Structural sites specific to citrus viroid groups

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Synthesis of cDNA probes by random-priming of a viroid template displays the unusual property of specificity to all members included within a single citrus viroid Group. The specificity of hybridization reactions was influenced by the structural conformation of the viroid RNA template, reaction conditions for reverse transcription and hybridization protocols. Mapping the loci for probe transcription from the CEVd, CVd-IIb, and CVd-IV genomes suggests that a similar structured conformation may be responsible for group specificity. A stem–loop configuration in the viroid template and hybridization target sites can be proposed to be responsible for the availability of the group-specific sequences.

Introduction

The genus Citrus harbours the largest collection of different viroids of any single plant group. The characterization of five distinct groups of citrus viroids was accomplished by molecular size, host range, and symptom reaction on the citrus indicator citron (Citrus medica L.) (Duran-Vila et al., 1988; Semancik & Duran-Vila, 1991).

Citrus exocortis viroid (CEVd) has been well described and constitutes a single viroid ‘group’ distinct from the remaining citrus viroids. The citrus viroid I Group (CVd-I) contains viroids related to the apple scar skin viroid (ASSVd) family. CVd-Ia and CVd-Ib, which was renamed as citrus bent leaf viroid (CBLVd) following sequence determination (Ashulin et al., 1991), are in the size range of 327–315 nucleotides (nt) (unpublished data). Citrus viroid II Group includes CVd-IIa, CVd-IIb or citrus cachexia viroid (CCaVd) (Semancik et al., 1988), and CVd-IIc in the size range of 302–297 nt and are all related in the hop stunt viroid (HSVd) family.

The citrus viroid III Group (containing CVd-IIIa, CVd-IIIb, CVd-IIIc and CVd-IIIId) with an estimated size range of 295–280 nt (Semancik & Duran-Vila, 1991) has been poorly described until the recent determination of nucleotide sequence of CVd-IIIa (297 nt) and CVd-IIIb (294 nt) as related to the ASSVd structure (Rakowski et al., 1994). The single member of the citrus viroid IV Group has been sequenced at 284 nt by Puchta et al. (1991).

In an attempt to determine group relatedness, molecular hybridization among the CVd groups was tested (Semancik & Duran-Vila, 1991). The analyses exploited the novel finding that cDNA probes generated by a random-priming protocol displayed specificity to all known members of the respective citrus viroid Groups.

This report describes factors influencing the synthesis and hybridization of cDNA probes generated with random hexamers. Structural features of the viroid templates which may function to confer specificity among the citrus viroid Groups are presented.

Methods

Viroid sources. Single viroid isolates of CEVd, CVd-IIb, and CVd-IV were selected for study from the Citrus Viroid Collection of the University of California, Riverside, as representative of three CVd groups with the common feature of being related to the PSTVd family. Mixed viroid isolates E804 (CVd-IIIa and CVd-IV) and E821 (CVd-Ib, CVd-IIa, CVd-IIb, and CVd-IIIb) provided a broad range of citrus viroids for hybridization tests. The viroids were propagated by graft inoculation to citron (Citrus medica L.) and maintained at elevated daily glasshouse temperatures (35–40°C) for extended intervals (10–15 h) to maximize symptom expression and viroid titre.

Nucleic acid extraction and viroid purification. Leaf tissue was extracted as previously reported (Semancik et al., 1988). Viroid RNA was extracted from alternate herbaceous hosts Gymara aurantiaca for CEVd and Cucumis sativus cv. Suyo for CVd-IIb and CVd-IV. Detection and isolation of viroid RNA employed sequential polyacrylamide gel electrophoresis (sPAGE) (Semancik & Harper, 1984; Rivera-Bustamante et al., 1986). Purified circular and ‘natural’ linear forms of the viroids recovered from plant extracts were utilized as templates for cDNA synthesis after electrophoresion from 8 M-urea-containing gels using an IBI unidirectional apparatus. The concentration of the purified viroid was estimated by the intensity of the silver-stained band (Igloi, 1983).

