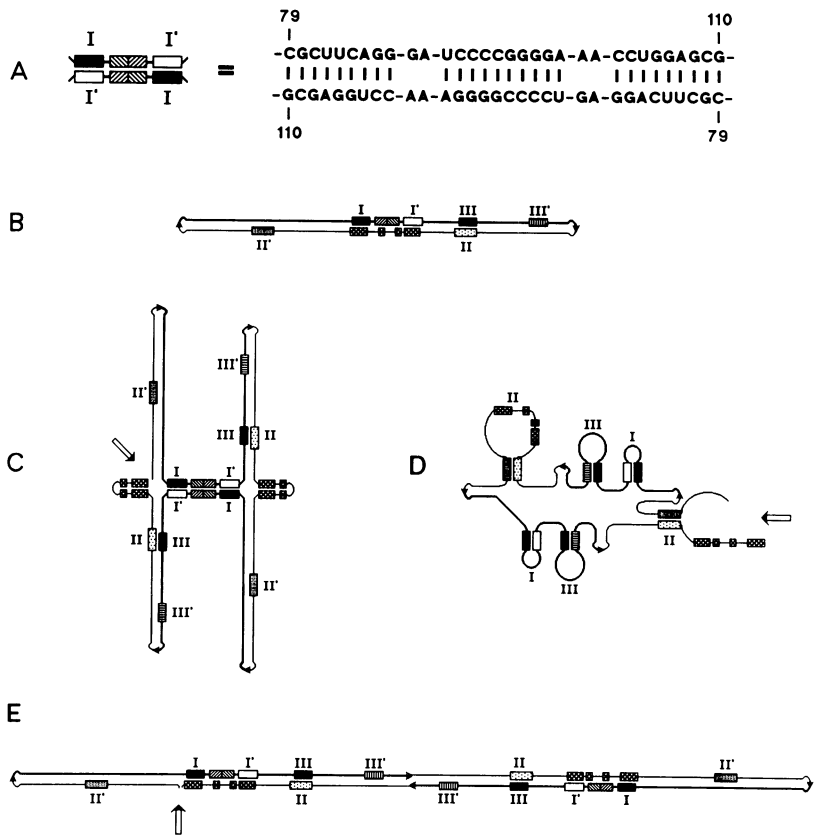

Fig. 3. Structural models of dimeric (+) strand transcripts. Complementary sequences are symbolized by boxes with corresponding graphic patterns. The 5′ to 3′ direction of the strand is indicated by arrowheads that are positioned in the hairpin loops at the left and the right ends of the native structure. **A**, Schematic representation of the upper central conserved region of potato spindle tuber viroid (PSTVd) (nucleotides 79–110) and of the arrangement of two conserved regions in three successive helices. Segments I and I′ form hairpin I during the thermal denaturation of the circular PSTVd (cf. Fig. 1). **B**, Secondary structure of the mature circular viroid (see Fig. 1) showing the position of the segments from **A**, segments II and II′ which form hairpin II, and segments III and III′ which form hairpin III. The various shaded boxes are complementary sequences (cf. **C**) in the lower central conserved region. **C**, Structure of the dimeric transcript with the three successive helices, also called the trihelical structure. **D**, Transient or multihairpin structure with stable hairpins (I, nucleotides 79–87 and 102–110; II, nucleotides 227–236 and 319–328; III, nucleotides 127–135 and 160–168; hairpin at the left end of the native structure, nucleotides 3–28 and 334–357; and hairpin at the right end of the native structure, nucleotides 169–177 and 182–192). **E**, Extended structure similar to that of the native circular viroid. The thick arrows with the open shafts in **C**–**E** indicate the beginning and end locations of the start and stop of the linear transcript. This figure is reproduced from Riesner (1990).

plasmids carrying two units of tandemly repeated viroid cDNA are infectious, whereas plasmids carrying only a monomeric viroid cDNA unit are not infectious. Meshi and his colleagues (1985), Tabler and Sänger (1984), and Visvader and her colleagues (1985) concluded from their experiments with clones of variable but longer than unit length that the minimal entity which had to be present twice (i.e. at the 5′ and the 3′ end of the linear molecule) 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 I is relevant for processing the viroid precursor to the exact monomeric length circle, probably by cellular endonucleases and ligases. It was suggested by Riesner *et al.* (1979), Visvader *et al.* (1985), and Diener (1986) that the structure containing three successive helices (cf. Fig. 3C) could be formed in that region and that this structural element is relevant for processing. Steger *et al.* (1986) showed experimentally that a structure with three consecutive stable helices did, in fact, exist in solution.

