SEQUENCE-SPECIFIC PRIMING OF THE IN VITRO SYNTHESIS OF DNA COMPLEMENTARY TO CITRUS EXOCORTIS VIROID

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1. Introduction

Viroids, the smallest known agents of infectious disease, are single-stranded, circular, covalently closed RNA molecules characterized by a high degree of base pairing [for review see ref. 1]. An understanding of the functional significance of the unique primary and secondary structure as displayed by the viroid genome is of central importance to the understanding of the mechanisms of viroid replication and pathogenicity. In this sense, the synthesis of DNA complementary to viroid RNA has been a valuable approach (1) to detect replicative intermediates of viroid biosynthesis by molecular hybridization [2–5], (2) to clone the genome in the form of a double-stranded DNA copy [6], and (3) to sequence the RNA genome with the aid of chain-terminating deoxyribonucleoside triphosphate analogs [7].

In this communication we report on the synthesis of DNA complementary to citrus exocortis viroid (CEV) RNA by using a sequence-specific oligodeoxyribonucleotide for priming complementary DNA (cDNA) synthesis by reverse transcriptase. It will be shown that the sequence specificity of DNA initiation dramatically increases the yield of full-length transcripts and additionally allows the rapid sequencing of part of the viroid genome.

2. Materials and methods

AMV reverse transcriptase was made available through the Office of Program Resources and Logistics at the NIH, Bethesda. The α-^{32}P-labeled deoxyribonucleoside triphosphates (400 Ci mmol^{-1}) and ^{3}H-labeled dTTP (48 Ci mmol^{-1}) were purchased from the Radiochemical Centre, Amersham. The 2′,3′-dideoxyribonucleoside 5′-triphosphates were products of PL-Biochemicals. Roth and Merck, respectively, supplied materials for polyacrylamide gel electrophoresis (PAGE).

Viroid RNA was purified to homogeneity by PAGE in the presence of 8 M urea [8]. Synthesis of the oligodeoxyribonucleotide primer p3 d(TTCTTTTTTCTTTTC), p3-primed reverse transcription, and DNA sequencing have been described previously [7]. Molecular hybridization by the Northern blot technique followed established procedures [5,9]. Separation of nucleic acids by PAGE under fully denaturing conditions at low salt and 40°C in the presence of 8 M urea was achieved according to the method of Sänger et al. [10].

3. Results

3.1. *Synthesis of DNA complementary to citrus exocortis viroid*

The pentadecadeoxyribonucleotide primer p3 used in this study may form 15 consecutive base pairs to the nucleotide sequence 49–63 of the potato spindle tuber viroid (PSTV) [7,11] as well as to sequence 50–64 of CEV RNA including one GT base pair at the 5′ end of the primer molecule (Gross, personal communication; see also fig.3). We have, therefore, tested the potential of this synthetic DNA to serve as a primer for CEV-specific cDNA synthesis by reverse transcriptase. Table 1 summarizes the total amount of DNA synthesized in a representative reaction at

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Table 1
Reverse transcription of CEV RNA

Template	[^{3}H]dTMP incorporated (pmol)	Template copying (%)
Primer p3	0.1	0.01
CEV RNA	10.2	0.7
CEV RNA + p3	49.4	3.4

Experimental conditions for cDNA synthesis were as described [7]. The enzymatic reaction was carried out for 4 h at 37°C in a final volume of 50 μl containing 500 ng of CEV RNA template and 100 units of AMV reverse transcriptase. Concentrations of primer p3 and deoxyribonucleose triphosphates were 7.4 μM and 100 μM, respectively, with [^{3}H]dTTP (23 Ci/mmol) as the radioactive precursor

saturating deoxyribonucleoside triphosphate concentrations (100 μM) after 4 h of incubation, at which time DNA synthesis has reached a plateau (data not shown). The amount of template copying (μg cDNA per μg CEV RNA template) varied between 1 and 4% for the p3-primed reaction and appears to depend on the specific viroid batch used for reverse transcription.