Production of cDNA probes. Reverse transcription was accomplished by the random-priming procedure described by Sambrook et al. (1989).
Table 1. Identification of primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' → 3'</th>
<th>Viroid nucleotides</th>
</tr>
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<tbody>
<tr>
<td>CEVd-1</td>
<td>CGGGGATACCTGAAGGAC</td>
<td>80–98</td>
</tr>
<tr>
<td>CEVd-2</td>
<td>GGAAACTCTGAGGAAGTGC</td>
<td>99–117</td>
</tr>
<tr>
<td>CEVd-3↑</td>
<td>GAGGACCGTGAAGAATCCAGGAGA</td>
<td>307–330</td>
</tr>
<tr>
<td>HSVd-1</td>
<td>GTGCCCGGGGCTCTCTTCTTCTGAGAACGA</td>
<td>72–88</td>
</tr>
<tr>
<td>HSVd-2</td>
<td>TCTTCTCGAAACACCCGAGG</td>
<td>89–106</td>
</tr>
<tr>
<td>CVD-IV-1</td>
<td>GAGCATGGATGTGAGCCACGC</td>
<td>160–181</td>
</tr>
<tr>
<td>CVD-IV-2</td>
<td>GCCCTTCCTCCGCGGACCTG</td>
<td>182–201</td>
</tr>
</tbody>
</table>

* Nucleotide number in the viroid sequence for CEVd (Gross et al., 1982), HSVd (Sano et al., 1989) and CVD-IV (Puchta et al., 1991).
† We acknowledge this primer as a gift from Prof. R. Symonds. The primer is constructed with a 12 nucleotide (5' CCCGATTCTAA) EcoRI tail attached to the 5' end of the CEVd sequence.

The viroid template (0.5–1 μg) and random hexamer primer (1–5 μg) were held at 24 to 100 °C for 2 min, snap cooled in ice and annealed at room temperature for 1 h. The random-primed cDNA probes were synthesized using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco-BRL) and labelled with [γ-32P]ATP. The cDNA was purified through Qiagen tip 5 columns, and concentrated by ethanol precipitation.

Characterization of cDNA. The cDNA product was analysed by 15 % PAGE containing 8 M-urea. Synthetic oligonucleotides (50-mer, 35-mer and 20-mer) used as molecular size markers were end-labelled with [γ-32P]ATP. The cDNA was purified through Qiagen tip 5 columns, and concentrated by ethanol precipitation.

Reverse transcription (RT) and polymerase chain reactions (PCR). The primers used for the specific viroid amplifications are presented in Table 1. An aliquot of a viroid-enriched 2 M-LiCl-soluble nucleic acid preparation equivalent to 20 mg of fresh tissue plus 50 pmol of first-strand primer in 20 μl reactions were heated to 80 °C for 1 min, snap-cooled in ice, and annealed for 10 min at room temperature. The reverse transcription (RT) reaction was essentially performed as described above. Added to the mix were 40 units of RNasin and 20 units of M-MLV reverse transcriptase. The reaction was incubated at 37 °C for 1 h.

An aliquot (2 μl) from the RT reaction (20 μl) was added to a 20 μl reaction mix containing: Taq buffer, 1.5 mM-MgC2O, 0.25 mM-dNTP mix, first- and second-strand primers at 10 pmol per reaction (Innes et al., 1990). Taq DNA polymerase (1 unit) was added, mixed, and overlaid with mineral oil. The PCR reaction was accomplished in an Ercopp thermocycler for 30 cycles at 92 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min. A final extension was done for 15 min at 72 °C.

Evaluation of the PCR reactions was done in 2 % agarose gels. When samples were used for blotting, 10 μl of the reaction was loaded on a 5 % polyacrylamide gel and electrophoresed for 3 h at 50 mA. The size of the PCR products was estimated with pGEM (Promega) molecular weight markers. The gel was stained with ethidium bromide and observed under UV light. The PCR products subjected to restriction enzyme mapping or sequence analysis were fractionated in a 2 % low melting agarose gel and purified using a Magic Prep column according to the manufacturer’s instructions (Promega).

