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Multiple pathways of reversion in viroids for conservation of structural elements

Feng Qu[1], Cornelia Heinrich, Peter Loss, Gerhard Steger, Po Tien[2] and Detlev Riesner

Heinrich-Heine-Universität Düsseldorf, Institut für Physikalische Biologie, Universitätsstrasse 1, D-4000 Düsseldorf, Germany and [2]Academia Sinica, Institute of Microbiology, Beijing, PR China

[1]On leave from Academia Sinica, Institute of Microbiology, Beijing, PR China

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From site-directed mutagenesis of potato spindle tuber viroid (PSTVd) it had been concluded earlier that the formation of a thermodynamically metastable structure containing hairpin II (HP II) is critical for infectivity. In order to differentiate between structural and sequence effects, in the present work base pairs in HP II were exchanged by site-directed double mutations without significant alterations in the native rod-like structure of PSTVd. The mutants were viable and genetically stable in the first generation, but one of the two mutations reverted to the wild-type nucleotide in the second generation. Single-site mutations in the stem of HP II, which had been described as revertants to the wild-type sequence earlier, were analysed with respect to the time course of reversion and the sequence variation during reversion. All replicating sequence variants were separated by gel electrophoretic techniques and the sequences and their relative frequencies were determined. From both types of studies it can be concluded (i) that HP II is a functional element in the (−)strand replication intermediate, generated due to sequential folding during synthesis, and that it is essential for template activity of (+)strand synthesis; (ii) that G:U pairs are tolerated transiently in (−)strand HP II; the lower stability of such a HP II is compensated by additional mutations outside HP II which suppress the competition of a rod-like structure; and (iii) that the reversions are generated spontaneously during (−)strand synthesis. Furthermore, the double-stranded structure of HP II is the essential element for short term replication of PSTVd but the exact sequence of the wild-type proves to be superior with regard to fitness and replicability of PSTVd.

Key words: frequency of reversion/metastable secondary structure/separation of sequence variants/site-directed mutagenesis/(−)strand replication intermediates of potato spindle tuber viroid

Introduction

Viroids are plant pathogens distinguished from viruses by the absence of a protein coat and by their small size. They are circular single-stranded RNA molecules consisting of a few hundred nucleotides, the smallest having ~240 and the largest ~600 nucleotides. No experimental evidence for a viroid-encoded translation product could be found, and it is generally assumed that viroid replication and pathogenesis depend completely on the enzyme systems of the host (Riesner and Gross, 1985; Diener, 1987; Semancik *et al.*, 1987). Thus, their genetic information is the RNA structure, the ability to undergo structural transitions, and the capability to interact with host cell factors. Recently, several elements of the viroid structure could be correlated to well defined steps in viroid function. Since this work concentrates on the biology of a particular structural element, the so-called hairpin II (HP II), we will summarize briefly the structural and functional background.

During thermal denaturation, as shown in Figure 1, viroids undergo a highly cooperative transition from their rod-like, native structure to a branched structure, in which all base pairs of the native state are disrupted and particularly stable hairpins are newly formed (HP I, II, III). At higher temperatures the stable hairpins dissociate independently from each other in the order of their individual stabilities (reviews by Riesner, 1987; Riesner and Steger, 1990).

The hairpin structures are not part of the native structure but are stable only at higher temperature. Thus, any functional role of these hairpins has to be attributed to metastable structures which contain these hairpins even under native conditions. It has been discussed in several reports and was also concluded from *in vitro* experiments that such metastable structures might be generated during synthesis of viroid replication intermediates (Hecker *et al.*, 1988; Loss *et al.*, 1991; Riesner *et al.*, 1992; Steger *et al.*, 1992). A section of such a replication intermediate is depicted in Figure 1B. During replication (reviews by Branch and Robertson, 1984; Diener, 1987) the circular [by definition (+)strand] viroid is transcribed into an oligomeric (−)strand RNA. The (−)strand acts as template for the synthesis of an oligomeric (+)strand RNA. Both transcription steps are catalysed by a host enzyme, the DNA-dependent RNA polymerase II (Mühlbach and Sänger, 1978; Schindler and Mühlbach, 1992). The (+)strand oligomeric RNA is cleaved enzymatically to unit length molecules which are then ligated to the mature viroid circles. HP I may be involved in these cleaving and ligation reactions. It could be shown that a (+)strand transcript of potato spindle tuber viroid, which is still infectious, consists of a monomeric molecule with only 22 additional nucleotides thus doubling part of the HP I forming region of the viroid (Tsagris *et al.*, 1991). This transcript was processed, i.e. it was cleaved and ligated to infectious circles *in vitro* by RNase T1 (Tabler *et al.*, 1992). By analysis of several site-specific mutants by *in vitro* processing, infectivity studies, temperature-gradient gel electrophoresis, and structure calculations, a detailed model for the structures and structural transitions involved in *in vitro* processing was derived and discussed as a mechanistic model for cellular processing of viroids (Steger *et al.*, 1992). HP III was only found in PSTVd and is therefore of marginal interest.

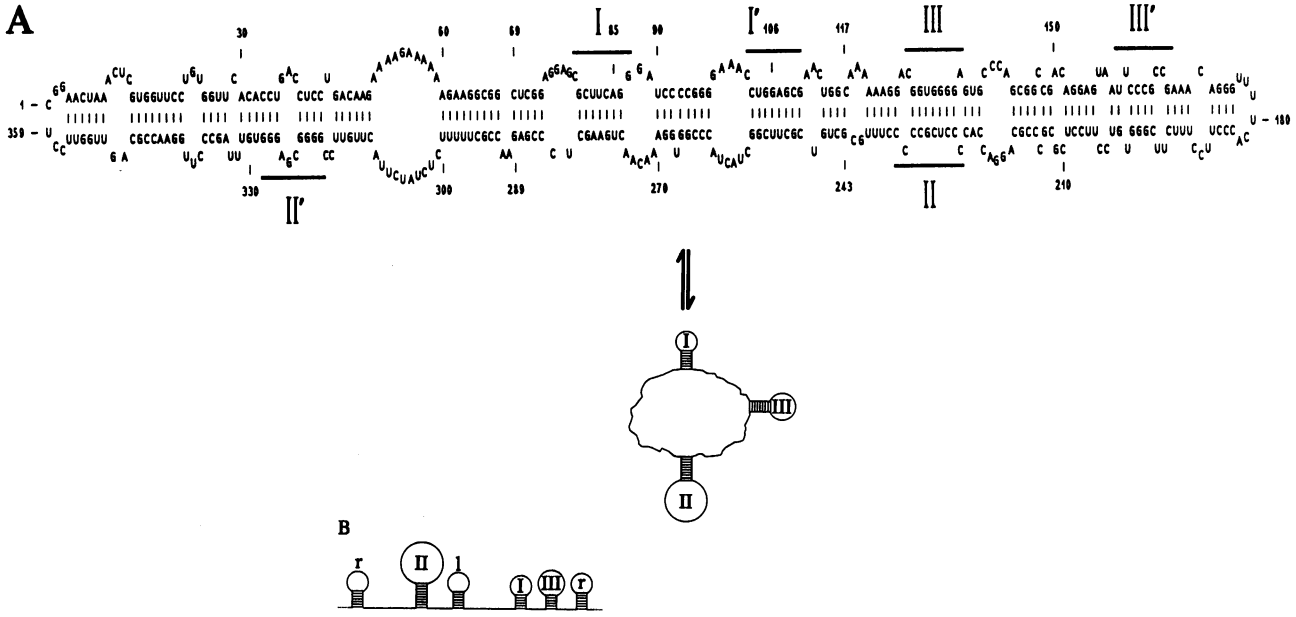

Fig. 1. Secondary structures of PSTVd. Sequence and nucleotide numbering is according to Gross *et al.* (1978). (**A**) Native, rod-like secondary structure of PSTVd (top) and partially denatured structure of PSTVd (bottom) which is stable at higher temperature (Henco *et al.*, 1979). The regions, marked by I and I′, II and II′, and III and III′, are able to form hairpins (HP) I, II and III, respectively, which are thermodynamically stable during thermal denaturation of PSTVd or are metastable during synthesis. (**B**) Section of an oligomeric (+)strand replication intermediate in a metastable secondary structure directly after synthesis. The hairpins of the left and right termini of the native structure are designated by l and r, respectively.

