Generation of viroid conformational isomers that are stable to incubation with magnesium ions and in a nuclear extract from tomato plants

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ABSTRACT

We identified conditions for heating and quick cooling viroid RNAs in the presence of salt which lead to the production of conformational isomers stable to incubation for at least 45 minutes at 30° in the presence of magnesium ions. Elution in 0.3 M NaCl allowed the purification of an electrophoretically slow form of an *in vitro* transcript carrying a complete copy of the potato spindle tuber viroid RNA sequence. Slow forms of this transcript and of kinase-labeled linear viroid RNA persisted for longer than 20 minutes when incubated with a protein-rich extract prepared from the nuclei of uninfected tomato plants, although both were slowly cleaved by a nuclease present in this extract. The fast form of the transcript was highly resistant to this tomato ribonuclease. The slow form of the transcript was much more susceptible to cleavage by RNase T1 than the fast form of this RNA, suggesting that the reduced gel mobility of the slow forms results from their relatively open structure. The ability to purify viroid conformational isomers from polyacrylamide gels will facilitate biochemical studies aimed at identifying host components interacting with RNAs of the viroid replication complex, which may not all be present in the most thermodynamically favored rodlike structure of mature viroids.

INTRODUCTION

Viroids are minute pathogens with single-stranded, circular RNA genomes (1). So far, viroids have been found only in plant systems; however, they have a number of features in common with other subviral pathogens with circular genomic RNAs, including the agent responsible for delta hepatitis in man (2,3). Viroids, which do not require helper viruses and contain fewer than 400 nucleotides, are ideal RNAs for structural analyses.

Purified viroid RNAs form a highly characteristic, unbranched

rodlike secondary structure, which has been studied under a variety of experimental conditions *in vitro* (4–6). While the stability and nuclease resistance of viroid RNA suggest that the rodlike structure predominates *in vivo*, several lines of evidence suggest that conformations differing from the rodlike structure are present in populations of actively-replicating viroid molecules. For example, conformations other than the full-length rodlike structure would appear to result inevitably from the rolling circle replication process proposed for viroids and related infectious RNAs (7). Furthermore, alternative conformations may result from the binding of cellular factors to specific sites in viroid RNA. The central conserved region of viroid RNA contains an element of local tertiary structure with features similar to the TFIIB binding site on 5S rRNA (8). Protein binding to such sites could induce small, local conformational changes or could have more global effects. Both direct analysis of purified viroid RNA and comparative sequence analysis reveal the potential of viroid RNA to form several helices not present in the rodlike secondary structure (9). One of these helices, 'hairpin I', has been associated with the processing of multimeric viroid transcripts (10–12), and another, 'hairpin II', is proposed to form a cis-active promoter element (13). Since many protein-RNA interactions are studied in reactions containing both magnesium ions and cellular proteins, we investigated the ability of gel-purified viroid conformational isomers to persist during incubation with these components.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England BioLabs; SP6 RNA polymerase, DNase, and RNasin from Promega; RNase T1 from Boehringer-Mannheim; T4 polynucleotide kinase and unlabeled nucleoside triphosphates from Pharmacia; and alpha- and gamma-[³²P]nucleoside triphosphates from New England Nuclear. Other chemicals were obtained from Sigma.

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Purification of circular and linear viroid RNA extracted from tomato plants

Circular and linear potato spindle tuber viroid (PSTV) RNAs were extracted from infected tomato plants in the presence of phenol and bentonite, and then were purified by electrophoresis in polyacrylamide gels, followed by CF-11 cellulose chromatography (14,15) and ethanol precipitation.

End-labeling of linear PSTV RNA

Gel-purified linear PSTV RNA (0.01–0.05 mg/ml) was incubated with T4 polynucleotide kinase (20 U/ml) in 80 mM Tris-HCl, pH 7.6; 12 mM MgCl₂; 6 mM DTT; and 0.25 mCi/ml gamma-[³²P]ATP (specific activity 3000 Ci/mol) for one hour at 37°. Purification involved extraction with phenol and chromatography on CF-11 cellulose. No pretreatment with alkaline phosphatase was performed since a high proportion of the linear viroid RNA we obtain from tomato plants has a 5' hydroxyl terminus.