Direct PCR sequencing. Viroid PCR products reported here were sequenced using the fmol sequencing system (Promega) with the 5' end-labelled primer protocol. The amplification was done for 30 cycles at 95 °C for 20 s, 55 °C for 30 s, and 70 °C for 30 s.

Asymmetric PCR amplification. The ability of the random-primed viroid-specific cDNA probe to function as a PCR primer was evaluated. Full-length viroid PCR products were used as templates for linear amplification (Innes et al., 1988). The template was combined with 32P-labelled cDNA probe, Taq buffer, 3 mM-MgCl2, 0.25 mM-dNTPs, 1.5 units of Taq DNA polymerase and water to a final volume of 20 μl. As controls, end-labelled synthetic oligonucleotides were assayed in parallel reactions. The products were evaluated on 5 % polyacrylamide 7 M-urea sequencing gels using a full-length viroid sequencing ladder for reference.

Fig. 1. Autoradiographs after slot blot (a, b) or electrotransfer (d, e) hybridization with 32P-labelled random-primed cDNA probes from citrus viroids, CEVd, CVD-IV, CVD-Ib, CVD-Ib, CVD-Ib, and CVD-Ib. A section from an 8 M-urea gel with citrus viroids, CEVd, CVD-IV, CVD-Ib, CVD-Ib, and CVD-Ib after silver staining is also shown (c).
for reverse transcription, the cDNA obtained was highly specific to CEVd [Fig. 1a (i), slots 1 to 3]. No reactions were observed on slot blots with isolates composed of heterologous viroids (CVd-IIIa, CVd-IV) [Fig. 1a (i), slots 4 to 6]; (CVd-Ib, CVd-IIa, CVd-IIb, CVd-IIIb) [Fig. 1a (ii), slots 1 to 3]; and healthy citron samples [Fig. 1a (ii), slots 4 to 6]. Specificity was also demonstrated with samples electrotransferred from denaturing gels (Fig. 1d).

However, when the linear form of CEVd was used as a template, specificity was lost in slot blot assays (Fig. 1b). Strong hybridization signals were detected with extracts containing all the viroid isolates as well as the healthy citron. Nevertheless, when the same samples were electrotransferred from 8 M-urea gels (Fig. 1e), the specificity was again observed. This distinction in specificity noted with the different hybridization protocol might suggest that denaturation of the viroid target molecules was not maintained with electrotransfer from the 8 M-urea-containing gel.

This interpretation was supported by strong hybridization signals obtained when cDNA probes synthesized from both circular and linear CEVd templates were utilized with samples electrotransferred from 5% polyacrylamide gels in the absence of any denaturing agents.

Restriction enzyme analysis of viroid PCR products. Full-length viroid PCR dsDNA products obtained from CEVd, CVd-IIb, and CVd-IV were separated on 2% low melting agarose gels. The restriction enzymes were selected using the GCG (Genetic Computer Group) mapping program (Schroeder & Blattner, 1982). The restriction enzyme patterns were analysed on a 7% polyacrylamide gel. The nucleic acids were electrotransferred to nylon membranes (MagnaGraph; MSI) with 0.1 μm pore size and the DNA was denatured with 0.4 M-NaOH.

The restriction fragments were hybridized with each specific cDNA probe at 42 °C for 24-48 h and washed twice in 6 × SSC and 0.1% SDS at room temperature followed by two washes in 1 × SSC at 37 °C.

Sequence analysis. The sequence data obtained with the fmol sequencing system was analysed using the GCG program. The region in which the specific cDNA probes mapped was analysed for the probability of stem-loop configurations in the secondary structure.