The experimental work on the potential processing structure was extended by conducting more calculations and applying TGGE analysis (Hecker *et al.* 1988; G. Steger, M. Mörchen, D. Riesner, M. Tabler, M. Tsagris, and H. L. Sänger. Secondary structure requirements for PSTVd processing, (Abstr.) Cold Spring Harbor meeting on RNA processing, Cold Spring Harbor, NY, May 17-21, 1989). The studies on the dimeric transcripts (cf. above) clearly demonstrated that these molecules may be present in quite different conformations and, in particular, may change their conformation from synthesis to their action as template for further synthesis or as substrate for cleavage. Those experiments were performed not only with dimeric transcripts of the wild-type PSTVd sequence but also with shorter transcripts that were altered in sequence and structure by site-directed mutagenesis. From those experiments (unpublished data) it could be concluded that the trihelical structure in its originally published form (cf. Fig. 3C) is not the structure which is active in processing. Rather a combination of the trihelical and the extended structure was in agreement with both the structural and biochemical data.

Hairpin II and complementary strand synthesis. The experiments on hairpin II will be described in some detail because more firm conclusions can be drawn at present. For more details, the reader may refer to the original publication (Loss *et al.*, in press).

Experiments after site-directed mutagenesis in hairpin II. Site-specific mutations were introduced into the stems of hairpin II. These mutations have been added to the depiction of the structure and structural transitions of PSTVd (see Fig. 1). All these mutations obviously destabilize hairpin II, except for the mutation at site 318 that stabilizes due to the formation of an additional base pair adjacent to the stem. The sites of mutation were selected carefully to introduce as little perturbation as possible into the native structure (cf. Fig. 1A). Only the mutation at position 318 transforms a mismatch between two helical regions into a base pair.

Thermodynamic analysis. Using standard procedures the mutations were introduced in monomeric and dimeric cDNA clones, and from these templates monomeric and dimeric RNA transcripts were synthesized. Although the main interest was directed toward analysis of the biological effects of the mutations, the thermodynamics of the mutants was also studied. Monomeric RNA transcripts were analyzed by thermodynamic calculations and by experimental TGGE. Without describing the details it can be stated that the mechanism of the conformational transitions as depicted in Figure 2 could be confirmed quantitatively: destabilization of hairpin II by destroying a base pair led to a stabilization of the rodlike structure, that is the switch from the extended to the branched structure was shifted to a higher temperature. Other details may be found in the original literature (Loss *et al.*, in press).

Infectivity and genetic stability. All mutants were tested for their infectivity on tomato plants. For this purpose the plants were inoculated with the corresponding double-stranded DNA of dimeric length or with its linear RNA transcripts. Both types of inoculation gave identical results. Surprisingly, all mutants except 321 were infectious. The sequences of all progeny viroids were determined. Mutant 321 contained a second mutation due to a cloning artifact and, therefore, will not be discussed further. Only some of the other mutations were stably maintained in the progeny, and other mutations reverted to the wild-type sequence after the first passage through the host plant. In Figure 4 the mutations in hairpin II are classified according to the results of sequencing as stable (S) or revertant (R). Reversion must have occurred in the plant and could not have come from transcription errors during synthesis of the transcripts because inoculation with cloned DNA or with RNA transcripts gave identical results.

Hairpin II as a regulatory element for replication: Indispensable core and variable periphery. The results allowed us to correlate the position of the mutation with the biological activity. Obviously, the stable mutations affect the structure in a way that is tolerated during replication.