The functional relevance of HP II was investigated by site-directed mutagenesis, thermodynamic studies and infectivity tests (Loss *et al.*, 1991). In PSTVd eight single-site mutations were generated in the segments which form HP II (cf. Figure 2A). Infectivity tests showed that mutations in the core region of HP II reverted to the wild-type sequence whereas mutations in the peripheral regions of HP II remained genetically stable. It was concluded that formation of a thermodynamically metastable structure containing HP II is critical for infectivity of potato spindle tuber viroid RNA. Since HP II exhibits a strong similarity in sequence as well as in three-dimensional structure to certain DNA GC clusters found in the 5′-upstream regions of some genes in man, animals, viruses and plants, a hypothesis about function of HP II as a binding site for host cell transcription factors was proposed.

In the present work we turned to two questions, which are a direct consequence from our earlier results on HP II.

(i) From the single-site mutations it could be concluded that the structural integrity of the core is essential, but it could not be decided whether the structural integrity alone or also the detailed sequence is involved. This question leads us directly to the simultaneous introduction of two compensatory mutations where a base pair of HP II is exchanged for another base pair. Genetic stability of an exchanged base pair would emphasize the importance of the double-stranded structure, slow reversion would show that the sequence also is involved.

(ii) In order to elucidate the mechanism of reversion, the kinetics of the reversion in the plant cell will be analysed at least semi-quantitatively. It will be of particular interest whether only the final product of reversion, i.e. the wild-type sequence, is detectable or whether reversion might proceed stepwise or even follow multiple pathways. These studies include separation of circular viroids which might differ only in one nucleotide exchange. Due to the technique of non-denaturing polyacrylamide gel electrophoresis (ndPAGE) which was recently developed in our laboratory particularly for this purpose (Zimmat *et al.*, 1990), it is possible to separate viroids differing in one nucleotide only, to amplify the isolated sequences by PCR and to sequence them. Thus cloning and sequencing of a high number of clones for determination of a sequence distribution can be circumvented.

Both types of studies will confirm the earlier results on HP II, but in addition they will elucidate that for short term replication of viroids the secondary structure of HP II is essential and sufficient, while in the long run the exact wild-type sequence will overgrow transient mutations with an intact double-stranded HP II but an altered sequence. All data fit into a mechanistic model in which spontaneous reversions are generated during (−)strand synthesis; G:U pairs are tolerated in HP II of the (−)strand, and a diminished stability of the hairpin structure is compensated by additional mutations outside HP II which destabilize the rod-like structure too.

Results

Compensatory mutations in the core region of HP II

Compensatory mutations in the core region, i.e. exchange of a base pair in HP II, guarantee the intactness of the double-stranded structure but change the sequence. Thus, one might expect from studying the infectivity and genetic stability of those mutants not only confirmatory results of our earlier single-site mutation studies but also a clear differentiation between the functional impact of the structure and of the sequence of HP II.

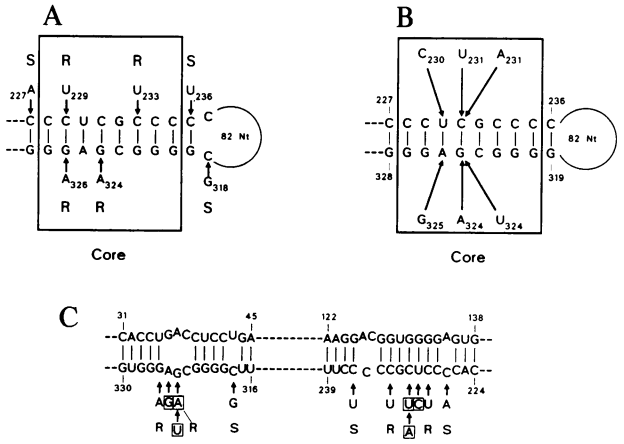

Fig. 2. Positions of single-and double-site mutations in PSTVd. (**A**) The single-site mutations are stable (S) or revert (R) to the wild-type sequence *in planta* (Loss *et al.*, 1991). The core of HP II, depicted in a box, contains the reversions; its border extends to the position of the stable mutants. (**B**) Position of the double-site mutations 230U→C/325A→G, 231C→U/324G→A and 231C→A/324G→U, which exchange base pairs in HP II. (**C**) Position of the single- and the double-site (boxed) mutations in the native, rod-like secondary structure of PSTVd. The single-site mutations are stable (S) or revert (R) to the wild-type (cf. A).

Choice of the mutations. Three different double mutations were introduced leading to base pair exchanges in the core region of HP II as depicted in Figure 2B. The influence of the mutations on the native rod-like structure of PSTVd is shown in Figure 2C (boxed-in letters). Only by the mutation 231C→A a base pair of the rod-like structure is destroyed; all other mutations are located in loops or exchange G:C for G:U pairs and vice versa; i.e. they leave the base pairing scheme unaltered. Apart from the structural considerations the double mutations of Figure 2B were chosen for the following reasons. Mutant 230U→C/325A→G replaces the single A:U pair in HP II by a G:C pair. Thus, the functional consequence of a 100% G:C HP II might be studied. With mutant 231C→U/324G→A it will be seen whether the reverting single-site mutation 324G→A will be changed into a genetically stable mutation by adding the compensatory mutation 231C→U. That mutation introduces two A:U pairs into HP II, which is a significant thermal destabilization. In fact, in nature a viroid does exist (chrysanthemum stunt viroid, CSVd) with a HP II containing two A:U pairs in the same position. Since CSVd is infectious to tomato (Niblett *et al.*, 1980), one might expect that two A:U pairs will be tolerated also with PSTVd. In CSVd, however, this second pair is of other polarity, i.e. 231A and 342U. Therefore, this polarity of an A:U pair was introduced as the third mutation (231C→A/324G→U), well realizing the resulting distortion of the rod-like structure.

Infectivity of the mutants. Tomato seedlings were inoculated with dimeric cDNAs of the wild-type and of the three double mutants, and 5 weeks later the plants were tested for viroid infection by bidirectional gel electrophoresis. The double mutants 230U→C/325A→G and 231C→U/324G→A were infectious, whereas no viroid could be detected after inoculation with the double mutant 231C→A/324G→U.

The double mutant 230U→C/325A→G was used also for a second plant passage. Viroid RNA was detected again after the second passage.

Genetic stability of the mutations. The sequences of the progenies from the infectious mutants were determined by direct RNA sequencing. For sequencing the segment 319–328 the primer RGV 1, for segment 227–236 the primer RGV 3 was used. To test for additional mutations in the rod-like structure opposite to the segments of HP II, the upper strand of the rod-like structure was sequenced using primers RGV 1 and RGV 5. Both double mutations were present in the first progeny; no clear indication for a reversion could be found. During the second plant passage, the mutant 230U→C/325A→G showed predominantly a reversion 325G→A, whereas the mutation 230U→C remained stable also in the second generation of progenies. Additional mutations in the upper strand of the rod-like structure could not be detected. Since the co-existence of stable mutations and reversions could be deduced only from sequencing gels, sequence variations of minor concentrations would not have been detected.

Mechanism of reversion of single-site mutations

As mentioned above it had been shown by Loss *et al.* (1991) that four single-site mutations introduced into the core region of HP II reverted to the wild-type sequence within the first passage, while three mutations in the periphery of HP II remained stable. Although the results could be interpreted in terms of the functional importance of HP II as a structural motif, the mechanism of this very fast reversion could not be elucidated. In particular, why non-viable mutations lead to fast reversions in some cases and do not grow at all in other cases (Owens and Hammond, 1990), is still an unsolved question.