Preparation of *in vitro* transcripts containing viroid sequences

A full-length PSTV cDNA segment with the sequence of the Holland strain (16,17) was excised from a dimeric PSTV cDNA clone (17) with the restriction enzyme *Hae* III and was transferred to a modified pSP6 plasmid (Promega) to give the construct pHD1. *In vitro* transcription of *Eco* RI-cleaved pHD1 with SP6 polymerase produced RNAs about 415 nucleotides in length, with the 359 nucleotides of the PSTV sequence flanked by vector sequences at both the 5' and the 3' ends. Transcription was carried out in 40 mM Tris-HCl, pH 7.5; 6 mM MgCl₂; 2 mM spermidine; 10 mM NaCl; 10 mM DTT; 2000 U/ml RNasin; 0.5 mM each of ATP, GTP, UTP, and CTP, with 2.5 mCi/ml of the desired alpha-[³²P]nucleoside triphosphate (usually GTP); 0.05 mg/ml of the linearized pHD1 plasmid; and 750–1500 U/ml SP6 RNA polymerase. The transcription reaction was carried out at 37° for one hour followed by treatment with RNase-free DNase for 5 minutes. The reaction mixture was then extracted with phenol. The RNA was purified by chromatography on cellulose CF-11 (14,15), concentrated by precipitation in ethanol, and stored in water at –20°. Radiolabeled transcripts were used for 2–3 weeks following synthesis.

Generation of viroid conformational isomers

Circular PSTV extracted from tomato plants, end-labeled linear PSTV prepared as described above, and internally-labeled pHD1 transcripts were placed in eppendorf tubes in 5 µl of a Tris-salt-magnesium ion buffer solution containing 10 mM Tris-HCl, pH 7.6; 100 mM NaCl; and 10 mM MgCl₂. The concentration of the RNAs varied, but the results were essentially concentration independent. The eppendorf tube was dipped in boiling water for 30 seconds and quickly transferred to an ice bucket. The RNA was left on ice for at least 10 minutes before being used for gels or subsequent reactions.

Elution of viroid conformational isomers from polyacrylamide gels

Following electrophoresis in polyacrylamide gels containing no urea, bands of conformational isomers were excised and the RNAs were eluted by gently shaking the gel bands overnight in 300 µl of 0.3 M NaCl. The volume was reduced to 100 µl by evaporation carried out in a speed-vac lyophilizer. The solution was then desalted by filtering it through a spin-column of

Sephadex G-50 equilibrated with water. If needed, the volume was further reduced by evaporation in the speed-vac.

The preparative gels and their analytical counterparts were 1.5 mm thick and contained 4% polyacrylamide; 45 mM Tris-borate, pH 8.0; and 1 mM EDTA. These gels were run with an accelerating voltage of 150 volts so that a bromophenol blue dye marker migrated at the approximate rate of 4.5 cm per hour.

Preparation of the nuclear extract from uninfected tomato plants

Tomato plants (*Lycopersicon esculentum*, cv. Rutgers) were grown in an EGC growth chamber with 12 hours of illumination at a constant temperature of 30°. Leaves from 16–20 day old plants were harvested in 30–50 gram batches. Nuclei were prepared according to Schumacher *et al.* (18) and then were stored as frozen pellets at –70° for up to four months. Nuclei from several preparations were combined and a clear nuclear extract was prepared, following the procedure of Tsagris *et al.* (19). The nuclear extract was brought to a final protein concentration of 0.8 mg/ml and stored in small aliquots at –70°.