Results

Viroid structure affects probe synthesis and hybridization

The structure and conformation of the viroid was critical to template activity as well as hybridization reactivity. When circular CEVd molecules were used as the template for reverse transcription, the cDNA obtained was highly specific to CEVd [Fig. 1a (i), slots 1 to 3]. No reactions were observed on slot blots with isolates composed of heterologous viroids (CVd-IIIa, CVd-IV) [Fig. 1a (i), slots 4 to 6]; (CVd-Ib, CVd-IIa, CVd-IIb, CVd-IIIb) [Fig. 1a (ii), slots 1 to 3]; and healthy citron samples [Fig. 1a (ii), slots 4 to 6]. Specificity was also demonstrated with samples electrotransferred from denaturing gels (Fig. 1d).

However, when the linear form of CEVd was used as a template, specificity was lost in slot blot assays (Fig. 1b). Strong hybridization signals were detected with extracts containing all the viroid isolates as well as the healthy citron. Nevertheless, when the same samples were electrotransferred from 8 M-urea gels (Fig. 1e), the specificity was again observed. This distinction in specificity noted with the different hybridization protocol might suggest that denaturation of the viroid target molecules was not maintained with electrotransfer from the 8 M-urea-containing gel.

This interpretation was supported by strong hybridization signals obtained when cDNA probes synthesized from both circular and linear CEVd templates were utilized with samples electrotransferred from 5% polyacrylamide gels in the absence of any denaturing agents.
or conditions (data not shown). The positive homologous reaction to CEVd indicates a highly structured configuration which remains available for hybridization even under non-denaturing conditions.

**Template–primer binding conditions and probe specificity**

The effect of temperature on the template–primer interaction was evaluated using both circular and linear forms of CEVd. It was possible to generate cDNA probes by random-priming reverse transcription at 24 °C. The probes produced from both circular and linear structures at this temperature displayed specificity for CEVd in slot blot hybridization (Fig. 2a, b). However, the total incorporation of [α-32P]dCTP at 24 °C was 10-fold lower than in the cDNA product following denaturation at 100 °C.

With increasing temperatures of template–primer denaturation (60–100 °C) the specificity of probes from the circular template was retained (Fig. 2c). Probes generated from the linear template lost all specificity and reacted with sample containing heterologous viroids as well as healthy control extracts (Fig. 2d). As a result, only circular viroid preparations were employed as templates in the succeeding studies, with denaturing conditions of 100 °C for 1 min.

**Size and mapping of specific cDNA probes on viroid genomes**

When the molecular size of the cDNA probes generated from different viroids was estimated by relative mobility, the product from the CEVd template appeared as a close doublet (Fig. 3 lane 1) migrating in the region of the 50 base standard. Both CVd-IIb (Fig. 3 lane 2) and CVd-IV (Fig. 3 lane 3) templates resulted in a cDNA product in the range of the 35 base oligonucleotide standard.

Hybridization of restriction fragments from the three viroid PCR products with the corresponding cDNA probes permitted the mapping of each locus for synthesis of the specific probes. The principal hybridization signals obtained with each digestion pattern identified the region of the viroid genome from which the cDNA had been transcribed. The patterns presented in Fig. 4 display restriction enzyme digestion (a) and hybridization signal (c) for CVd-IV and illustrate the data obtained from this

Fig. 4. Ethidium bromide-stained polyacrylamide gel (a, b) and matching autoradiographs (c, d) of full-length CVd-IV dsDNA PCR product (lane 1) digested with *AvaI* (lane 2), *BstNI* (lane 3), *SmaI* (lane 4), *TaqI* (lane 5), *BstNI + TaqI* (lane 6), *BstNI + SmaI* (lane 7) after hybridization with random-primed cDNA to CVd-IV template (c) or end-labelled synthetic oligonucleotide CVd-IV-2 (d). Full-length CVd-IV product (285 bp) and the smallest restriction fragment (66 bp) are indicated.
approach. An identical membrane (Fig. 4b) hybridized with the end-labelled synthetic oligonucleotide primer Cvd-IV-2. Fig. 4(d) demonstrates the efficacy of this protocol with very small restriction fragments.