In order to achieve a more mechanistic understanding of the behaviour of mutants in plants, the following strategy was applied. (i) Plants were inoculated with dimeric PSTVd cDNAs containing the mutations as shown in Figure 2A. The mutants designated as revertants (R) were inoculated to eight plants each; the mutants designated as stable (S) were inoculated to four plants each. (ii) From 3–6 weeks after inoculation the plants were checked individually for the presence of viroids by bidirectional gel electrophoresis. (iii) The samples containing viroids were applied to ndPAGE (and in one case to SSCP) in order to identify different sequence variants. (iv) The viroids in different bands were eluted from nd-2D-PAGE (Figure 3), reverse transcribed, PCR-amplified and their total sequence was determined.

Detection of viroids at different times after inoculation. The time course of the detectability of viroid RNA in individual plants inoculated with wild-type and with the mutant cDNA is listed in Table I. In plants inoculated with dimeric cDNA of wild-type sequence, viroids were detected in high concentration already 3 weeks after inoculation. This is in contrast to inoculations with the mutated dimeric cDNAs. For example in none of the plants inoculated with mutant 229, could viroids be detected 3 weeks after inoculation (cf. Figure 4). After 4 weeks viroids were detected in three plants (nos 4, 5 and 7), after 5 weeks viroids were detected in six of eight inoculated plants. In plant no. 3 viroids could not be detected reliably even 6 weeks after infection (data not

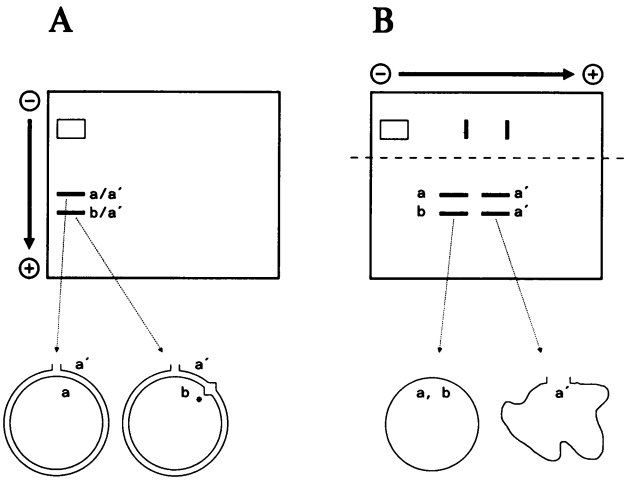

Fig. 3. Non-denaturing polyacrylamide gel electrophoresis (ndPAGE) and ndPAGE-coupled two-dimensional gel electrophoresis. (**A**) A partially fractionated RNA extract containing a mixture of two PSTVd strains, a and b, differing by a mutation, *, are hybridized to the transcript of pRH716 with (−)strand PSTVd [strain intermediate (DI)] sequence, a′. The RNA mixture containing the hybrids is applied to the single slot (upper left corner). During electrophoresis under native conditions, the duplexes of circles and linear transcripts are separated according to their different hydrodynamic stiffnesses. (**B**) For the second dimension, the gel from (A) was electrophoresed under denaturing conditions from left to right. Thus, circular molecules, a and b, are separated from the linear transcript a′. For identification of the horizontal position of circular and linear molecules, a mixture of circular and linear PSTVd was applied to the single slot; the upper part of the gel (above dashed line) was cut after electrophoresis and stained with silver. The vertical position of the different strains, a and b, was identified due to the visualization of the ^{32}P-labelled transcripts a′ after exposing the lower part of the gel to X-ray film.

Table I. Time course of infection in individual plants after inoculation with dimeric cDNA of wild-type and of single-site mutated sequences

Mutant	(Plants containing viroid)/(Plants inoculated)			
	3 weeks after inoculation	4 weeks after inoculation	5 weeks after inoculation	6 weeks after inoculation
Wild-type	8/8	8/8	8/8	8/8
229 (R)	0/8	3/8	6/8	6/8
233 (R)	0/8	8/8	8/8	8/8
324 (R)	4/8	8/8	8/8	8/8
326 (R)	0/8	2/8	8/8	8/8
227 (S)	2/4	3/4	4/4	4/4
236 (S)	3/4	4/4	4/4	4/4
318 (S)	1/4	3/4	4/4	4/4

(R) and (S) refer to reverting and stable mutations, respectively, as described in Figure 2 (cf. also Loss *et al.*, 1991). Presence of viroid was analysed by bidirectional gel electrophoresis. When plants in the same greenhouse were inoculated with buffer not containing viroid or viroid cDNA, no infections were observed; thus cross-contamination could be excluded.

shown). Although it cannot be confirmed by a statistical evaluation, the data of Table I 3 weeks after inoculation suggest as a general tendency that the infectivity of dimeric cDNA decreases gradually from the wild-type to stable mutants, and from stable mutants to revertants.

Analysis of sequence variants by ndPAGE. Mutant 229: after inoculation with mutant 229 well separated bands appear in

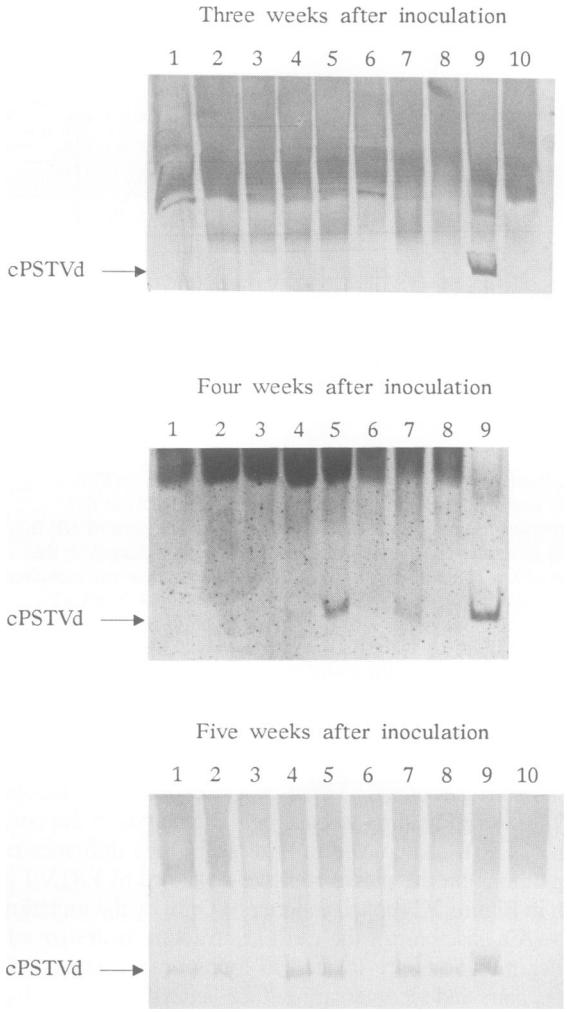

Fig. 4. Detection of viroid in plants inoculated with mutant 229. Bidirectional gel electrophoresis was applied for detection. Slots 1–8 show eight different RNA extracts derived from eight different plants. As a positive control, slot 9 contains an RNA extract from a plant inoculated with dimeric cDNA of wild-type DI PSTVd sequence. As a negative control, slot 10 contains an RNA extract from a non-inoculated plant. The position of circular PSTVd is indicated.

the analysis of viroid progenies by ndPAGE (Figure 5). Their relative intensities are different in each of the eight individual plants inoculated and vary with time (4, 5 and 6 weeks) after inoculation. Furthermore, different bands were detected in different plants. For an easier discussion, the bands are designated with numbers at the side of the gels. Plants Nos 4 and 7 exhibit two bands, plants Nos 1, 2 and 5 three bands and plant No. 8 only one band. The slowest band co-migrated with wild-type, from which mutant 318 as a control is clearly separated.