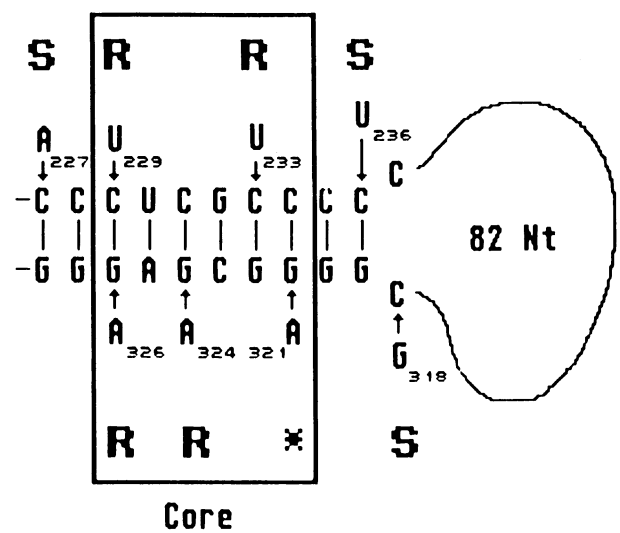

Fig. 4. Stable mutations (S) and reversions (R) in hairpin II of potato spindle tuber viroid. The core of the hairpin (boxed) contains all the reversions. For mutation 321, see the text. This figure is reproduced from Loss *et al.* (in press). (Reprinted by permission of Oxford University Press.)

The reversions, however, have to be part of a structural element that is indispensable for replication. A high selection pressure in favor of the wild-type sequence has to be assumed, because the revertants quickly overgrow the mutants during replication in the cell.

Whereas the genetic stability of the mutation could not be correlated with its influence on the native structure of the viroid, such a correlation with the position of the mutations in hairpin II is quite convincing. In Figure 4, hairpin II is divided into a core and two peripheral segments. The mutations in the core are reverted to the wild type; the mutations in the peripheral segments remain stable. Thus, it may be generalized from the present data that the structural integrity of the core is indispensable for replication, whereas mismatches or wobble pairs in the peripheral segments are tolerated.

Constant core and variable periphery in other viroids. As mentioned above, hairpin II is also present in the other viroids of the PSTVd class. PSTVd, tomato planta macho viroid, tomato apical stunt viroid, citrus exocortis viroid, chrysanthemum stunt viroid, and columnea latent viroid belong to this class. Although the structures of hairpin II in the different viroids vary slightly (Fig. 5A), they may be aligned so that the core is identical in all of them. This is demonstrated by the box in Figure 5A. The only exception is an AU pair in chrysanthemum stunt viroid compared to a GC pair in all other viroids. Thus, the core region of hairpin II that cannot be altered in PSTVd without