A qualitative analysis of ^{32}P-labeled cDNA synthesized in a pulse-chase experiment is shown in fig.1A. As a first observation the appearance of distinct cDNA size classes is noteworthy. Secondly, several cDNA species appear to be transcriptional intermediates (left-hand arrowheads in fig.1A), while others accumulate under chase conditions and, therefore, represent final reaction products (righthand arrow heads in fig.1A). The largest of these DNA transcripts exhibit a slightly higher electrophoretic mobility as compared to the linear PSTV RNA marker (359 nucleotides) and thus are full-length DNA transcripts of CEV RNA (371 nucleotides; Gross, personal communication).

3.2. *Characterization of CEV complementary DNA*

Molecular hybridization by the Northern blot technique [9] confirmed the CEV specificity of the

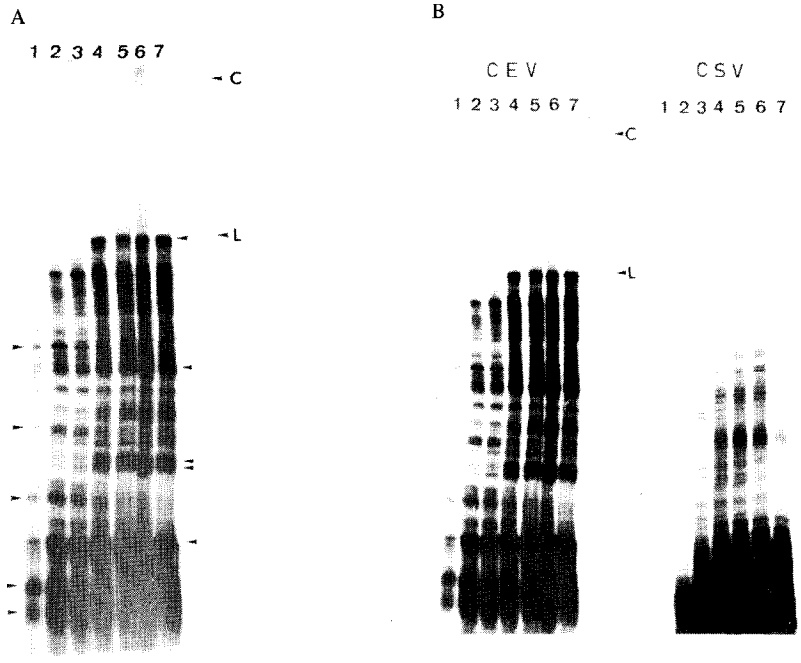

Fig.1. PAGE analysis of ^{32}P-labeled, viroid-specific cDNA. DNA synthesis proceeded in a total volume of 50 μl in the presence of 2 μM of each α-^{32}P-labeled deoxyribonucleoside triphosphate as described [7], and 5-μl aliquots were withdrawn at 1 (15 min), 2 (30 min), and 3 (1 h) after initiation of DNA synthesis. After the addition of unlabeled triphosphate to 1 mM, each 5-μl aliquot was withdrawn at 4 (2 h), 5 (3 h), 6 (4 h), and 7 (5 h) after the initiation of DNA synthesis. A. Primer p3-primed CEV cDNA synthesis. Left-hand arrowheads indicate apparent intermediates of transcription; right-hand arrowheads point out accumulating cDNA species. B. Primer p3-primed CEV and CSV cDNA synthesis. C. Circular PSTV RNA marker; L: Linear PSTV RNA marker.