The band intensities cannot be compared quantitatively between different samples because of the numerous preparational steps involved; since the inoculation varies from plant to plant and with the time after inoculation, the concentration of viroids and the yield of viroid preparation was also variable and leads to signals of quite different intensity, particularly with samples from the heavily infected plants after 6 weeks. In spite of this variability 6 weeks after inoculation the band at the wild-type gel position prevailed, and only in plants Nos 5 and 7 were faster bands still

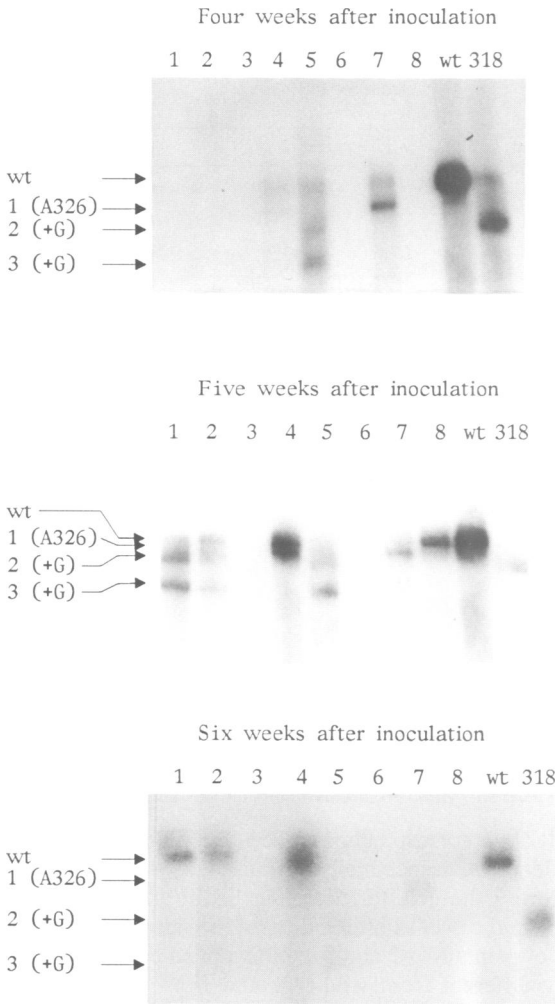

Fig. 5. Time course of appearance of mutated and reversed progeny as analysed by ndPAGE. Eight plants (slot numbers 1–8) were inoculated with dimeric cDNA of mutant 229. Partially fractionated RNA extracts were prepared at different times after inoculation as indicated, hybridized to ^{32}P-labelled transcript of pRH716, and the hybrids were analysed by ndPAGE. Slot wt shows hybridized RNA from a plant inoculated with dimeric cDNA of wild-type PSTVd sequence, slot 318 hybridized RNA from mutant 318. At the left side of the gels, the bands are designated as wt [position of hybrid of RNA of wild-type sequence and (−)strand transcript of pRH716], 1, 2 and 3. Inscriptions in brackets (left) designate the type of mutation (cf. below).

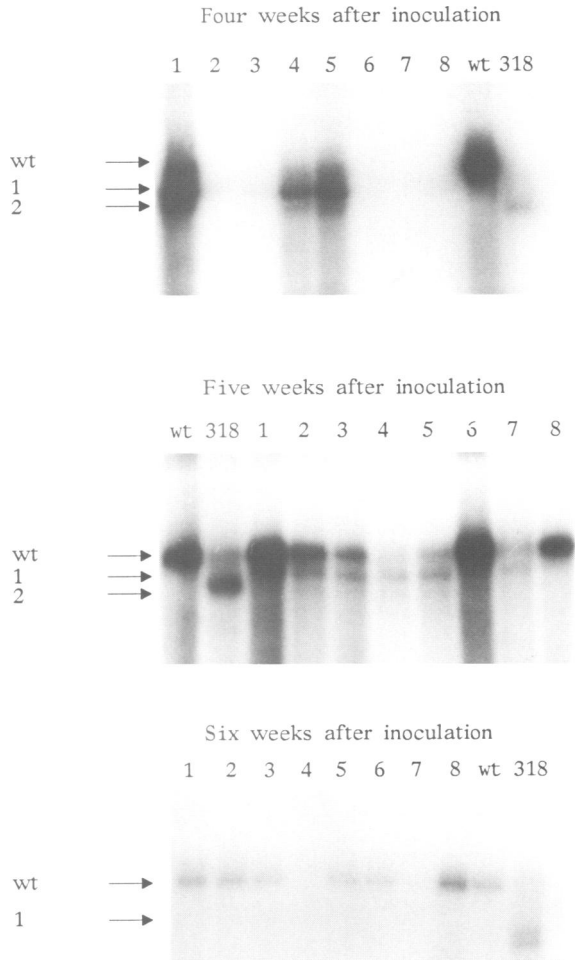

Fig. 6. Time course of appearance of mutated and reversed progeny after inoculation with dimeric cDNA of mutant 324 as analysed by ndPAGE. Otherwise see legend to Figure 5.

detectable. In summary, during the early course of infection multiple bands appeared and in the late phase only the band with the wild-type position remained.

Mutant 324: four weeks after inoculation extracts from plants Nos 1, 4 and 5 showed bands (1 and 2) running faster than the wild-type band (cf. Figure 6). During the following weeks bands 1 and 2 became fainter and the intensity of the band at the wild-type position increased. Six weeks after inoculation all samples except No. 4 showed only the wild-type band.

Mutant 326: in two from eight plants inoculated with mutant 326, a band running faster than the wild-type band was detected (data not shown). In samples from all other plants only bands with wild-type position were present. Six weeks after inoculation all plants exhibited only the band with wild-type position.

Mutant 227: in all samples only one band could be detected, and this band migrated slightly but significantly more slowly than that of the wild-type viroid (data not shown).

Mutant 318: in all samples only one band could be detected, and this band migrated faster than that of wild-type viroid. Thus, mutant 318 was used as a control (Figures, 4, 5 and 6).

Mutants 233 and 236: mutants 233 and 236 differ by a single C→U transition from wild-type viroid. When hybridized with the (−)strand transcript RH716, a G:C base pair is substituted by a G:U wobble pair, and this substitution was not expected to be detectable in ndPAGE. Thus, the experimental result of finding only bands at the wild-type position with mutants 233 and 236 (data not shown) is in accordance with the presence of the mutants as well as with wild-type viroid.

Since mutant 236 was described by Loss *et al.* (1991) as a stable mutant, the samples were used for sequence analysis without further attempts to test for sequence variations. Mutant 233, however, was described as a revertant and therefore we tried to follow the time course of reversion by other techniques than ndPAGE.