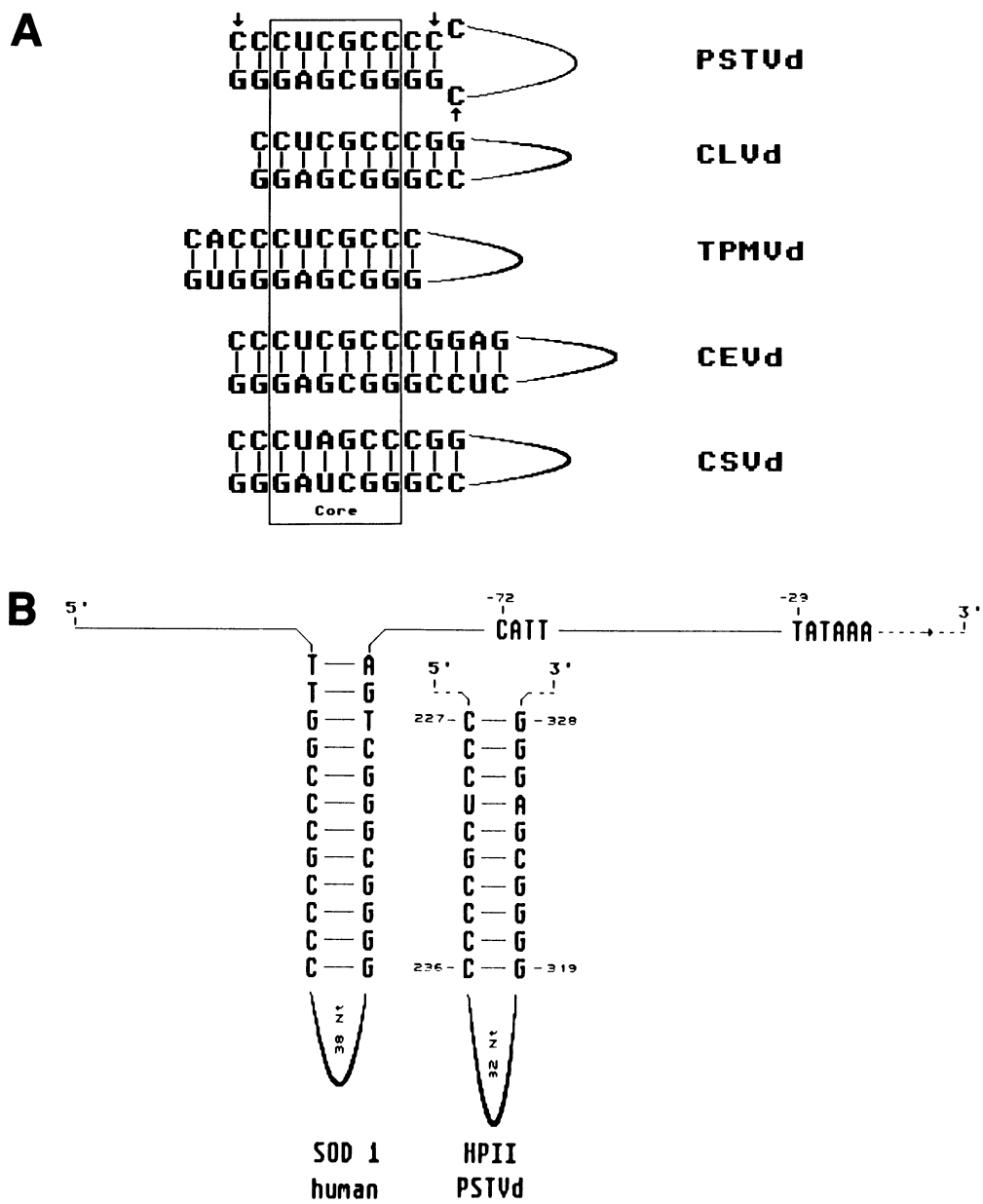

Fig. 5. Comparison of hairpin II (HPII) in different viroids (**A**) and with a 5′-upstream GC-rich segment in the gene encoding human superoxide dismutase (SOD1) (**B**). PSTVd indicates potato spindle tuber viroid; CLVd, columnea latent viroid; TPMVd, tomato planta macho viroid; CEVd, citrus exocortis viroid; and CSVd, chrysanthemum stunt viroid. This figure is adapted from Loss *et al.* (in press). (Reprinted by permission of Oxford University Press.)

loss of infectivity is also constant if different viroids are compared. The variable segments in hairpin II of PSTVd vary significantly from viroid to viroid. In summary, the variations in the structure of hairpin II, which were tolerated after site-directed mutagenesis of PSTVd, are at very similar sites as the natural variations.

Homology with recognition sites of host genes. Do structural elements similar to hairpin II exist also in the host? In fact, GC-rich segments are found in the 5′ untranscribed regions of eukaryotic genes. Unfortunately, those have been reported so far mostly for human (Levanon *et al.* 1985), animal (Gidoni *et al.* 1984), and viral systems (McKnight *et al.* 1984), whereas corresponding regions from plants have been analyzed only in one case (Matsuoka and Minami 1989). A striking similarity between hairpin II from PSTVd and a GC-rich segment from the 5′-upstream region of the gene encoding human superoxide dismutase (Levanon *et al.* 1985) is shown in Figure 5B. Although depicted in Figure 5B as a hairpin structure within one strand, the double-stranded GC-rich region may also be formed in the host DNA by the (+) and the (−) strands. Thus, a hairpin structure in the viroid could correspond to a segment of the genomic double-stranded DNA of the host. Other examples of GC-rich segments could be given with similar homology. Most of those GC segments belong to so-called housekeeping genes and have been described as acting as binding sites for transcription factors (Gidoni *et al.* 1984).

Hairpin II in viroids and homologous DNA segments in host genes: Both form an A-double-helix. An RNA structural motif in viroids has just been compared with a DNA motif in host genes. Is this comparison legitimate? Recently, several structures of double-stranded DNA oligomers with a high GC content were studied by X-ray analysis (Heinemann *et al.* 1987). It was found that those fragments of DNA assume an A-form double helix rather than the typical B-form. For an easier comparison the structures of A-RNA, B-DNA, and a GC-rich deoxyoligonucleotide are shown in Figure 6. Although the A-form of DNA and the A-form of RNA are not identical, they are much more similar than the A-form of RNA and B-form of DNA. If recognition of GC-rich segments would be interpreted merely on the basis of their A-form in an otherwise B-form of DNA, one may speculate that viroids mimic a cellular recognition structure in which RNA and DNA assume nearly the same structure.

