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Circularization of linear viroid RNA via 2'-phosphomonoester, 3', 5'-phosphodiester bonds by a novel type of RNA ligase from wheat germ and *Chlamydomonas*

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Summary

A novel type of RNA ligase activity in extracts of wheat germ or Chlamydomonas requires 2',3'-cyclic phosphate and 5'-phosphate ends for ligation to form a 2'-phosphomonoester, 3',5'-phosphodiester bond. Using 5'-^{32}P-labeled linear PSTV, we demonstrate that RNase T_1-nicked viroid predominantly forms $-G_{3'}^{2'}{}_{-P}-$5'U-bonds. Natural linear PSTV, however, forms mainly $-C_{3'}^{2'}{}_{-P}-$5'A-bonds upon enzymatic circularization. We show that natural linear PSTV RNA has nicks between C_{181} and A_{182}, or between C_{348} and A_{349}, and that consequently C_{181} and C_{348} carry 2',3'-cyclophosphate termini.

INTRODUCTION

Previously we have shown that viroids are single-stranded covalently closed circular RNA molecules (1). This was confirmed by establishing the complete primary and secondary structure of potato spindle tuber viroid, PSTV (2). However, it remained obscure how viroids replicate and by which mechanism they are circularized in the host plant cell. We have recently described a novel type of RNA ligase from wheat germ, which forms 2'-phosphomonoester, 3',5'-phosphodiester ($-N_{3'}^{2'}{}_{-P}-$5'N-) bonds (3) and needs 2',3'-cyclic phosphate termini and 5'-phosphate ends for ligation (4). We suggested that this enzyme may be involved in tRNA splicing and viroid circularization (3). In fact, Branch et al., using uniformly labeled PSTV, have recently shown that the wheat germ enzyme indeed circularizes natural linear viroid RNA or viroid RNA linearized by mild RNase T_1 treatment (5). However, the nature of the newly formed bond and the location of the nick(s) in natural linear viroid RNA were not determined.

In this report we show that this type of wheat germ RNA ligase is also present in Chlamydomonas, that ligase preparations from both sources circularize linear viroid RNA, and that the nick in natural linear PSTV is predominantly located in one of two defined positions.

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MATERIALS AND METHODS

RNase T_1 and T_2 were products from Sankyo, Tokyo. Calf intestinal alkaline phosphatase was from Boehringer, Mannheim. PSTV was prepared as described (1). Extracts (S23) from wheat germ were prepared as described (3). Extracts of Chlamydomonas reinhardtii were prepared as follows: The organism was cultured for 48 hr (log phase) under standard conditions. 1000 ml medium contained (grams in brackets) $K_2HPO_4 \cdot 3H_2O$ (1.82); KH_2PO_4 (0.72); $MgSO_4 \cdot 7H_2O$ (0.2); NH_4Cl (0.05); $CaCl_2 \cdot 2H_2O$ (0.01); Na-acetate (1.0); yeast extract (Difco) (2.0); peptone (Difco) (2.5), and 1 ml of a solution with trace elements, containing in 100 ml (grams in brackets): citric acid (5.0); $ZnSO_4 \cdot 7H_2O$ (5.0); $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (1.0); $FeCl_2 \cdot H_2O$ (0.5); $CuSO_4 \cdot 5H_2O$ (0.25); $MnSO_4 \cdot H_2O$ (0.05); H_3BO_3 (0.05); $Na_2MoO_4 \cdot 2H_2O$ (0.05). Cells harvested by centrifugation were washed with medium without yeast extrakt, peptone and trace elements. 1 g of wet cells was suspended in 0.5 ml buffer containing 10 mM Tris acetate pH 7.5, 3mM $Mg(OAc)_2$, 50 mM KOAc and 1 mM DTT, crushed with a Polytron homogenizer for 1-2 min at 4°C and centrifuged at 26.000g for 15 min. The supernatant was directly used as ligase source in the experiments described below.

Linearization of unlabeled PSTV by RNase T_1

Purified PSTV was cleaved by RNase T_1 to obtain the corresponding linear molecules. The reaction mixture (5µl) contained 20 mM Tris HCl (pH 8.0), 1 mM EDTA, 1 x 10^{-4} unit/µl RNase T_1 and 1 µg of purified PSTV. The mixture was incubated at 37°C. Aliquots (1 µl) were withdrawn from the mixture into 20 µl of phenol, saturated with 50 mM acetate buffer (pH 5.0) at 0.5, 2, 5, 15 and 30 min. The preparation was phenolized three times, precipitated with ethanol and labeled at the 5'-end with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Partial alkaline hydrolysis of labeled circular PSTV

To prove the circular form of labeled PSTV, isolated circular molecules were incubated in 50 mM NaOH for 3 min at 80°C in the presence of 6M urea and 5 µg of yeast tRNA and analysed by electrophoresis on 5 % polyacrylamide gels (6).