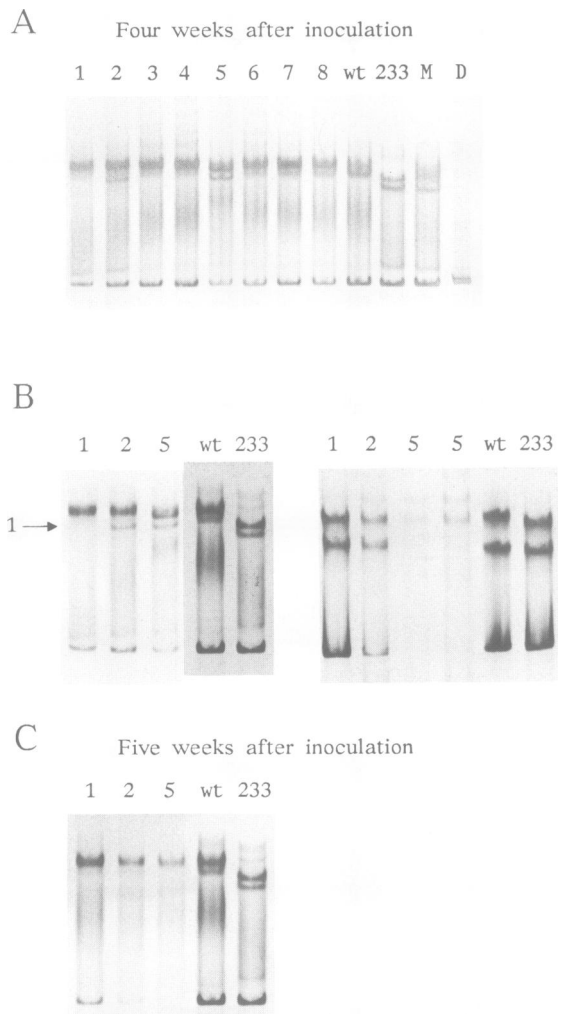

Fig. 7. The time course of appearance of mutated and reversed progeny after inoculation with dimeric cDNA of mutant 233 as analysed by SSCP. The viroid progenies were reverse transcribed and PCR-amplified. The resulting cDNAs were denatured with NaOH and applied to native gel electrophoresis. wt refers to cDNA amplified from wild-type PSTVd DI, 233 refers to mutant 233 cDNA, M refers to the mixture of both, D refers to the undenatured double-stranded cDNA. (**A**) and (**B**) show the analysis of samples prepared 4 weeks after inoculation. The samples of (A) and (**C**) were amplified with the primer pair RGV 5/QFV 6. In (B) the band patterns of DNA from plants Nos 1, 2 and 5 amplified with primer pair RGV 5/QFV 6 (left) and pair RGV 3/RZV 4 (right) are compared proving distinct DNA species if amplified with either primer pairs. At the left side of the gel in (B) the arrow marked 1 designates the bands in slots 2 and 5 which are sequenced. In (C) the analysis after 5 weeks is shown for viroids in plants 1, 2 and 5 proving the disappearance of the additional bands in plants Nos 2 and 5.

Analysis of sequence variants of mutant 233 by SSCP. Another simple method by which single-site mutations can be detected, is the single-strand conformation polymorphism (SSCP) (Orita *et al.*, 1989). Single-stranded cDNA is analysed instead of double-stranded RNA as in ndPAGE, thus the difficulty of differentiating between a G:C and a G:U pair may be avoided. Samples were reverse transcribed and amplified with primer pair RGV 5/QFV 6. Thus, the start and stop sites of the amplified cDNAs were positions 209 and 208, respectively. As a second primer pair RGV 3/RZV 4 corresponding to 355 and 354 as start and stop nucleotides, respectively, was used.

Table II. Sequences of PSTVd progenies

RNA (DNA) eluted			Sequence change				
Figure	Band	Slot	wild-type →		cDNA →		progeny
5	1	7	^{229}C	→	U	→	U
			^{326}G	→	G	→	A
5	2	5	^{229}C	→	U	→	U
5	3	5	^{322}GGGG	→	GGGG	→	GGGGG (insertion of G)
5	wt	8	^{229}C	→	U	→	C
7	1	5	^{233}C	→	U	→	U
			^{126}A	→	A	→	U
7	wt	1	^{233}C	→	U	→	C
6	1	4	^{324}G	→	A	→	A
6	2	4					
6	wt	2	^{324}G	→	A	→	G
data	1	5	^{326}G	→	A	→	A
not			^{144}C	→	C	→	U
shown	wt	8	^{326}G	→	A	→	G
	1	all	^{227}C	→	A	→	A
	1	all	^{236}C	→	U	→	U
	1	all	^{318}C	→	G	→	G

Sequence changes were determined in each case from one band of one gel slot; in each slot viroid from only one plant was analysed.

In Figure 7 the gels for detecting SSCP are shown for samples 4 and 5 weeks after inoculation with mutant 233. Samples of plants Nos 2 and 5 exhibit a band pattern which is clearly different from wild-type and from mutant 233. Samples from all other plants correspond to the wild-type band pattern although with varying relative intensities. Since different positions of bands correspond in some cases to different conformations and not to different sequences, the relative distribution of conformations may vary from sample to sample. The distinctly different positions of bands for samples of plants Nos 2 and 5 were confirmed with both sets of primers (cf. Figure 7B). If samples were prepared 5 weeks after inoculation, a pure wild-type band pattern was detected in the SSCP analysis of all plants (cf. Figure 7C).

Sequence analysis of PSTVd variants after inoculation with different mutants

From all bands, which were present in significant intensities in the gel analysis, RNA (or DNA after SSCP) was eluted, reverse transcribed, PCR-amplified and the amplified cDNA sequenced. Since in most cases more than one variant existed in a single isolate, nd-2D-PAGE was used to isolate a unique sequence variant of PSTVd. For the sequence analysis the isolate from a single plant was always used.

In Table II all results from the sequence analysis are listed. Designation of bands and plants, from which the RNA was eluted, was taken from Figures 5–7; in the other cases only one band (1) was obtained which migrated differently from the wild-type. Since the complete viroid sequence was determined from every band, a sequence change not only at the site of mutation or direct neighbourhood but also in distant segments of the viroid could be detected. In two cases the sequences from two different bands (bands 2 and 3 of 229C→U and bands 1 and 2 of 324G→A) were

found to be identical. This is possibly due to different mismatch arrangements in the heteroduplex of the viroid and the transcript.

The stable mutations in positions 227, 236 and 318, and the reversions in positions 229, 233, 324 and 326 as reported by Loss *et al.* (1991) could be confirmed in the present study. Among the revertants only mutant 324G→A could be detected with the mutated sequence up to 5 weeks. In the other revertants the site-directed mutation was detected in the plant always together with additional nucleotide changes (cf. bands 1–3 of 229C→U).

As an obvious summary of the sequence analysis it may be emphasized that not only stable mutations or direct revertants are viable, but also additional nucleotide changes introduced during plant infection lead to transiently multiplying viroids. In the case of mutant 229C→U three different types of infectious viroids were clearly generated, whereas the mutation originally inoculated is not viable.

Discussion

Functional relevance of structure versus sequence

In our earlier report (Loss *et al.*, 1991) we showed that site-specific mutations in the core region of HP II (see Figure 2) reverted to the wild-type sequence during the first plant passage, whereas mutations in the periphery remained stable in the progeny. From this finding we concluded that the structural integrity of the core of HP II is critical for infectivity. The stability of a site-directed mutation was determined by sequencing RNA extracts of 20 inoculated plants, i.e. the sequence of the dominant RNA species was determined. In this work the fate of the mutations was followed in individual plants, and not the average sequence but individual sequences which were present only in minor concentrations could be determined. Thus, more detailed information was expected. All sequence variations during reversion are presented in Table III together with their influence on the structure of HP II for the (+)strand as well as for the (−)strand.

In order to compare the present with the earlier results, the data of the sequence analysis at the end of the first passage, i.e. 6 weeks after inoculation, have to be considered. Mutations 227, 236 and 318 described in the earlier study as stable mutants (Loss *et al.*, 1991) were confirmed as such in every individual plant (cf. Table II). Mutations 229, 233, 324 and 326, revertants in the earlier study, were confirmed also in the present work as revertants in the majority of all plants (cf. Table III). A minor sequence heterogeneity, e.g. a low concentration of a different sequence in one out of eight plants, was not detectable in the earlier study.

