Detection of complementary RNA intermediates of viroid replication by Northern blot hybridization

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Molecular hybridization by the Northern blot technique in combination with 125I-labeled PSTV (+) RNA and 32P-labeled PSTV cDNA as probes has been applied to detect viroid-specific sequences in healthy and viroid(PSTV)-infected tomato plants. Conditions are described which allow differentiation of (+) and (-) viroid sequences on the basis of the different thermostabilities of the corresponding hybrid molecules. By this experimental approach, it is documented that no viroid-specific DNA sequences can be detected and that viroid replication proceeds via complementary RNA intermediates. Out of the seven (-) RNA species found, six are apparently larger than the circular viroid (+) RNA and one is about the same size as the linear (+) RNA molecule.

The replication mechanism of viroids has been a matter of speculation for some time. These pathogens infecting higher plants are single-stranded covalently closed circular RNAs of low molecular weight (about 120 000 daltons, corresponding to about 360 nucleotides) which exist as highly base-paired, rodlike structures (1). Because of the limited genetic information contained in the viroid genome it is generally assumed that their replication is dependent upon enzymes preexisting in the infected host cell. From inhibition studies with α-amanitin in vivo (2) we have previously concluded that DNA-dependent RNA polymerase II plays an important role in the biosynthesis of viroid RNA at early stages of infection. Because of the DNA-like structure of viroid RNA (3), RNA polymerase II was considered to possibly transcribe infecting viroid (+) RNA into its complementary form (2). (The polarity of the infecting viroid RNA has been arbitrarily designated (+).) This earlier assumption has been substantiated by our recent finding that in vitro the purified enzyme synthesizes full-length transcripts of potato spindle tuber viroid (PSTV) (4) as well as citrus exocortis viroid (CEV) and chrysanthemum stunt viroid (CSV) with high specificity and appreciable efficiency (Rackwitz et al., unpublished results).

These in vivo and in vitro data clearly implicate complementary RNA molecules as intermediates in viroid replication. Such (-) RNA species have been detected in CEV-infected plant tissue by the
technique of molecular hybridization in solution (5,6). Previous postulates on the existence of viroid-complementary DNA in infected (7) and even in healthy plant cells (8) were based on host-cell RNA contamination in the radioactively labeled viroid probes used for molecular hybridization (9,10). We have, therefore, carefully examined this latter technique with nucleic acids covalently linked to diazobenzylloxymethyl (DBM) paper (11) (Northern blot) and using as hybridization probes highly purified, $^{125}$I-labeled PSTV RNA as well as $^{32}$P-labeled PSTV complementary DNA (PSTV cDNA). In this communication we report on the specificity of the hybridization probes and on the nature of the replicative RNA intermediates of viroid replication detected by this approach.

Materials and Methods

Extraction of nucleic acids from healthy and PSTV-infected tomato plant tissue, separation into soluble and ribosomal RNA fractions by 2 M LiCl, and treatment with RNase A and DNase I, respectively, followed established procedures (12). Separation of nucleic acids on 3.5% and 5% polyacrylamide gels under fully denaturing conditions ($40^\circ$C; low ionic strength and 8 M urea) was achieved as previously described (13). Northern blot hybridization was performed according to the technique of Alwine et al. (11) by blotting nucleic acids after gel separation and alkali treatment onto freshly prepared DBM paper in 25 mM sodium phosphate buffer, pH 6.8, and prehybridization of the blot in hybridization buffer (50% formamide/0.75 M sodium chloride/0.075 M sodium citrate/0.02% w/v Ficoll/0.02% w/v polyvinylpyrrolidone/0.5 mg/ml calf thymus DNA/1% w/v glycine) for 48 h at 42°C. Molecular hybridization was done under identical conditions using either $^{125}$I-labeled viroid (+) RNA or $^{32}$P-labeled viroid cDNA as radioactive probes. For discriminating between the different types of hybrids the autoradiographed blots were washed for 10 min at 65°C in 0.01 x SSC and autoradiographed again (SSC = 0.15 M NaCl, 0.015 M sodium citrate). PSTV RNA was labeled with $^{125}$I by the method of Commerford (14) to a specific radioactivity of $2\cdot10^8$ dpm/µg. PSTV cDNA transcripts were synthesized by reverse transcriptase to a specific radioactivity of $2.5\cdot10^9$ dpm/µg using the $\alpha$-$^{32}$P-labeled deoxyribonucleoside triphosphates (400 Ci/mmol) and a viroid-specific primer (15). For autoradiography, Kodak X-Omatic films and Dupont Cronex intensifying screens were used. Radiochemicals were purchased from the Radiochemical Centre, Amersham. Reverse transcriptase was kindly supplied through the Viral Oncology Program, NIH, Bethesda.