Hairpin II as a hypothetical binding site of transcription factors. The homology between a structural element in viroids and one in host genes suggests that both structures may act as a recognition structure for the same host protein. Examples of recognition structures that are essential for transcription and/or replication are promoters, enhancers,

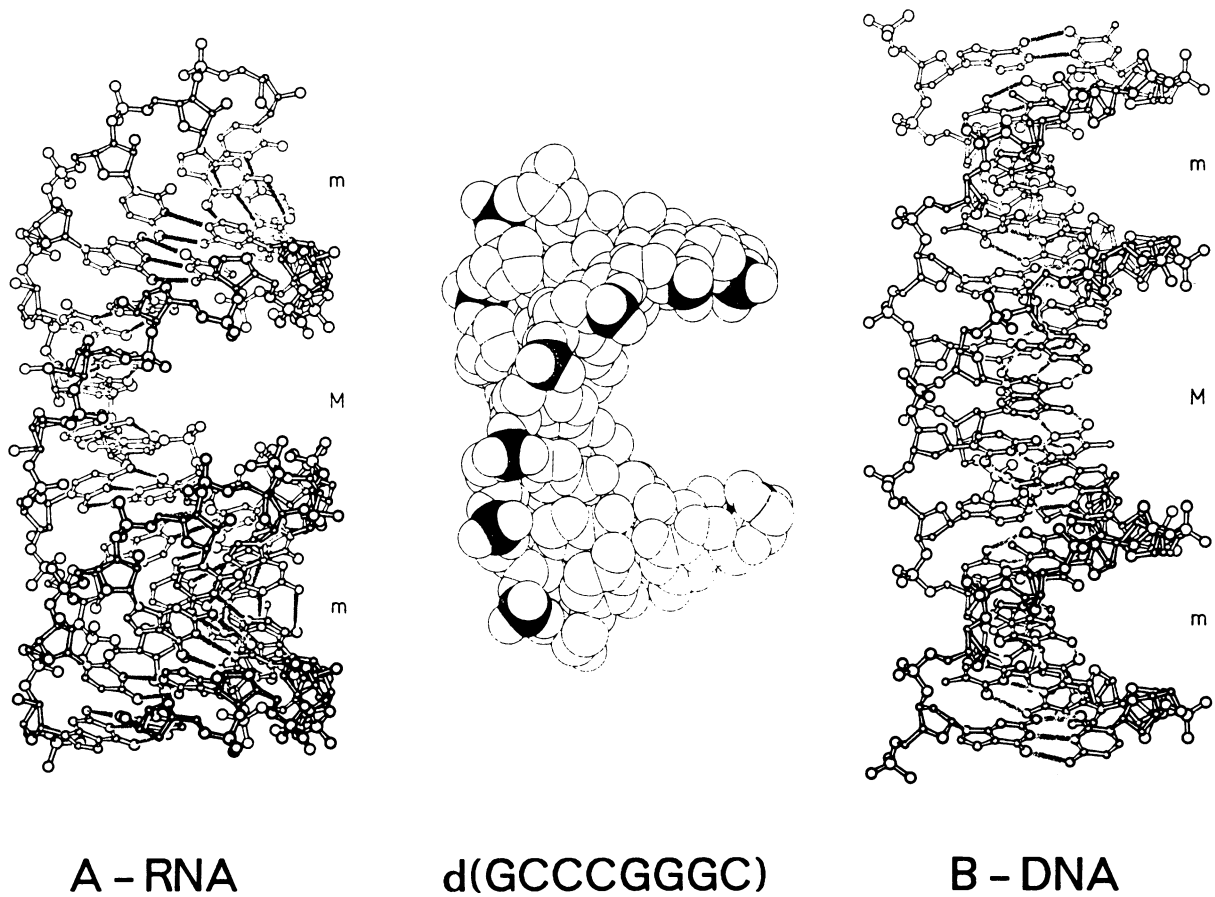

Fig. 6. Structures of A-RNA (left), B-DNA (right) (reproduced from Saenger 1984; reprinted by permission of Springer-Verlag, Heidelberg), and the GC-rich oligodeoxynucleotide (middle) (reproduced from Heinemann *et al.* 1987; reprinted by permission of Oxford University Press). The structures were determined from X-ray analysis.