Base pair exchanges (cf. Figure 2) were introduced in order to differentiate between the influence of changes in the structure and in the sequence. The double mutant 231C→A/324G→U was not infectious, most probably because the mutation 231C→A seriously disrupted a helix of the native rod-like structure (cf. below). The other two double mutants, 230U→C/325A→G and 231C→U/324G→A, are viable and their sequences are stable in the progeny of the first generation. Obviously, it has to be concluded that the structure is more critical than the sequence. In the second generation, however, which was

Table III. Schematic drawings of HP II of the mutants used for inoculation, of the resulting progeny, and the time course of their appearance during infection

Site-directed mutation[1)]		Progeny		number of plants / 8 inoculated plants after	
(+)strand	(-)strand	(+)strand	(-)strand	4 weeks	6 weeks
229C→U				2	1
				3	1
				6 (wt)	6 (wt)
233C→U		+126A->U	+126U->A	2	1
				7 (wt)	8 (wt)
324G→A				7	1
				8 (wt)	8 (wt)
326G→A		+144C->U	+144G->A	2	0
				8 (wt)	8 (wt)
231C→U/324G→A					
230U→C/325A→G		1. generation 2. generation			

[a]A cDNA of this sequence was used for inoculation of the plants.

analysed only for mutant 230U→C/325A→G, a single-site reversion 325G→A could be detected. Preliminary data (Hollstein, 1992) indicate that also in the double mutant 231C→U/324G→A a single-site reversion 231U→C occurs as the first step of reversion. Both results lead us to expect that complete reversion to the wild-type sequence might happen in future generations. We may argue from these considerations, and we will strengthen the arguments below, that the first level of selection or indispensable requirement inside the plant is the structural intactness of HP II, and that the reversion to the exact sequence of the wild-type may be regarded as an additional optimization.

Regarding the different types of revertants from one and the same single-site mutation 229C→U, the original mutation could not be detected at all in the progeny, probably because the structural distortion is too serious, especially in the (−)strand (cf. below). With the revertants three different ways to re-establish the structural intactness of HP II were found (cf. Table III), either in different plants or co-existing in one and the same plant. Regarding the (−)strand the A*C mismatch is either reverted to a G:C base pair, or transformed to an A:U base pair due to a compensatory mutation. As a third possibility an insertion of a C in the (−)strand extends the stem of HP II by 1 bp into the loop

region, thus compensating for the disruption of the stem due to the A*C mismatch at the opposite end of HP II. In this way an uninterrupted helix of 8 bp is re-established which is exactly the size of the indispensable core (cf. Figure 2). All these sequence variants are viable for some weeks, but after 6 weeks the revertant to wild-type has overgrown all other variants.

Reversion of the other mutants follows a somewhat different pathway. The single-site mutant 324G→A is viable as inoculated in most of the plants for some time. Considering the (−)strand HP II the G:U pair evidently keeps the structure intact for replication. Also in the (−)strand of the double mutant 230U→C/325A→G a G:U pair is generated in the second generation, most probably as an intermediate on the pathway to the wild-type sequence. With the mutant 326G→A the G:U pair in (−)strand HP II is tolerated too, but only in combination with an additional mutation 144G→A. In the case of mutant 233C→U even an A*C mismatch is tolerated, which is, however, a clear exception among all mutants studied.

If mutants with a G:U pair in the (−)strand HP II are viable without sequence change for some time before they are overgrown by the revertants, one should expect that they are viable in all infected plants. This is indeed (almost) the case for mutant 324G→A, where in seven out of eight inoculated plants the original mutant sequence was found. With the other mutants, 223C→U and 326G→A, only two out of eight plants show the original mutations in HP II; exactly in these mutants, however, an additional mutation was found which does not affect HP II. Obviously, the requirement of an additional mutation is the reason for the low frequency of the original mutation in the progeny.

We may summarize at this point, that (i) the earlier results (Loss *et al.*, 1991) could be confirmed quantitatively, (ii) the structural intactness of HP II, including G:U pairs, is the first prerequisite for infectivity, and (iii) the wild-type sequence of HP II indeed appears as the result of an optimization process leading to its long-term dominance. These conclusions are in good accordance with the suggestion mentioned earlier, that HP II mimics a cellular recognition signal, probably a GC-rich transcription factor binding site, although such a binding site could not yet be identified in the tomato genome.

Relevance of structural elements in the (+) and in the (−)strand during replication

As seen from Table III, three variants (324G→A, 326G→A, double mutant 230U→C/325A→G in the second generation) contain A*C mismatches in the (+)strand. In the (−)strand, however, only mutant 223C→U contains an A*C mismatch which has already been discussed above and needs further attention because of the additional mutation 126A→U (cf. below). The A*C mismatch in mutant 229C→U has not to be considered as a distortion of the (−)strand HP II because it was compensated by the extra G as discussed above. Therefore, the most simple and most systematic interpretation of all structures which replicate in the plant at least transiently would be (i) that the smallest deviation from an intact double-stranded HP II is a G:U pair,(ii) that G:U wobble pairs but not A*C mismatches are tolerated in the (−)strand and (iii) that HP II is most critical as a structural element of the (−)strand replication intermediate. This had been suggested earlier (Loss *et al.*, 1991) from mere functional considerations.

Competition of alternative secondary structures in replication intermediates

After inoculation with single-site mutants, additional mutations in other regions than the stem of HP II appeared, inferring an interdependence of both mutations via the rod-like structure. In Figure 8 the effect of the additional mutation 126A→U on the (+)strand (A) as well as on the (−)strand (B) is shown, for 144C→U only the (−)strand (C) with the corresponding G→A mutation is shown because no effect can be seen in the (+)strand.

The native structure of the (+)strand (Figure 8A, left side) is shifted due to the site-directed mutation 233C→U to an alternative base pairing scheme (right side) and is shifted back as an effect of the additional mutation 126A→U. The G:C-rich helix 128−134/234−228 would be distorted in the alternative base pairing scheme; distortion of the same helix in the double mutant 231C→A/324G→U led to non-viability of that mutant. This G:C-rich helix of native PSTVd might correspond to HP II of the (−)strand. One G:C and one A:U pair of HP II (G320−G327) are replaced by two G:U pairs in native PSTVd (G128−G134). From the studies of the transient structures during reversion one knows that those replacements do not destroy the function. Consequently, the G:C-rich helix in native PSTVd might act as transcription factor binding site for (−)strand synthesis in the same way as HP II for (+)strand synthesis.

Minus-strands are generated as multimeric intermediates in metastable structures containing HP II. These intermediates serve only as templates for (+)strand synthesis and should not fold into a thermodynamically stable structure. Thus, for the influence of the additional mutations on the (−)strand structures one has to consider a competition between the HP II-containing structure and a rod-like structure, especially that of lowest free energy. Since HP II is markedly destabilized by an A*C mismatch in one case (233C→U) and a G:U instead of a G:C pair in the other (326G→A), a stable rod-like structure could compete out the functionally important HP II. As can be seen from Figure 8B and C, both additional mutations drastically destabilize the rod-like (−)strand structure and thereby prevent the functionally unfavourable competition presented by this structure.

Thus, the additional mutations tend to restore the native rod-like structure of the wild-type (+)strand and compensate for a destabilized HP II in the (−)strand by destabilization of a rod-like (−)strand structure.