With analysis of hybridization signals from digestion with different enzymes (Fig. 5), the location delimited by the AvaII site at nucleotide 20 and the BamHI site at nucleotide 64 was identified as the template locus for synthesis of the cDNA probe to Cvd-IV. The limits of the hybridization signal for CEVd were the HhaI fragment at nucleotide 233 and the AvaII site at nucleotide 366. With Cvd-IIb, the probe template site was localized between the PvuI site at nucleotide 171 and the DdeI site at nucleotide 260.

To confirm these data, asymmetric PCR amplifications were performed using each specific cDNA probe as extension primer against the full-length dsDNA PCR products from each viroid. As internal controls, end-labelled synthetic oligonucleotides were similarly assayed (data not shown).

With CEVd, one amplification product (about 300 nt) was obtained with the specific cDNA probe. Primer CEVd-3 produced a larger extension product with the priming site located at nucleotide 330, while the CEVd-1 primer used for first-strand synthesis of the PCR dsDNA product gave a full-length product. Cvd-IIb produced a single main band at nucleotide 250, while the Cvd-IV product was located at nucleotide 45. These sites in the three viroid genomes were contained within the regions identified as the loci for synthesis of the cDNA probes by the restriction digest assay.

Structural configurations and specific probe synthesis

Loci for cDNA probe synthesis within each viroid genome from restriction digest hybridization and asymmetric amplification are depicted in Fig. 6. The sites are located in the left portion of the viroid sequence spanning the pathogenicity and central conserved domains (Keese & Symons, 1985). For CEVd and Cvd-IIb, the lower strand of the model is implicated and for Cvd-IV, the upper.

Regions for synthesis of specific cDNA localized in the overall viroid rod-like structures were analysed for proximity to conformational features, like stem-loop configurations, which might influence the reactions noted.

![Fig. 5. Pattern of restriction enzyme fragments with hybridization against Cvd-IV-specific probe (shaded). The position of the primers Cvd-IV-1 (P-1) and Cvd-IV-2 (P-2) used for PCR amplification are indicated.](image-url)
Fig. 6. Positions of sites for random priming cDNA (bar) for CEVd, CVd-IIb, and CVd-IV. Nucleotides included in boxes represent the palindromic sequences in the stems delimiting the loop structures.
Citrus viroid-specific probes

![Diagram showing predicted stem and loop nucleotide sequences for CEVd, CVd-IIb, and CVd-IV]

Loop sequences

(a) G-C C-G G-C C-G C-G C-U 249 G-C 329
(b) G-C C-G G-C G-C C-G 221 G-C 273
(c) G-U U-G 239 C-G 339

(d) AGCUUCUCUCUGAGAUUACCAGGAAACUACCCGAAGCUUACCCCAAACGCCUUUCUUAUAUCUUCACUGCUCU

(e) UGGCAUCACCUCUGGUUCGUCGUCCAUACCUGCUUUGUCUAUCUGAGCC

(f) AGUUUUCUCUGCGGAACCAUAUACAGCUGCUGGAGGAACAUACCUGAGAGGGA

Fig. 7. Depiction of predicted stem and loop nucleotide sequences in CEVd (a, d), CVd-IIb (b, e), and CVd-IV (c, f). The asterisk represents the priming site derived from asymmetric amplification.

Discussion

The importance of site-specific nucleotide sequence variation within the viroid molecule has been well substantiated and led to the convenient partitioning of the viroid genome into domains (Keese & Symons, 1985). From analysis of secondary structures, expression of biological activity, and site-directed mutagenesis, the left side of the viroid molecule assumes a prominent role in the biology of viroids (Loss et al., 1991).

Data presented here indicate that this region is also preferentially available for reverse transcription and synthesis of viroid-specific cDNA. Thus, some site-specific relaxation of the minimum free energy rod-like structure induced by components of the RT reaction should be considered. The hairpin II structure located in this region for enzyme recognition may also be linked to the initiation of viroid infection as a binding site for transcription factors (Loss et al., 1991) and (-) strand template activity (Qu et al., 1993).