RESULTS

A stable isomer of circular viroid RNA

To seek alternative conformations of viroid RNA which might provide substrates for future biochemical studies, circular PSTV was heated in a Tris buffer containing 100 mM NaCl and 10 mM MgCl₂ for 30 seconds as described in Materials and Methods. Samples were quickly cooled on ice and then were fractionated by electrophoresis in gels containing a Tris-borate-EDTA buffer, but with no urea. As shown in Figure 1, the heating and quick cooling produced a form of viroid RNA (panel A, lane b, band #2) which migrated much more slowly than untreated control samples (panel A, lane a, band #1). The slower form did not rapidly revert to the native structure; rather, samples heated and quick cooled and then incubated for 45 minutes at 30° in the Tris-salt-magnesium buffer retained the slow gel mobility characteristic of the alternative conformation (panel A, lane c, band #2), indicating its ability to persist under conditions commonly used for biochemical assays.

Since the absence of free ends might be expected to limit production of conformational isomers of circular viroid RNA and to favor their prompt conversion into the rodlike structure, it was important to verify that the treated samples actually contained circular RNA and not linear molecules produced while the circular RNAs were heated in the presence of magnesium ions. Electrophoresis in gels containing urea confirmed that control samples; samples heated with magnesium ions and quick cooled; and samples heated, quick cooled, and then incubated in the presence of magnesium ions for an additional 45 minutes at 30° all contained circular viroid RNA as the major species (see Figure 1B, lanes a–c). These samples also all contained a small amount of linear PSTV, which migrates much more rapidly than circular PSTV in gels containing urea.

Production of stable isomers of linear PSTV and a linear transcript containing a complete copy of the PSTV RNA sequence

Like its circular counterpart, linear PSTV purified from tomato plants could be converted into a slowly-migrating conformation by heating and quick cooling (Figure 2A, lane b, band L-2). Once formed, this conformation survived further incubation at 30° for

Figure 1. A stable conformational isomer of circular PSTV. Circular PSTV was either resuspended in water and kept on ice (lane a); or heated and quick cooled in a standard buffer containing 10 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 10 mM MgCl₂ (lane b); or heated and quick cooled in the Tris-NaCl-MgCl₂ buffer and then incubated at 30° for 45 minutes (lane c). Prior to analysis in the 4% polyacrylamide gel containing 45 mM Tris-borate, pH 8.0, 1 mM EDTA, but no urea, shown in panel A, RNAs were mixed with one volume of glycerol and 1–2 μ l of a dye mixture containing 0.25% bromophenol blue and 0.25% xylene cyanol; while RNAs to be fractionated in the 4% polyacrylamide gel containing 45 mM Tris-borate, pH 8.0, 1 mM EDTA and 7 M urea shown in panel B were prepared for electrophoresis by the addition of three volumes of a solution that was 90% formamide, 10 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, followed by heating for 3 minutes at 90°. Gels were stained with ethidium bromide. Fast (band #1) and slow (band #2) electrophoretic forms of circular PSTV are identified in panel A. 'O' marks the origin of electrophoresis

Figure 2. Conformational isomers of end-labeled linear PSTV. In studies carried out in parallel to those described in the legend to Figure 1, end-labeled linear PSTV was either stored on ice (lane a); heated and quick cooled in Tris-NaCl-MgCl₂ (lane b); or heated, quick cooled and then incubated at 30° for 45 minutes (lane c) prior to electrophoresis in a 4% polyacrylamide gel containing either no urea (panel A) or 7 M urea (panel B). Fast (band L-1) and slow (band L-2) electrophoretic forms are identified in panel A. 'O' marks the origin of the gels.

45 minutes in the presence of magnesium ions (Figure 2A, lane c, band L-2). Electrophoresis in gels containing urea demonstrated that the linear viroid RNA remained intact during the heating and incubation treatments (Figure 2B, lanes a–c). Other experiments revealed that 100 mM NaCl, but not magnesium ions, was required to produce the slowly-migrating form of end-labeled linear viroid RNA (data not shown).