Mechanistic model of reversion

All experimental data of this work may easily be interpreted in mechanistic terms. We assume, as in our earlier work (Loss *et al.*, 1991), that from a double-stranded cDNA invading the cell a (−)strand RNA is transcribed which assumes a HP II-containing structure during synthesis. This transcription step is not affected by the mutations. The viability of the (−)strand RNA as replication intermediate inside the nucleus is not in contrast with the finding of very low infectivity when the inoculation was carried out with unprotected (−)strand RNA (Tabler and Sänger, 1985). The (−)strand with a heavily distorted HP II does not act as template for (+)strand synthesis. Only one G:U pair in HP II, as in 324G→A or in the second generation progeny of 231C→U/324G→A, is tolerated and leads to synthesis of progenies without further mutations. In all other cases the replication cycle stops at the stage of the (−)strand. It

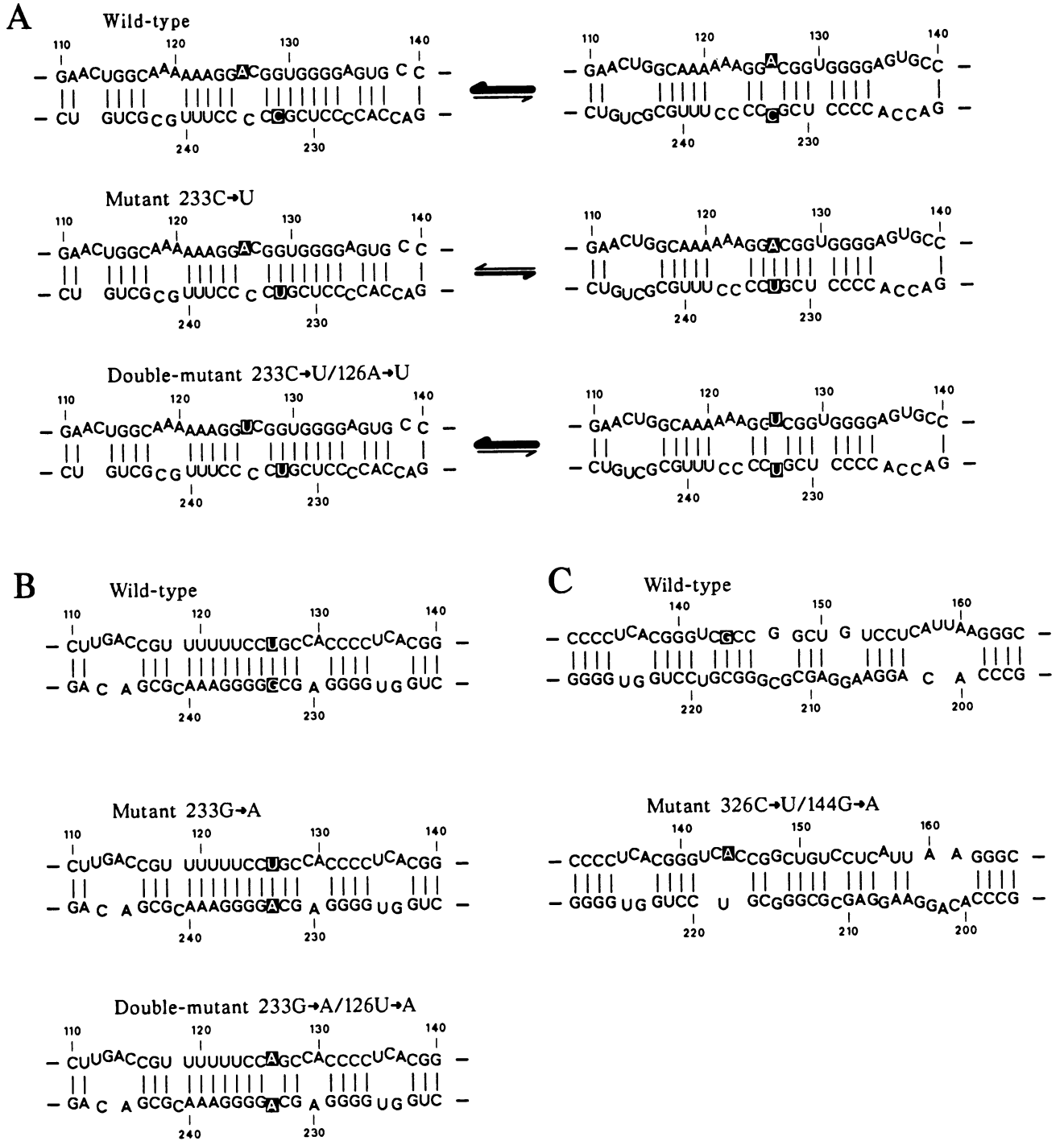

Fig. 8. Influence of single-site and additional mutations on the rod-like structure of the (+)strand (**A**) and of the (−)strand (**B** and **C**). White letters on a black background designate the positions of the mutations; the thick arrows in (A) indicate to which structure the equilibrium is shifted.

might proceed after two different types of events: first, due to the error rate of the synthesizing enzyme (DNA-dependent RNA polymerase II) a reversion to wild-type is introduced and thereby a functional HP II is re-established. Secondly, compensating mutations might happen which lead to a functional form of HP II. To these belong compensatory mutations (Table III, upper structure of 229C→U), insertions extending the length of the stem (middle structure of 229C→U), and additional mutations compensating for the lower stability of HP II by destabilization of the competing rod-like structure of the (−)strand (233C→U, 326G→A). One should emphasize that the stop of the replication cycle is overcome in all cases by a single-site mutation. The rare event of a double mutation after inoculation has never been observed. For reversion of a double mutant it was found that only the first step had occurred in the second generation.

The pause of the replication cycle and the wait for the reversion or a compensatory mutation could indeed be observed experimentally. Although not followed continuously, it is obvious from the data 3 weeks after inoculation (cf. Table I) that the wild-type replicates fastest, and that the stable mutations also produce progenies already 3 weeks after infections. The revertants 229, 233 and 326 do not exhibit infections because they are still waiting for reversion

or compensatory mutation. Only revertant 324 produced progenies because it grows without reversion or compensatory mutation (cf. Table III).

One cannot assume that mutants with additional mutations revert to the wild-type, which would indeed be a double mutation. Because of the better adaptation of the wild-type HP II to cellular recognition structures, probably those of transcription factors, the wild-type grows fastest and all other sequence variants die out.

Since inoculation was carried out with double-stranded cDNA, (−)strand RNA as well as (+)strand RNA might be transcribed in the first step. Both processes lead to infections as described by Tabler and Sänger (1984). The possibility of (+)strand transcription does not change our mechanistic considerations outlined above. The (+)strand would be processed to (+)strand circular viroids carrying the site-directed mutation without the need for reversion or compensatory mutations, because HP II is not involved in the processing reactions (cf. Steger *et al.*, 1992). For the amplification of this type of circular viroid, which carries the mutation but is generated only by processing not by replication, all considerations about the mechanism of reversion outlined above are equally true.

Materials and methods

Buffers and enzymes

Enzymes were obtained from Boehringer Mannheim, Pharmacia and New England Biolabs and were used according to the suppliers' instructions.

Synthetic oligonucleotides

In total six primers for site-directed mutagenesis and five primers for sequencing were used.

Mutagenesis primers [5′→3′, numbers indicate the corresponding nucleotides in the (+)strand]:
(230 C) ^{244}GCGCAAAGGGGGCGGGGGGTGGTCC^{220},
(231 U) ^{244}GCGCAAAGGGGGCAAGGGGTGGTCC^{220},
(231 A) ^{244}GCGCAAAGGGGGCTAGGGGTGGTCC^{220},
(324 A) ^{338}GGGCTAAACACCCTTGCCCCGAAGC^{314},
(324 U) ^{338}GGGCTAAACACCCTAGCCCCGAAGC^{314} and
(325 G) ^{338}GGGCTAAACACCCCCGCCCCGAAGC^{314}.

DNA sequencing primers (5′→3′): (M13 primer) GTAAAACGACGGCCAGT and mutagenesis primer 325 G.

RNA sequencing primers (Gruner, 1992) (5′→3′):
(RVG 1) ^{87}CCTGAAGCGCTCCTCCGAG^{69},
(RGV 3) ^{354}CCAACTGCGGTTCCAAGGGCTAAACACC^{327}, and
(RGV 5) ^{208}GGAAGGACACCCGAAGAAAGGAAGGGTGAAA^{178}.

The following synthetic oligonucleotides were used for reverse transcription, PCR amplification of PSTVd sequence variants and sequencing of PCR products (5′→3′):
(RZV 2) ^{88}GATCCCCGGGGAAACCTGGAGCGAA^{112}, RGV 3,
(RZV 4) ^{356}TCCTCGGAACTAAACTCGAGGTTCCTGTGG^{26}, RGV5, and (QFV 6) ^{209}TCGCGCCCGCAGGACCAC^{226}.
RGV 3/RZV 4 amplified cDNA with ends at the left terminal region of PSTVd, RVG 5/QFV 6 amplifid cDNA with ends at the right terminal region of PSTVd.