Factors affecting viroid-specific probe reactions

Observations made here indicate the importance of viroid conformation in defining the template for synthesis...
of cDNA probes as well as the target for hybridization. A specific cDNA probe was produced from the circular viroid template but an apparently mixed population of specific and non-specific probes resulted from the linear template. Although the possibility for contamination of the template with host RNA must be considered in the interpretation of data obtained with the linear template, no evidence for a similar case with the circular template was observed even though both templates were purified in an identical manner.

Strong hybridization could be obtained after the viroid was electrophoresed from gels in the absence of any denaturation agent. Viroid RNA could be reverse transcribed at 24 °C and in the absence of any apparent denaturation of the template. Thus, viroid structure permits not only reverse transcription of cDNA probes but also hybridization under non-denaturing conditions.

Prominence of a relatively homogeneous cDNA probe is a striking feature of the product synthesized by reverse transcription with random hexamer primers. The cDNA probes generated from viroid templates ranged from 35 nt for CVD-IIb and CVD-IV to 50 nt for CEVd.

**Viroid genome sites for specific cDNA synthesis**

Restriction fragment hybridization identified loci containing the template for synthesis of cDNA by random priming. In all cases tested, this locus included portions of the pathogenic and central conserved domains. A feature common to locations on the CEVd and CVD-IIb sequence is the proximity to the poly(U) tract which is used to characterize the pathogenicity domain.

A portion of this site for specific cDNA probe synthesis was included in the region described by Sano et al. (1988) for production of a synthetic oligonucleotide probe used to discriminate CEVd from other members of the PSTVd family.

These data indicate that viroid-specific probes are synthesized from regions that are not highly conserved among the different citrus viroid groups. They also substantiate the empirical observations (Duran-Vila et al., 1988; Semancik & Duran-Vila, 1991) for definition of the citrus viroid grouping by specific cDNA probes produced by random-priming.

**Structure and configuration of the specific probe sites**

For the three viroids reported here, size and location of a stem–loop configuration is compatible with: (i) the capacity to synthesize the observed size of the viroid-specific cDNA probe, (ii) the mapping site obtained by hybridization of the restriction nucleases fragments, and (iii) the locus derived from asymmetric amplification.

Formation of the secondary structure in CEVd as proposed by Gross et al. (1982) included hairpin structures I and II detected during thermal denaturation and renaturation studies of the viroid molecule. The hairpin II structure forms a loop of 75 nt and a nine bp stem between nt 239 to 251 and 339 to 327. Loss et al. (1991) in comparing the hairpin II structure in six viroids of the PSTVd family presented an identical core sequence of 8 bp which formed a stem and a single-stranded loop. It was further proposed that hairpin II functions as a binding site for host transcriptional factors.

The importance of native as well as metastable conformations in the structure and function relationship of viroids has been proposed by Riesener (1991). Infectivity studies of Loss et al. (1991) with PSTVd mutants demonstrated that mutations in the core (stem) of the hairpin II structure resulted in reversion to wild-type, whereas mutations in the peripheral (loop) sequence were genetically stable. This evidence for selection pressure for stability in the stem of hairpin II reinforces the importance of this conformation in the structure and function of the viroid.

In the CEVd-A constructs from which infectious monomeric viroid could be transcribed in vitro (Rigden & Rezaian, 1992), nt 300–330 were utilized as favourable primer locations. This sequence is contained within the region mapped for synthesis of the cDNA probes specific to CEVd.

The pathogenicity domain in CEVd appears to be involved in the expression of biological activity by modulating host symptom production (Visvader & Symons, 1985). This observation coupled with the similar domain detected for the primer hybridization site for the three citrus viroids studied here supports the possibility of a specific sequence or conformational locus for direct host–viroid interaction.

As a practical application for the novel observations presented here, the probes also offer a complementary tool for biological indexing and diagnosis of specific citrus viroids. Mapping of the sequence on the viroid molecule for the synthesis of viroid-specific cDNA could be exploited to design synthetic oligonucleotide probes for more subtle diagnostic purposes. These might include the discrimination of pathogenic viroids from closely related viroids which simply replicate in symptomless carrier hosts.

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References


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