5'-End labeling of PSTV with [γ-^{32}P]ATP and T4 polynucleotide kinase

5'-End labeling was performed in 10 µl reaction mixtures containing 1 µg of either untreated or RNase T_1 treated PSTV, 0.1 mCi of [γ-^{32}P]ATP (1000 Ci/mmol), 2.5 units of T4 polynucleotide kinase, 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1.7 mM spermine and 15 mM 2-mercaptoethanol. The mixture was incubated for 30 min at 37°C. Labeled materials were separated by 5 % polyacrylamide slab gel electrophoresis under fully denaturing conditions as

previously described (6).

Circularization of 5'-end labeled linear PSTV with the extracts of wheat germ and Chlamydomonas reinhardtii.

The reaction mixture (20 µl) contained 50 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 4 mM $MgCl_2$, 2.5 mM ATP, 5'-end labeled linear PSTV (5000 cpm) and 5 µg wheat germ or Chlamydomonas reinhardtii extract protein. The mixture was incubated for 10 min at 30 °C. The reaction was terminated by addition of 20 µl 50 mM Na-acetate buffer (pH 5.0)-saturated phenol. Phenolization was repeated twice. The sample was precipitated with ethanol and analysed by 5 % polyacrylamide slab gel elektrophoresis.

One sample from complete reaction was treated with 0.2 units of calf intestine alkaline phosphatase in 5 µl 10 mM Tris-HCl (pH 8.0) at 50°C for 1 hr.

Incubation of unlabeld PSTV with [γ-^{32}P]ATP and wheat germ extract.

PSTV (2 µg) were incubated at 30°C in a mixture (10 µl) containing 0.1 M Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 5 mM dithiothreitol, 50 µCi [γ-^{32}P]ATP (1000 Ci/mmol) and 5 µg wheat germ extract protein. 2 µl of 20 mM unlabeled ATP was added to the mixture at 3 min and the incubation was continued. The reaction was terminated at 15 min by addition of 20 µl 50 mM acetate (pH 5.0) saturated phenol. The phenolized and ethanol precipitated sample was treated with 1 µg of proteinase K (Merck) in 0.2 % SDS for 1 h at 37°C in order to eliminate phosphorylated proteins.

Nucleotide sequence analysis

The sequence at the 5' termini of natural linear PSTV molecules was established by the two-dimentional mobility shift method (7, 8). The 5' end labeled linear PSTV was boiled in 10 µl of 50 mM $NaHCO_3$ (pH 9.1)/0.5 mM EDTA for 15 min (9, 10) followed by treatment with 0.1 N HCl at 37°C for 5 min to hydrolize 2',3'-cyclic phosphates (10).

The oligonucleotides were separated by electrophoresis on cellulose acetate at pH 3.5 in the first dimension and homochromatography on a DEAE-cellulose thin-layer plate in 20 mM KOH homomix in the second dimension (8).

Analytical procedures

The nucleotide composition of $N^{2'-P}_{3'-P}$-5'N was determined as follows: the nuclease P_1 or RNase T_2 resistant dinucleotides were treated with 0.02 units of calf intestinal alkaline phosphatase in 5 µl 10 mM Tris-HCl (pH 8.0). After 1h of incubation at 50°C, 1 µl of 40 mM nitrilotriacetic acid was added to the mixture and the sample was incubated for 10 min at 50°C and then for 2 min in boiling water to inactivate phosphatase (11). The pH of