Results

Characterization of the hybridization probes

First we wanted to characterize the purity and specificity of the radioactive probes used in our study in order to avoid misinterpretation of hybridization data previously encountered by other groups (7,8). For this, highly purified PSTV RNA after iodination with $^{125}$I was subjected to polyacrylamide-gel electrophoresis under fully denaturing conditions (13), and only the circular, covalently closed RNA mole-
Fig. 1. Molecular hybridization of $^{125}$I-labeled PSTV RNA (A and B) and $^{32}$P-labeled PSTV cDNA (C) to nucleic acids separated on a 5% polyacrylamide gel and blotted onto DBM paper. Lanes 1, 500 µg of total RNA from healthy tomato plants; lanes 2, 500 µg of total RNA from PSTV-infected tomato plants; lanes 3, 1 µg of purified PSTV RNA. Conditions of hybridization were as described in the 'Materials and Methods' section. B represents blot as in A, but after washing in 0.01 x SSC at 65°C. C PSTV, circular PSTV RNA; L PSTV, linear PSTV RNA.

cules were used for hybridization after reisolation and partial alkali hydrolysis. Secondly, $^{32}$P-labeled PSTV cDNA was obtained by reverse transcription of PSTV RNA in the presence of the oligodeoxyribonucleotide primer $d(TTCTTTTTTCTTTTC)$ (15) complementary to sequence 63-49 of the PSTV RNA (16). The cDNA was characterized as a genuine PSTV RNA transcript by DNA sequencing with chain-terminating inhibitors (15). Subsequently, both probes were hybridized to nucleic acids covalently linked to DBM paper. The results of such an analysis are shown in Fig. 1. Lanes 3 contain purified, unlabeled PSTV RNA separated into its circular (C) and linear (L) forms. Under the hybridization conditions employed (42°C), $^{125}$I-labeled (+) RNA hybridizes to the unlabeled viroid RNA of the same (+) polarity.
(Fig. 1A, lane 3) as previously observed (4). These (+)(+) RNA hybrids, however, are relatively thermolabile and dissociate when the blot is washed in 0.01 x SSC at 65°C (Fig. 1B, lane 3), which is well below the TM of 81°C described for the (+)(-) viroid RNA hybrid (5).

Furthermore, this probe hybridizes not only to PSTV RNA, but also to the two viroid 'species' CSV and CEV RNA (data not shown), which were isolated from non-solanaceous hosts and which differ markedly from PSTV in their fingerprints (17). Similarly, 32P-labeled PSTV cDNA forms hybrids not only with the homologous PSTV RNA as expected (Fig. 1C, lane 3; Fig. 2, lane 3), but also with the heterologous CSV and CEV RNA (Fig. 2, lanes 1 and 2, respectively), as was evident from overexposed autoradiograms (data not shown). Such cross-hybridization is also apparent when CEV-specific 32P-labeled cDNA is applied as the radioactive probe for molecular hybridization (Fig. 3, lanes 1 and 3). Thus, base sequence homology between the three viroid species PSTV, CEV, and CSV is high enough to allow detection by heterologous hybridization probes under our standard experimental conditions. It is not possible, however, to quantitate sequence homology on Northern blots because for technical reasons the nuclease treatment necessary for quantitation will give unreliable results.

At 42°C 32P-labeled viroid-specific cDNA does not hybridize to itself or to the cDNA of another viroid, which is in contrast to the formation of (+)(+) double-stranded viroid RNA as described above. When, however, the hybridization temperature is lowered to 25°C, such (-)(-) DNA hybrids are formed (Fig. 2, lane 4). This observation is in accordance with the calculated lower stability of the hypothetical (-) viroid molecule (3), which would melt about 13°C below the TM of 50°C as determined for the (+) strand in our hybridization buffer (Riesner et al., unpublished results).

All these findings can be explained by the unique structural features of the viroid molecule. Its high degree of self-complementarity is not only the basis for its intramolecular base-pairing but also allows the formation of intermolecular hybrids between viroid-specific (+)(+) and (-)(-) RNA or and DNA molecules. From thermodynamic data (3) it can be expected that the melting temperature (TM) of the (-)(-) RNA hybrids is about 13°C lower than that of the (+)(+) RNA combination. Moreover, from published data for base-pairing in DNA (18) and RNA (19) it can be expected that the TM of a DNA with the GC content of the PSTV molecule is about 10°C lower than that of the corresponding RNA. The addition of these two temperature differences agrees well with the observed difference between the stability of the (+)(+) RNA hybrids at 42°C and that of (-)(-) DNA and (-)DNA(-)RNA hybrids at 25°C. Despite the identical stability of the latter two hybrids it is possible to discriminate between viroid-specific (-) RNA and (-) DNA intermediates by treating the corresponding nucleic acid samples with DNase and RNase, respectively, before electrophoretic separation and blotting.