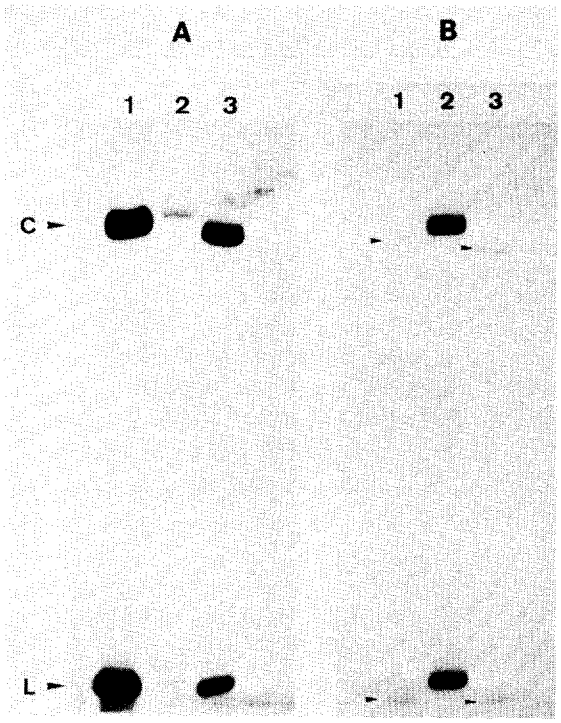

Fig.2. Molecular hybridization of (A) ^{32}P-labeled PSTV cDNA and (B) ^{32}P-labeled CEV cDNA to 1 μg each of unlabeled PSTV (lane 1), CEV (lane 2), and CSV (lane 3) RNAs separated into circular (C) and linear (L) forms and covalently linked to diazobenzyloxymethylpaper [5,9].

DNA transcripts as well as the previously described [5] existence of base sequence homologies between different viroid species. ^{32}P-labeled CEV cDNA was hybridized to PSTV, CEV, and chrysanthemum stunt viroid (CSV) RNAs separated by PAGE under fully denaturing conditions [10] and blotted to diazobenzyloxymethyl-paper [5,9]. The autoradiogram of such a hybridization analysis shows that ^{32}P-labeled CEV cDNA not only hybridizes to the linear (L) and circular (C) forms of the homologous CEV RNA (fig.2B, lane 2), but also, though to a rather low extent, to PSTV as well as CSV RNA (fig.2B, lanes 1 and 3, respectively). Such base sequence homologies are also detected when the identical blot after removal of ^{32}P-labeled CEV cDNA by washing in sterile water at 80°C for 15 min is hybridized with ^{32}P-labeled PSTV cDNA as shown in fig.2A. Base sequence homologies, though detectable by this approach, cannot be quantitated for reasons discussed in detail elsewhere [5].

3.3. *Specificity of CEV complementary DNA synthesis*

While non-specifically primed synthesis of DNA complementary to viroid RNA yields heterogeneous products of predominantly low size [12], sequence-specific priming remarkably increases the yield of large DNA transcripts (fig.1A; [7]). This observation is clearly documented when the primer p3 is used for priming the synthesis of DNA complementary to CSV RNA. This viroid RNA may form only a very unstable hybrid with p3 due to the presence of two internal mismatches (fig.3). Consequently, the CSV-specific DNA transcripts obtained under experimental conditions identical to those of CEV cDNA synthesis, fall into small size classes with no full-length transcripts discernible (fig.1B).

Sequence-specific priming additionally allows rapid sequencing of part of the viroid genome with the aid of chain-terminating inhibitors of DNA synthesis [7,13]. The partial sequence obtained for CEV RNA by this approach is depicted in fig.4 and reveals two prominent features: (1) the CEV isolated used in this study is a mixture of point mutants (arrowheads in fig.4 point out most obvious mutations), and (2) for this particular nucleotide sequence which corresponds to PSTV RNA sequence 1–47 [11], CEV and PSTV RNA exhibit a base sequence homology of approximately 75%.