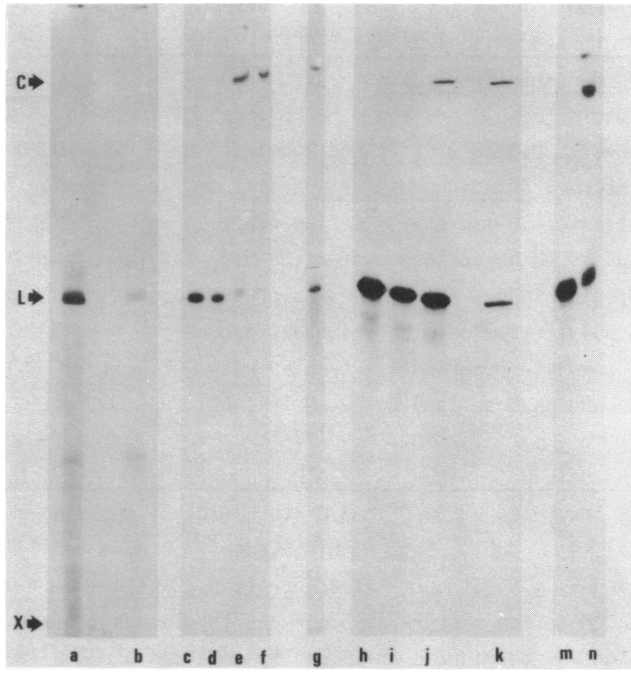

Fig. 1. Linearization and labeling of PSTV, and its circularization with extracts of wheat germ and Chlamydomonas reinhardtii.
RNase T_1-treated (lane a) or untreated (lane b) PSTV was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled linear PSTV from lane a was used as substrates for RNA ligase from wheat germ (lane c - f) and Chlamydomonas (lane h - k). Linear PSTV from lane a was incubated at 30°C for 10 min in the absence of extract (lane c and h), with extract in the absence of ATP (lane d and i) or in the complete mixture (lane e and j). The RNA from the complete mixture was treated with phosphatase (lane f and k). To prove the circular form, the upper band of lane e was eluted, partially hydrolysed by NaOH and electrophoresed (lane g). All other circular PSTV products were also checked for circularity in the same way (not shown). [^{32}P]- labeled natural linear PSTV was incubated with wheat germ extract as above; m: without ATP; n: complete mixture with ATP. C = circular PSTV; L = linear PSTV; X = marker dye xylene cyanol blue.

this mixture was adjusted to 5.0 by the addition of 0.7 µl of 0.5 M acetate (pH 5.0). This mixture was treated with 1 µg of nuclease P_1 or 1 unit of RNase T_2. Digests were analysed by thin-layer chromatography on cellulose plates in solvent A [isobutyric acid/NH_4OH/H_2O, pH 4.3, 577:38:385 (vol/vol)]. All other nuclease digestions were carried out as described (3). For electrophoretic analyses, 5% polyacrylamide slab gels (20 x 40 cm, 0.5 mm thick) were used. The compositions of gel and electrode buffer were as described (6).

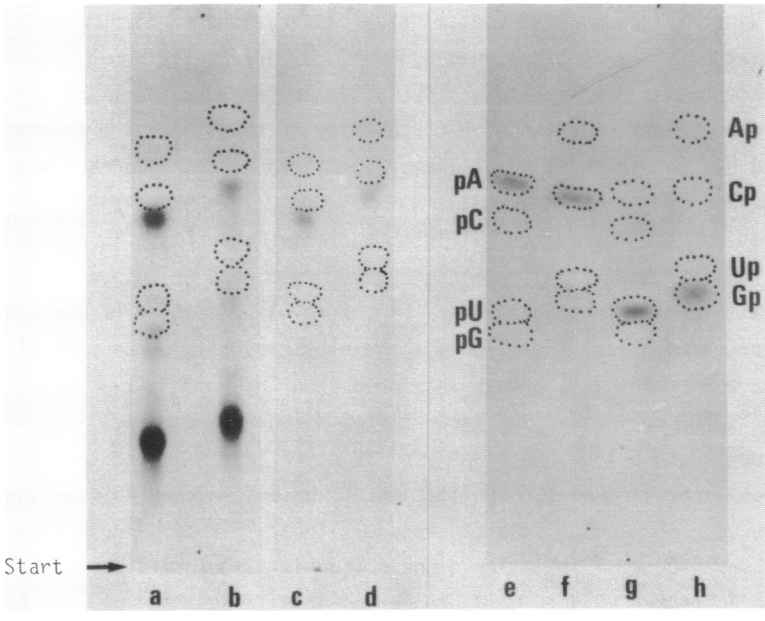

Fig. 2. Analyses of the bond formed by wheat germ ligase upon circularization of linear PSTV.
Circularized molecules (upper bands of Fig. 1, lane e and n) were eluted from the gel and digested with nuclease P1 (lanes a and c) or RNase T2 (lanes b and d).
Lanes a and b are digests of circularized PSTV of Fig. 1, lane e. Lanes c and d are that of Fig. 1, lane n. Spots of lane a and b were eluted from the plate and treated with phosphatase followed by digestion with nuclease P1 (lanes e and g) or RNase T2 (lanes f and h). Lanes e and f are products from the upper spots of lane a and b. Lanes g and h are from the lower spots of lane a and b.
Digests were chromatographed on cellulose thin-layer plates with authentic nucleoside 5'-phosphates or nucleoside 3'-phosphates (dotted circles) using solvent A. Expected nuclease-resistant dinucleotide products in lane a and b are pC$<^{P}_{P^{*}}$A and C$<^{P}_{P^{*}}$Ap (upper spots of lane a and b, respectively), and pG$<^{P}_{P^{*}}$U and G$<^{P}_{P^{*}}$Up (lower spots of lane a and b, respectively) (* specifies [^{32}P] phosphate).