Table 1 summarizes the pertinent characteristics of the hybridization probes. It is obvious that by our approach we are in a position to differentiate unequivocally between viroid-specific RNA and DNA sequences of (+) and (-) polarity.
Fig 2. Molecular hybridization of $^{32}$P-labeled PSTV cDNA to nucleic acids separated on a 5% polyacrylamide gel and blotted onto DBM paper. Conditions of hybridization were as described except that the hybridization temperature was lowered to 25°C and the time of hybridization was increased to 96 h. Lane 1, 1 μg of CSV RNA; lane 2, 1 μg of CEV RNA; lane 3, 1 μg of PSTV RNA; lane 4, 0.5 μg of PSTV cDNA; lane 5, 500 μg of total DNA from healthy tomato plants; lane 6, 500 μg of total DNA from PSTV-infected tomato plants. C, circular PSTV RNA; L, linear PSTV RNA.
Fig. 3. Molecular hybridization of $^{32}$P-labeled CEV cDNA to PSTV, CEV, and CSV RNA separated on a 5% polyacrylamide gel and blotted onto DBM paper. Lane 1, 1 µg of PSTV RNA; lane 2, 1 µg of CEV RNA; lane 3, 1 µg of CSV RNA. Molecular hybridization at 42°C was as described in the 'Materials and Methods' section. Preparation of $^{32}$P-labeled CEV cDNA followed the protocol for the synthesis of PSTV-specific, $^{32}$P-labeled complementary DNA (15).
RNA INTERMEDIATES OF VIROID REPLICATION

Table 1. Hybridization characteristics of $^{125}$I-labeled PSTV (+) RNA and $^{32}$P-labeled PSTV-specific (-) DNA (PSTV cDNA)

<table>
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<tr>
<th>Hybridization Probe</th>
<th>Hybridization to</th>
<th>T (°C)</th>
<th>PSTV (+) RNA</th>
<th>PSTV (-) RNA</th>
<th>PSTV (-) DNA</th>
<th>CEV (+) RNA</th>
<th>CSV (+) RNA</th>
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<tr>
<td>$^{125}$I-labeled (+) RNA</td>
<td>42</td>
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<tr>
<td>$^{32}$P-labeled (-) DNA</td>
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Molecular hybridization was performed at the indicated temperatures under the conditions described in the 'Materials and Methods' section. +, Hybrids are formed which are stable at 65°C in 0.01 x SSC; [+], hybrids are formed which are unstable to heating to 65°C in 0.01 x SSC; -, no hybrids are formed.

Nature of replicative intermediates

When DNase-treated RNA or RNase-treated DNA from healthy or PSTV-infected tomato plant tissue is subjected to molecular hybridization analysis, it can be concluded without any ambiguity that not even trace amounts of viroid-specific (+) or (-) sequences are present in the RNA or DNA fraction from healthy tomato plants (Fig. 1A,B,C, lanes 1; Fig. 2, lane 5; Fig. 4, lanes 1 and 4). As for PSTV-infected tomato, we do not detect any viroid-specific sequences in total DNA (Fig. 2, lane 6) even with the highly sensitive $^{32}$P-labeled PSTV cDNA probe under the less stringent hybridization conditions of 25°C, thus confirming recently published data on the absence of viroid-specific sequences in the DNA of infected plants (9,10).

When, however, total DNase-treated RNA from PSTV-infected tomato tissue is analyzed, the following picture evolves. Besides detecting low levels of circular and linear PSTV (+) RNA, hybridization with $^{125}$I-labeled PSTV RNA reveals at least four additional viroid-related RNAs after separation on a 5% polyacrylamide gel (Fig. 1A, lane 2); these form double-stranded RNA hybrids stable to heating to 65°C in 0.01 x SSC and thus represent (-) forms of PSTV RNA (Fig. 1B, lane 2). Three of these RNA species are evidently larger in size than circular PSTV RNA, while one exhibits an apparent molecular weight slightly higher than that of L (+) PSTV. The upper band is located at the top of the 5% polyacrylamide separation gel and represents at least four species as is obvious from an analysis on a 3.5% polyacrylamide gel (Fig. 4): two of these RNAs are soluble in 2 M LiCl (Fig. 4, lane 5), while the remaining viroid-specific (-) RNAs show different solubilities on treatment with 2 M LiCl and are predominantly recovered from the LiCl-insoluble fraction (Fig. 4, lane 2). In addition, they have lower apparent molecular weights than the LiCl-soluble (-) RNAs.
Fig. 4. Molecular hybridization of $^{125}$I-labeled PSTV RNA to nucleic acids separated on a 3.5% polyacrylamide gel and blotted onto DBM paper. Conditions of hybridization were as described in the 'Materials and Methods' section. Lane 1, 500 μg of LiCl-insoluble RNA from healthy tomato plants; lane 2, 500 μg of LiCl-insoluble RNA from PSTV-infected tomato plants; lane 3, 1 μg of purified PSTV RNA; lane 4, 500 μg of LiCl-soluble RNA from healthy tomato plants; lane 5, 500 μg of LiCl-soluble RNA from PSTV-infected tomato plants. C, circular PSTV RNA.