Bacterial strains

Escherichia coli strains BMH71-18, BMH71-18mutS and MK30-3 were used to propagate and mutagenize M13 clones. They were a gift from Drs L.Altschmid and W.Hillen, University of Erlangen. The *E.coli* strain DH5a (BRL) was used in combination with the phagemid vector pBluescript KS(+) (Stratagene).

Vectors

The M13mp9/M13mp9rev system (Kramer *et al.*, 1984) was used for site-directed mutagenesis. The phagemid pBluescript KS(+) (Stratagene) was used for cloning the generated mutants as dimer molecules in head-to-tail orientation.

Infection of plants and collection of PSTVd samples

Dimeric cDNA molecules of seven different PSTVd mutants with single-site mutations (Loss *et al.*, 1991) and of wild-type [strain intermediate (DI)] were used to inoculate tomato plants (*Lycopersicon esculentum cv.* Rutgers) with 1 μg of cDNA per plant.

For each mutant either four or eight plants were inoculated. Plants inoculated with wild-type cDNA or just with inoculation buffer were taken as positive or negative controls, respectively. The plants were placed in such a way that a direct contact between neighbouring plants was avoided. From 3 to 6 weeks after inoculation, 1−2 g plant leaves were collected weekly from every plant. These leaves were subjected separately to nucleic acid extraction and PSTVd analysis by bidirectional gel electrophoresis (Schumacher *et al.*, 1983).

Dimeric cDNA molecules of the three different PSTVd mutants with double mutations were used to inoculate tomato plants (*L.esculentum cv.* Rutgers) with 1 μg dimeric cDNA per plant. After 5−6 weeks 1 g of tomato leaves was analysed for the presence of circular PSTVd (Schumacher *et al.*, 1983), and 1−2 g of systemically infected tomato leaves (carrying ~1 μg extractable viroid RNA) were homogenized, and new tomato seedlings were inoculated with 100 ng viroid per tomato seedling.

Preparation of PSTVd RNA

Tests for PSTVd infections in tomato plants were carried out by bidirectional gel electrophoresis (Schumacher *et al.*, 1983). For RNA sequencing a larger amount and greater purity of PSTVd was achieved by a preparation procedure including a HPLC purification step (Colpan *et al.*, 1983; Riesner *et al.*, 1987).

Resolution of multiple sequences in PSTVd samples by ndPAGE

The ^{32}P radioactively labelled monomeric (−)strand of PSTVd, RH716, was transcribed from plasmid pRH716 (Hecker, 1989). The PSTVd samples were hybridized with the ^{32}P-labelled RH716 (Hecker *et al.*, 1988) and electrophoresed in a ndPAGE (Zimmat *et al.*, 1990). If the sequences of the circular PSTVd and the linear (−)strand transcript are exactly complementary, the corresponding homoduplex migrates more slowly than a heteroduplex formed by a sequence variant of PSTVd with the transcript of the wild-type. The slab gel contained 5% acrylamide (30:1), 8 M urea, 10% glycerol, TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA), 0.13% TEMED and 0.07% ammonium peroxodisulfate. The electrophoresis was carried out for 12−16 h at 25°C and 150 V in TBE buffer using a thermostated apparatus. In spite of the presence of 8 M urea native conditions for double-stranded RNA were guaranteed, but it was found that the resolution of homo- and heteroduplices increased due to the presence of 8 M urea. The gel was exposed to X-ray film for 1−2 days.

Isolation and sequence analysis of single PSTVd strains

For isolation of a single PSTVd sequence variant, a ndPAGE-coupled two-dimensional gel electrophoresis (nd-2D-PAGE) was developed (Figure 3). In the first dimension, the different hybrids formed between RH716 and different PSTVd strains were separated by ndPAGE. In the second dimension, electrophoresis was carried out under denaturing conditions, in which the hybrids were denatured to linear RH716 and circular viroid strains. Circular PSTVd molecules migrate much more slowly than the linear PSTVd molecules including the *in vitro* transcripts, so that both can be separated. In order to identify the positions of circular and linear molecules, a mixture of circular PSTVd and linear *in vitro* transcript was loaded on to the marker slot prior to electrophoresis of the second dimension. After autoradiography of the transcripts and silver staining of the upper part of the gel, the positions of different PSTVd sequence variants can be defined. The gel fragments containing PSTVd variants were excised and the RNAs were eluted. They were reverse transcribed and PCR-amplified into full length cDNA (Innis and Gelfand, 1990). For sequence analysis of the PCR products, the TAQence (version 2.0) sequencing kit purchased from USB (OH, USA) was used with minor modifications.

Single-strand conformation polymorphism (SSCP)

If the PSTVd progenies of a mutant could not be resolved from wild-type in ndPAGE, the SSCP technique was applied to check for the existence of sequence variant(s). The viroid progenies were reverse transcribed and PCR-amplified (Innis and Gelfand, 1990), the PCR products were subjected to analysis with SSCP (Orita *et al.*, 1989). For our purpose the SSCP procedure was modified in the following steps: (i) the PCR products were denatured with NaOH instead of heating to achieve a high yield of single strands, (ii) a gel of 1.5 mm instead of 0.4 mm thickness was used for easier handling,(iii) silver staining instead of radioactive labelling was used to visualize the signals.

DNA isolation

Small scale plasmid isolation was done according to the method of Birnboim and Doly (1979). Large scale preparations of plasmids and M13 RF-DNA

were carried out as described by Hillen *et al.* (1981). M13 single-stranded DNA template for mutagenesis and dot blot analysis was isolated following the procedure of Messing (1983).

Site-directed mutagenesis

Site-directed mutagenesis of the monomeric PSTVd cDNA was carried out using the gapped-duplex method described by Kramer *et al.* (1984). For introducing double mutations the PSTVd cDNA was hybridized with two mutagenic oligonucleotides. As a modification to the procedure cited above a 2000 times molar excess of the first oligonucleotide and a 4000 times molar excess of the second oligonucleotide over the phage was used for inducing the double mutation in a one-tube reaction. The molar excess was 10 and 20 times, respectively, higher than described by Kramer *et al.* (1984). The rate of mutation ranged from 20 to 50%. The potential mutants were prescreened by dot blot analysis (Dalbadie-McFarland *et al.*, 1982) and verified by sequencing.

Molecular cloning of monomeric and dimeric PSTVd cDNA

The monomeric PSTVd (intermediate strain) cDNA (Cress *et al.*, 1983) was cloned into the *Sma*I site of an M13mp9amE vector (Kramer *et al.*, 1984) by using standard techniques (Maniatis *et al.*, 1982). After mutagenesis the altered cDNAs were separated by agarose gel electrophoresis according to the procedure described by Lizardi *et al.* (1984) and self-ligated. Resulting dimers were purified using agarose gel electrophoresis. The mutant head-to-tail dimers were cloned into the *Sma*I site of the vector pBluescript KS(+).

Sequence analysis

After mutagenesis the double mutations were analysed in the M13 vector. For sequencing the T7 sequencing kit (Pharmacia) and the appropriate primer (cf. above) were used. PSTVd isolates from infected plants were sequenced according to Zimmern and Kaesberg (1981) using a 10 times higher concentration of primers (Gruner, 1992).

Theoretical structure analysis of RNA

The theoretical analysis of the secondary structures and structural transitions of the PSTVd sequence variants and of the (−)strand replication intermediates was carried out using an algorithm which was described in detail by Schmitz and Steger (1992). It is an extension of the algorithm of Nussinov *et al.* (1978) and Zuker (1989) and its modified version from Steger *et al.* (1984). All calculations were carried out with a VAXstation II (Digital Equipment Corporation).

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