RESULTS AND DISCUSSION:

Due to their small size, viroids seem to depend completely on host enzymes for their replication. Consequently, the last step of viroid maturation should be the circularization of linear unit length precursor viroids by an appropriate host RNA ligase. A possible candidate for viroid ligation is an enzyme which we found in wheat germ extracts (3), and which needs 2',3'-cyclophosphate ends and 5'-phosphate termini for the formation of -N$^{2'}_{3'}$—P—5'N- bonds (4). Fig. 1 shows that PSTV, either RNase T_1-treated or untreated, is readily labeled by T_4-kinase. In case of untreated PSTV, so-called

natural linear viroid, which contaminates preparations of circular viroid in trace amounts (1), becomes readily labeled. Circularization by wheat germ and Chlamydomonas extract, respectively, is achieved by short incubation in an ATP-dependent reaction. The circularity of the ligation products was confirmed by the regeneration of linear viroid upon controlled introduction of a single nick with alkali (6).

The digestion of circularized PSTV with nuclease P_1 or RNase T_2 produces labeled nucleotide material from the newly formed bond which does not co-migrate with normal mononucleotides (Fig. 2, a-d). Digestion of RNase T_1-nicked, re-circularized PSTV (lanes a and b) yields mainly one spot of low chromatographic mobility, and a minor product of high mobility. This latter material is the only digestion product from circularized natural linear PSTV (lanes c and d). Further analyses as described in legend to Fig. 2 unequivocally prove that the re-ligation of RNase T_1-nicked PSTV yields mainly $-G^{2'-P}_{3'-P}$-5'U-bonds, whereas natural linear PSTV is circularized via a $-C^{2'-P}_{3'-P}$-5'A-bond. This latter dinucleotide is also formed in case of the RNase T_1-nicked PSTV (Fig. 2, upper spots in lanes a and b), due to the presence of natural linear molecules. Corresponding analyses of PSTV circularized by Chlamydomonas extracts gave exactly the same results (not shown) as those in Fig. 2.

The exclusive presence of $-C^{2'-P}_{3'-P}$-5'A-bonds in circularized natural linear PSTV deserves special attention. From our dayly experience with RNA we know, that C-A bonds are the weakest bonds in RNA. Therefore we have to consider the possibility, that the so-called natural linear viroids may be an artefact of preparation. We have shown earlier (6), that preparation of viroid RNA in Mg^{++}-containing buffers, as used by other groups, may produce nicked viroid during isolation and purification.

The following results and arguments support the idea that, at least in our PSTV preparations, the trace amounts of linear molecules are in fact of natural origin and not an artefact:

(a) Viroid preparations were performed in EDTA-containing, Mg^{++}-free buffer (1, 6) in order to avoid any nicking.

(b) 5'-End group analysis of PSTV nicked with Mg^{++}-containing buffer yields pA, pC, pU and pG in a ratio of 38:17:25:20 (6). End group analysis of our 5'labeled natural linear PSTV (Fig. 1, lane b or m),however, gave only pA in over 90 % yield (not shown).

(c) If the trace amounts of linears in our batch of PSTV had been introduced during preparation, we would expect that not only C-A bonds were cleaved.

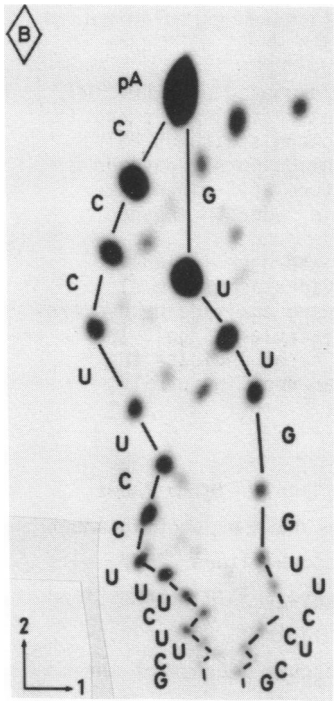

Fig. 3. Sequence analysis of [5'-^{32}P] labeled natural linear PSTV.
5'end labeled natural linear PSTV (Fig. 1 lane b) was partially hydrolized by mild alkaline treatment and analyzed by the two-dimensional mobility shift method (9, 10).
B indicates the position of blue dye marker, xylene cyanol.