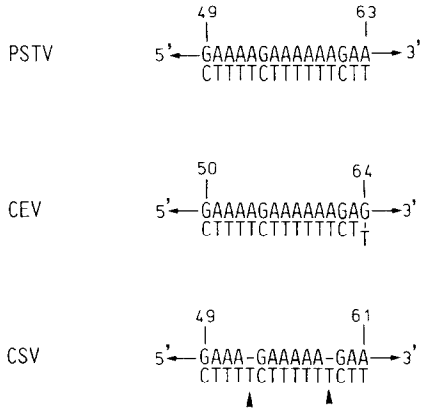

Fig.3. Scheme of potential base pairing between primer p3 and PSTV RNA [7,11] as well as CEV and CSV RNA. Numbers designate corresponding nucleotides in the primary sequences of PSTV [11] as well as CEV and CSV (Gross, personal communication).

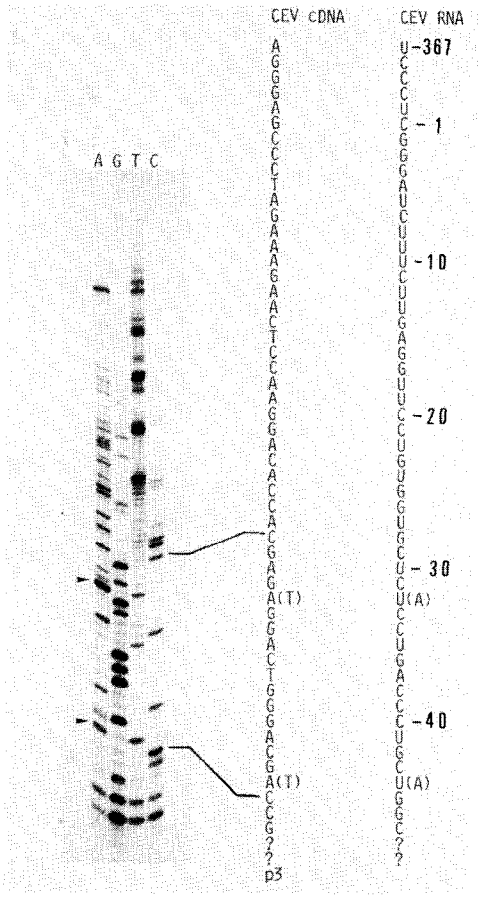

Fig.4. Partial sequence of CEV RNA as determined by p3-primed cDNA synthesis in the presence of the chain-terminating inhibitors ddATP (A), ddGTP (G), ddTTP (T), and ddCTP (C). Arrowheads indicate most prominent point mutations. Numbers designate nucleotide sequence as established by RNA sequencing (see legend to fig.3).

4. Discussion

This report describes the synthesis and characteristics of DNA complementary to the RNA genome of the citrus exocortis viroid. The use of a sequence-specific pentadecadeoxyribonucleotide for priming cDNA synthesis by reverse transcriptase bears important advantages over the previously described random DNA initiation by either DNAase I-digested calf thymus DNA [12] or oligo (dT) and 3′-polyadenylated viroid RNA [14]. First, sequence specificity of the priming event dramatically increases the production of full-length DNA transcripts as compared to randomly primed DNA synthesis [12,14], and will thus make feasible the molecular cloning of the entire viroid genome.

Secondly, the specificity of DNA initiation allows rapid sequencing of part of the viroid genome and, as a consequence, a comparative analysis of nucleotide sequences contained in different viroid species or viroid strains. Thus, DNA sequencing confirms the existence of base sequence homologies as obvious from molecular hybridization (fig.2): CEV and PSTV RNA exhibit a base sequence homology of 75% in the region of concern. In addition, rapid sequencing with a sequence-specific primer provides a diagnostic tool to document unambiguously the molecular homogeneity of a particular viroid isolate. While PSTV as analyzed by this approach [7] appeared to be an isolate consisting of only one molecular species, the CEV isolate studied in this report proved to be a mixture of point mutants. Additional sequence-specific primers synthesized to match selected regions conserved in different viroid species will facilitate future studies on the functional importance of particular primary sequences contained in these unique pathogens.

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