Figure 3. Conformational isomers of internally-labeled pH1 transcripts. RNA transcripts containing a complete copy of the PSTV sequence were either stored on ice (lane a); heated and quick cooled in the standard Tris-NaCl-MgCl₂ buffer (lane b); heated and quick cooled in water (lane c); or heated and quick cooled in water and then heated and quick cooled in the Tris-HCl-MgCl₂ buffer (lane d) prior to electrophoresis in a 4% polyacrylamide gel containing either no urea (panel A) or 7M urea (panel B). Fast (band H-1) and slow (band H-2) electrophoretic forms are identified in panel A. 'O' marks the origin of the gels.

Since linear viroid RNA purified from tomato plants is only available in limited amounts, experiments were carried out to determine whether RNA transcripts containing the full-length copy of the PSTV sequence could be induced to form large quantities of stable conformational isomers and thus potentially substitute for viroid RNA itself in certain studies. When the plasmid pH1, which contains one complete copy of PSTV cDNA, was transcribed and purified under the conditions described in Materials and Methods, a population of conformational isomers resulted which generated a number of bands upon electrophoresis in Tris-borate-EDTA gels containing no urea (Figure 3A, lane a). The most rapidly migrating species in this population was designated H-1. When samples of the pH1 transcripts were heated and quick cooled in the Tris-NaCl-MgCl₂ buffer described above, almost all of the RNA was converted into forms migrating more slowly than the RNA in band H-1, forming a prominent band, H-2, and other minor bands (Figure 3A, lane b). The slowly-migrating conformations were not present in samples heated and quick cooled in water; rather, transcripts receiving this treatment migrated rapidly as a uniform population, forming a band at the position of H-1 (Figure 3A, lane c). Such RNA could subsequently be induced to form the slowly-migrating conformation by heating and quick cooling in the Tris-NaCl-MgCl₂ buffer (Figure 3A, lane d). Electrophoresis in gels containing no urea demonstrated that the pH1 transcripts were of uniform length before and after the experimental manipulations used to alter the distribution of conformational isomers (Figure 3B, lanes a–d).

Purification of stable conformational isomers using polyacrylamide gels

Both internally-labeled pH1 transcripts and kinase-labeled linear PSTV were treated as described above to generate samples enriched for either the fast or the slow electrophoretic forms and then were fractionated in non-urea gels. RNAs were eluted from

Figure 4. Conformational isomers recovered from preparative scale gels. Transcripts of pHDI containing a full-length copy of the PSTV sequence and end-labeled linear PSTV were heated and quick cooled in the standard Tris-HCl-MgCl₂ buffer and fractionated in a 4% polyacrylamide gel with no urea. Gel bands representing various conformational isomers were excised and the RNA eluted in 0.3 M NaCl and further purified as described in Materials and Methods. RNA recovered from bands H-1 and H-2, of pHDI (lanes a and b, respectively), and RNA from bands L-1 and L-2, of linear PSTV (lanes c and d, respectively), were fractionated in a 4% polyacrylamide gel with no urea. 'O' indicates the origin of the gel.

excised gel bands in 0.3 M NaCl, desalted, concentrated (as described in Materials and Methods), and then were analyzed by electrophoresis in a second non-urea gel. The RNAs recovered from bands H-1 and H-2, composed of pHDI transcripts, and from band L-1, the fast form of linear PSTV, had the same mobility after these manipulations as they did in the preparative scale gel (Figure 4, lanes a–c), indicating that gel electrophoresis can be used as part of a procedure to isolate viroid conformational isomers in a highly purified form. In contrast, about 50% of the RNA in band L-2, the slow form of linear PSTV, converted to the fast form during the gel elution and purification process (Figure 4, lane d).

Incubation of viroid conformational isomers with a nuclear extract from tomato plants

Various forms of pHDI transcripts and linear PSTV RNA were treated with a nuclear extract from tomato plants at 30° in the standard Tris-NaCl-MgCl₂ buffer to determine whether the slow forms could maintain their distinctive structure under these conditions, and also to examine any digestion products which might result from cleavage by nucleases present in the extract. A substantial proportion of the RNA in bands H-2 and L-2, the slow forms, survived incubation in the nuclear extract for 20 minutes (Figure 5). While little of the RNA in these forms remained at the end of a 60 minute incubation, RNA in the fast forms (from bands H-1 and L-1) was highly stable through prolonged exposure to the nuclear extract (see Figure 5). Loss of RNA in the slow forms may have been due to both conversion to the fast forms and to nuclease cleavage, which was detected by fractionation of the reaction products in gels containing urea (see Figure 6).