At least all C-A bonds present in PSTV should be equally affected. This is not at all the case, as evident from Fig. 3. 5'End labeled natural linear PSTV from Fig. 1, lane b ("L") was partially digested by mild alkali treatment (9, 10) and the resulting fragments separated by two dimensional electrophoresis/ homochromatography (Fig. 3). The mobility shift from fragment to fragment clearly allows one to read two predominant sequences only:
(a) ACCCUUCCUUUCUUCG.... and
(b) AGUUGGUUCCUCG... . Hence the nick in natural linear PSTV is located between C_{181} and A_{182} or between C_{348} and A_{349} (Fig. 4). A careful analysis of the mobility shift pattern in Fig. 3 indicates, that nicks between other C-A bonds which are even more exposed in single-stranded loops (e.g., C_{273}- A_{274}), are present only to a low degree. This low level of nicks may represent those nicks introduced as an artefact during viroid preparation. However, we can not yet completely rule out the possibility that one of the specific nicks (or both) in so-called natural PSTV may be an artefact.
At the moment it seems too early to speculate about the significance of the specifically located nicks in natural linear viroids for viroid

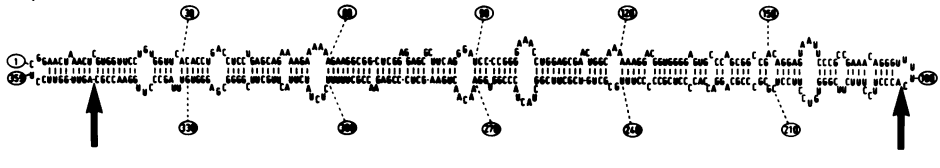

Fig. 4. Primary and secondary structure of PSTV and the main positions of nicks in natural linears. Arrows indicate the location of predominant nicks (C_{181}-A_{182} and C_{348}-A_{349}).

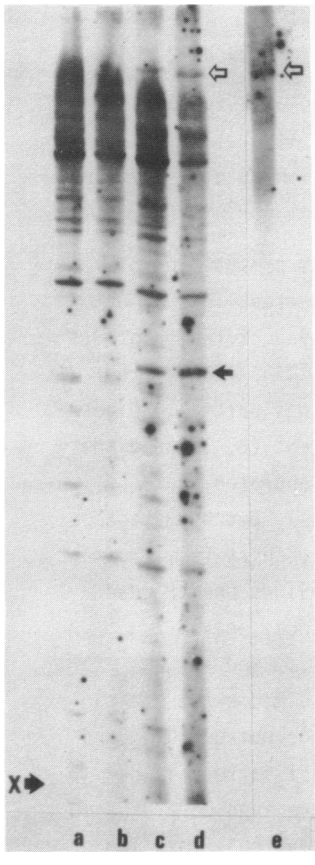

Fig. 5. Phosphorylation and circularization of unlabeled PSTV with wheat germ extract and [γ-^{32}P]ATP.
Unlabeled PSTV was incubated with [γ-^{32}P] ATP and wheat germ extract (lanes c and d). Lanes a and b are controls without PSTV. In lanes b and d, unlabeled ATP was added to the reaction mixture at 3 min as described in Methods in order to enhance the ligation.
The same reaction mixture as in lane d was treated with phosphatase (lane e). Incubated mixtures were analyzed by electrophoresis in 5 % polyacrylamide gel. The white and the black arrow indicate the positions of circular and linear PSTV, respectively.

ligation (or replication). Fig. 5 shows that, due to the 5'-kinase present in wheat germ extract (4), natural linear PSTV is phosphorylated with [γ-^{32}P]ATP at the 5'end and covalently circularized. Some circularization already occurs because of the presence of excess [γ-^{32}P]ATP (Fig. 5, lane c). As we have shown earlier (4), this step needs and is enhanced by added excess ATP (Fig. 5, lane d).

Yet we do not know whether the 2'-phosphate of the newly formed bond has any specific function, except that it renders this bond resistant to many RNases (3). The fact that no labeled mononucleotides are seen in digests of circularized PSTV (Fig. 2, lane a and b) indicates that the 2'-phosphate is completely stable at least during 10 min incubation in wheat germ extract.

In summary, the above observations may reflect the final steps in the maturation of viroid RNA in vivo.

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