and binding sites for transcription factors. Because viroids are replicated by DNA-dependent RNA polymerase II (Pol II), one may consider that hairpin II acts as a promoter for this enzyme. On the other hand, Pol II was shown by electron microscopy (Goodman *et al.* 1984) to bind to the left- and right-end hairpins of the native rodlike structure. Thus, the electron micrographs of viroid-Pol II complexes do not indicate an involvement of hairpin II in Pol II binding. Inside the cell, however, the situation is more complex as studied *in vitro* (Goodman *et al.* 1984).

Transcription factors bind to the template in addition to Pol II, and not only the circular viroids in the most stable conformation but also the oligomeric viroid intermediates in metastable conformations may act as templates for Pol II. Taking into account the finding that a host structural element which is homologous to hairpin II acts as a binding site for transcription factors (Gidoni *et al.* 1984), it seems appropriate to assume that an as yet unidentified transcription factor of the host binds to hairpin II. Since in circular viroids the transcription factor could bind to hairpin II only after the native structure is denatured, it is more obvious to assume that the transcription factor binds to hairpin II in the (−) strand oligomeric viroid RNA.

As shown above, this strand is synthesized in a multibranched structure and does not assume the rodlike structure during the replication cycle. Possibly, also the oligomeric (+) strand RNA may act as a template for further (−) strand RNA synthesis. Therefore, from the thermodynamic point of view the formation and thereby the functional relevance of hairpin II are more important in the oligomeric replication intermediates: these molecules are synthesized in a structure containing hairpin II and require the recognition of hairpin II by a host for synthesis of their counter strands.

Cellular location of viroids. Our knowledge of the structure of viroids and their replication intermediates was derived from *in vitro* experiments. Functional aspects, on the other hand, were studied in experiments with cellular extracts and by infections of whole plants. The intermediate step, however, that is the question of the cellular location of viroid replication intermediates, was missing.

Studies with *in situ* hybridization and confocal laser scanning microscopy. To fill this gap, the intracellular localization of viroids has been investigated by fractionation studies (Schumacher *et al.* 1983) and by viroid-specific *in situ* hybridization and analysis of the distribution of the fluorescent hybridization signal by digital microscopy (Harders *et al.* 1989). Only the more recent studies, that is those with *in situ* hybridization, will be described here.

Isolated nucleic acid from green leaf tissue of tomato plants infected with PSTVd was bound to microscope slides, fixed with formaldehyde, and hybridized with biotinylated transcripts of cloned PSTVd cDNA. The bound probe was detected with lissamine-rhodamine-conjugated streptavidin. Nucleoli were identified by immunofluorescence using the monoclonal antibody Bv96 (Frasch 1985) and a secondary fluorescein isothiocyanate-conjugated antibody. In plants infected with either a lethal or an intermediate PSTVd strain, the highest intensity of fluorescence arising from hybridization with the probe specific for the viroid (+) strand was found in the nucleoli, confirming results of previous fractionation studies (Schumacher *et al.* 1983). A similar distribution was found for (−) strand replication intermediates of PSTVd using specific (+) strand transcripts as hybridization probes.

To determine if viroids are located at the surface or in the interior of the nucleoli, the distribution of the fluorescent hybridization signals was studied with a confocal laser scanning microscope (CLSM). In Figure 7 the intensity distribution in an optical section with a focal depth of only 0.4 μm through an isolated nucleus is displayed as a three-dimensional isometric projection, with pixel gray values represented by vertical displacement in the form of a wire frame. The area in the cross section where the high viroid concentration was detected is exactly that of the nucleolus. It can be concluded that viroids are neither restricted to the surface of the nucleoli nor to a peripheral zone, but are instead homogeneously distributed throughout the nucleolus.

Structural implications. The particular subcellular localization of viroids clearly differentiates them from plant viruses. The latter generally accumulate and replicate in the cytoplasm and only rarely in the nucleus. No virus, viral RNA, or any other form of a pathogen is known to be targeted to the nucleolus, PSTVd being the notable exception. However, since this is the only viroid species and infected tomato leaves are the only tissue examined in this connection to date, it remains to be established whether the involvement of the nucleolus in the infection process is a general phenomenon.