Discussion

In this communication we provide evidence for the existence of PSTV-specific sequences in the RNA fraction of PSTV-infected tomato plants by exploiting the Northern blot technique for molecular hybridization studies. Such sequences are not detected in the purified DNA of infected plant tissue nor are they present in corresponding RNA or DNA preparations from healthy plants. These data unequivocally demonstrate that, in contrast to previous postulates (7,8), the PSTV genome is exogenously acquired by the tomato plant and that viroid replication proceeds via RNA intermediates.

Molecular hybridization by the Northern blot technique, i.e. after covalent linkage of denatured nucleic acids to derivatized paper as described in our study, has important advantages for the detection of viroid-specific sequences as compared to hybridization in solution. First, the Northern blot is a faithful replica of the original gel pattern
and, therefore, allows the immediate and precise identification and size determination of all those unlabeled nucleic acid species which form hybrids with the radioactive probes. Secondly, immobilization of the unlabeled denatured nucleic acids by covalent linkage to the cellulose matrix prevents inter- as well as intramolecular base-pairing among the nucleic acid species in this fraction. Since the formation of the first correct base pair(s) is the rate-limiting step in nucleic acid renaturation (20), intramolecular base-pairing of the highly self-complementary (+) or (-) viroid RNA molecules would be kinetically favored in solution over hybridization to a radioactive probe of opposite polarity. Moreover, (+) and (-) viroid-specific RNA sequences are present together in nucleic acids isolated from viroid-infected cells and cannot be separated from each other by conventional fractionation procedures. Therefore, in solution these unlabeled species will form intermolecular (+)*(−) hybrids and thus prevent quantitation of the hybridization data. This severe interference is avoided by covalent linkage of the nucleic acids to the derivatized paper. The third advantage of our approach is the ease with which the Northern blots can be processed under critical thermal conditions so that viroid-specific sequences of (+) and (−) polarity can be discriminated. Finally, once prepared, the Northern blots can be used repeatedly. The resolving power and reliability of our technique is based on the use of exactly characterized and highly labeled (+) viroid RNA and viroid-specific cDNA for probing, and on the unlabeled viroid RNA, DNase-treated RNA samples, and RNase-treated DNA samples, all of highest purity, as used for testing.

Our molecular hybridization analysis takes advantage of the different thermostabilities of the viroid-specific RNA and/or DNA hybrids which are potentially possible. Accordingly, the temperature during the hybridization and during the subsequent washing of the Northern blot has been applied for differentiation (see Table 1). The RNA-RNA hybridization is carried out at 42°C and analysed. The subsequent washing at 65°C removes all (+)*(+) RNA hybrids while the much more stable (+)*(−) RNA hybrids are visible on the autoradiograph. When, on the other hand, viroid-specific cDNA is hybridized at 42°C, only (-)DNA*(+)viroid-RNA hybrids are found, whereas due to their instability at this temperature neither (-)DNA*(−)RNA nor (-)DNA*(−)DNA hybrids are formed. If, however, this analysis is carried out at 25°C all these combinations are stable and can thus be detected on the blot. Again, the subsequent washing at 65°C removes all hybrids of identical polarity. On the basis of these data other strategies of discriminating the different viroid-specific hybrids can be visualized. It should also be noted that the Northern blot technique has a good potential to be used for routinely screening for viroids in cultured plants.

With the described probes and procedures at least seven molecular species of (-) viroid RNAs have been identified, six of which display apparent molecular weights higher than that of circular PSTV (+) RNA and one of which appears to be slightly larger than the linear (+) viroid molecule. The occurrence of the larger complementary RNAs may be easily explained by the circularity of the RNA template from which they are presumably transcribed. Thus, DNA-dependent RNA polymerase II, responsible for transcription of the infecting viroid
genome (2,4), may in vivo produce multiple, covalently linked copies of the covalently closed template RNA. Although such multimers have not yet been detected during in vitro transcription experiments (4), it is conceivable that additional proteins like an unwinding protein may form part of the in vivo transcription complex and bring about the production of concatemers. From the existence of multiple forms of complementary viroid RNA in the infected cell, it is obvious that the RNA template for the production of progeny viroid RNA as well as the enzyme(s) involved in this process remain to be determined.

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References