Figure 5. Persistence of conformational isomers incubated with a nuclear extract. An unfractionated population of pHDI transcripts and RNA eluted from either band H-1 or band H-2 (panel A); and an unfractionated population of linear PSTV and the RNA eluted from band L-1 and L-2 (panel B) were incubated at 30° in the standard Tris-NaCl-MgCl₂ buffer plus 1 µg/µl tRNA either without or with nuclear extract ('NEX') for the times indicated. The pHDI reactions contained 0.04 mg/ml of protein, while those of linear PSTV contained 0.16 mg/ml of protein. 'U' denotes untreated samples. Analysis was carried out in 4% polyacrylamide gels containing no urea.

Partial RNase T1 digestion of viroid conformational isomers

RNase T1 digestions were carried out under conditions leading to partial cleavage to see whether conformational isomers differing in gel mobility could also be distinguished by this approach. The mixture of conformational isomers present in pHDI transcripts, as well as RNA purified from bands H-1 and H-2, were partially digested by incubation with various concentrations of RNase T1 in the Tris-NaCl-MgCl₂ buffer for 20 minutes at 30°. As shown in Figure 7A, the RNAs eluted from bands H-1 and H-2 have very different patterns of partial digestion products, indicating that they have different structures. The RNA from band H-1 appears to be more resistant to RNase T1 than the RNA from band H-2, in keeping with the results obtained in experiments using the nuclear extract (see Figures 5A and 6A).

A parallel analysis was performed on RNA recovered from bands L-1 and L-2 of end-labeled linear PSTV. The presence of partial digestion products unique to RNA from band L-2 indicated that RNA in the slow form has different nuclease-sensitive sites than RNA in the fast form (see Figure 7B). However, since about half of the RNA excised from the position of the slow form undergoes conformational switching during the

Figure 6. Sensitivity of the slow conformations to cleavage by a nuclease in the nuclear extract from tomato plants. In experiments parallel to those presented in Figure 5, various forms of the pHDI transcript (panel A) and end-labeled PSTV (panel B) were incubated at 30° in the standard Tris-NaCl-MgCl₂ buffer plus 1 µg/µl tRNA either with or without nuclear extract ('NEX') [which yielded a final protein concentration of 0.04 mg/ml (panel A) or 0.16 mg/ml (panel B)]. Prior to electrophoresis in these 4% polyacrylamide 7 M urea gels, samples were mixed with three volumes of a formamide-EDTA-dye mixture and heated 3 minutes at 90° as described in the legend to Figure 1. 'O' marks the origin.

elution process, only RNA from band L-1 was available in pure form. Due to this complexity, it was not possible to determine the relative RNase T1 sensitivity of the slow and fast conformations from this experiment. However, the reduced gel mobility of the slow form suggests that it is less compact than the fast form.

DISCUSSION

We found that when circular PSTV RNA, as well as end-labeled linear PSTV, and RNA transcripts of clone pHDI (which contains a complete copy of the PSTV sequence) were heated and quick cooled in a buffer composed of 10 mM Tris-HCl, 100 mM NaCl, and 10 mM MgCl₂, conformational isomers with reduced electrophoretic mobility were formed. These slow forms were stable to incubation at 30° for at least 45 minutes in the Tris-HCl-MgCl₂ buffer that was the standard for our experiments. We used this buffer for virtually all of the heating and quick cooling treatments, although studies carried out on end-labeled linear PSTV revealed that the 100 mM NaCl, but not the 10 mM MgCl₂, was required to generate the slow form. Studies of end-labeled linear PSTV and pHDI transcripts demonstrated that the slow forms of these RNAs could also survive at least 20 minutes of incubation at 30° in the standard buffer augmented by a protein-rich extract made from the nuclei of tomato plants.