Functional implications. The nucleolar localization of viroids has to be considered in the context of their replication and pathogenic action. At first glance, the

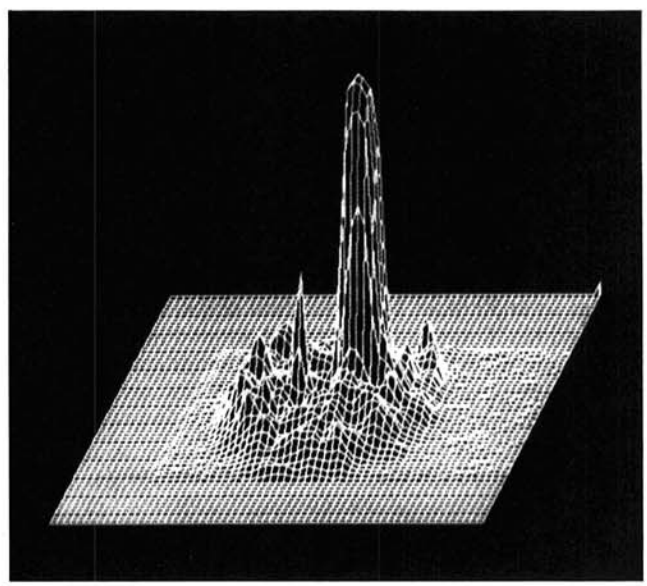

Fig. 7. Distribution of viroid concentration in a cross section through an isolated nucleus displayed as a three-dimensional isometric projection. Viroid concentration was measured as the intensity of fluorescence after *in situ* hybridization with a fluorescence-labeled RNA probe; the intensity of fluorescence was determined by scanning an optical section with a focal depth of 0.4 μm in a confocal laser scanning microscope. This figure is reproduced from Harders *et al.* (1989). (Reprinted by permission of Oxford University Press.)

localization of mature viroids and (−) strand and (+) strand replication intermediates in the nucleolus seems to contradict the presumed involvement of RNA polymerase II in the synthesis of these RNAs, particularly since the present work has shown that the viroid RNA is in the interior and not on the surface of the nucleolus. There are two logical possibilities for reconciling this apparent contradiction: either the RNA polymerase II or the viroid, including its replication intermediates, migrates between the nucleoplasm and the nucleolus. I am not aware of any experimental evidence for such a redistribution of RNA polymerase II. However, at least for one RNA species, snRNA U3, migration from the nucleoplasm to the nucleolus has been demonstrated (for a review, see Busch *et al.* 1982). A similar behavior may be postulated for the viroids. This model would be in accordance with our results inasmuch as circular viroid RNAs (as well as its replication intermediates) are found in the nucleoplasm, although at much lower concentrations than in the nucleolus.

One may also consider the possibility that the synthesis of RNAs by RNA polymerase II occurs in the nucleoplasm but that the (+) strand oligomers are processed to mature circular viroids only in the nucleolus. Thus, the high concentration of mature viroids observed in infected tissue may represent a reservoir or storage form of the products arising from a maturation mechanism topologically restricted to the nucleolus.

While viroid replication may well occur at sites of low steady state concentration of the template (i.e. in the nucleoplasm), it is tempting to speculate that the pathogenic action of the viroids may be exerted at the locus of highest concentration, namely the nucleolus. If so, it would appear that the mere presence of the viroids might not in itself be pathogenic, inasmuch as the same distribution and copy number were found for the RNAs of different PSTVd strains (Gruner 1987). That is, the difference in pathogenicity among viroid strains may originate from fundamental differences in the molecular interactions between viroid and host and not from differences in copy number or subcellular localization. Unfortunately, only hypotheses exist as yet regarding the mechanisms of viroid pathogenesis (for review, see Owens and Hammond 1987). Most of these cannot be related to the nucleolar location. Only one proposal clearly pointed to the nucleolus as the site of pathogenic action. As pointed out by Schumacher *et al.* (1983) and Kiss *et al.* (1983), PSTVd exhibits sequence homologies to snRNA U3. This RNA is thought to be involved in preprocessing of pre-rRNA inside the nucleolus (Busch *et al.* 1982). Different mechanisms for viroid interference with rRNA processing may be considered. However, a more detailed mechanism may be considered only if more data are available about rRNA processing in the host plants.

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