Figure 7. RNase T1 partial digestion products of conformational isomers of pHDI transcripts and end-labeled linear PSTV. In experiments parallel to those presented in Figure 6, various forms of the pHDI transcript (panel A) and linear PSTV (panel B) were incubated at 30° for 20 minutes in the standard Tris-HCl-MgCl₂ buffer plus 1 µg/µl tRNA and RNase T1. The RNase T1 concentrations used in the experiments shown in panel A were 0.0625 µg/ml (lane a); 0.125 µg/ml (lane b); 0.25 µg/ml (lane c); 0.5 µg/ml (lane d); 1.0 µg/ml (lane e); while the RNase T1 concentrations used in the experiments shown in panel B were 0.0 (lane a); 0.025 µg/ml (lane b); 0.05 µg/ml (lane c); 0.1 µg/ml (lane d); and 0.2 µg/ml (lane e). 'O' indicates the origin of electrophoresis of these 4% polyacrylamide gels containing 7 M urea.

Therefore, the slow forms have the requisite stability for use in biochemical assays.

We did not evaluate our ability to purify the slow form of circular PSTV because this RNA was available in limited amounts; however, we did find that the slow form of the pHDI transcripts could be recovered from gels in a nearly pure state. In contrast, about 50% of the slow form of the end-labeled PSTV RNA converted to the more rapidly migrating electrophoretic form during the purification process, which included an overnight elution step carried out at room temperature. A modified purification process might permit the slow form of linear PSTV to be obtained. Since linear PSTV terminates at a number of different positions (20, and our unpublished observations) it may be that the half of the population capable of maintaining the slow conformation has termini differing from those of the RNAs readily converted to the rapid conformation.

The slow form of pHDI was more susceptible to nuclease cleavage than the fast form when tested with either RNase T1 or a nuclease present in the tomato nuclear extract. This nuclease readily cleaved control single-stranded RNAs (our unpublished observations) and thus it appears to prefer single-stranded regions, as does RNase T1. However, the tomato nuclease favors different sites in viroid RNA than RNase T1 (compare pattern of gel bands in Figures 6 and 7). The greater sensitivity of the slow form of pHDI to nucleases directed against single-stranded RNA regions suggests that the slow forms contain highly accessible sites that are not protected by the unusual pattern of intramolecular base pairing that gives rise to the unbranched rodlike structure of native viroid RNA (4–6).

Further study would be needed to determine the relationship between the conformational isomers we have studied and those described previously. In an extensive series of studies, Riesner and collaborators have shown that metastable conformations are formed during thermal denaturation of circular viroid molecules (6,9). These denaturation intermediates contain three helices, in the case of PSTV, including hairpins I and II, which have been considered to serve functional roles (9–13,21). The rodlike structure reappeared in populations of fully-denatured or partially-denatured viroid molecules at rates which depended upon the final temperature (6). Riesner and colleagues trapped a conformational isomer by quick cooling partially melted PSTV (6) and carried out additional studies on dimeric viroid transcripts either prepared *in vitro* or isolated from infected plants (9,22). These transcripts contained secondary structure elements similar to those present in partially denatured circular viroid RNA. The presence of molecules having conformations other than the rodlike structure in populations undergoing replication raises the possibility that conformational switching is important during the viroid life cycle. Conformational switching out of a rodlike structure is required for ribozyme formation in several circular subviral RNA pathogens, such as the circular satellite RNA of lucerne transient streak virus (23) and the delta hepatitis agent (24). Multiple conformational states have been described for many additional RNAs. For example, 7S RNA undergoes conformational changes during cycling of the signal recognition particle (25). Furthermore, several conformational switches have been described in studies of 16S rRNA (26). Thus, the ability to form multiple biologically-active conformational isomers may occur frequently in RNAs. We have presented an approach for preparing conformational isomers of viroid RNA whose stability will allow them to be probed by techniques of structural analysis and by biochemical techniques aimed at identifying host factors capable of binding to viroid RNA.

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