# Cell-Free Circularization of Viroid Progeny RNA by an RNA Ligase from Wheat Germ

Abstract. *Linear, potato spindle tuber viroid RNA has been used as a substrate for an RNA ligase purified from wheat germ. Linear viroid molecules are efficiently converted to circular molecules (circles) which are indistinguishable by electrophoretic mobility and two-dimensional oligonucleotide pattern from viroid circles extracted from infected plants. In light of recent evidence for multimeric viroid replication intermediates, cleavage followed by RNA ligation by a cellular enzyme may (i) be a normal step in the viroid life cycle and (ii) may also reflect cellular events.*

Specific cleavage of RNA precursor molecules and the joining of newly created termini is a mechanism that has been recognized increasingly as a central step in many RNA processing pathways (*1, 2*). Several RNA ligase activities have been reported from both prokaryotic (*3*) and eukaryotic (*4–8*) sources, and—in the case of a yeast transfer RNA (tRNA) ligase—a function in tRNA splicing has been suggested (*5*). In seeking additional substrates for eukaryotic RNA ligase activities that participate in RNA processing in vivo, we consider plant viroids a logical choice because (i) their infectious, mature form consists predominantly of a small covalently closed circular RNA molecule (*9–13*); (ii) during synthesis, nascent RNA chains must be linear and, thus, must be ligated to form a circle; (iii) since no viroid-encoded proteins have been detected (*14–16*), it appears that enzymes of the host plant are used for viroid replication (*10, 17–19*). Together, these observations suggest that host plants contain an RNA ligase that circularizes linear viroid RNA. Thus, we have studied the ability of an RNA ligase activity from wheat germ to circularize linear potato spindle tuber viroid (PSTV) RNA.

Extracts of *Saccharomyces cerevisiae* splice tRNA precursors containing intervening sequences (*20*). Splicing by yeast extracts proceeds in two steps: (i) endonucleolytic cleavage produces two half tRNA molecules and an intervening sequence and (ii) adenosine triphosphate (ATP) -dependent ligation connects the two halves (*4*). The tRNA halves can also be joined (*21*) to produce mature sequence tRNA by an activity from wheat germ (Fig. 1A). Briefly, the purification (which leads to about a 250-fold enrichment in enzyme activity) involves the following steps (*21*): centrifugation of the crude wheat germ extract at 12,000*g*; polyethyleneimine precipitation to remove nucleic acids; Sephadex G-50 exclusion chromatography; ammonium sulfate precipitation; chromatography on Biorex-70, DEAE-cellulose, heparin-agarose, Sepharose 4B, LKB HA-Ultrogel, ATP-agarose, and tRNA-agarose. The preparation used here had no detectable ribonuclease, phosphatase, or adenosine triphosphatase activities.

PSTV-infected plants contain a number of viroid-specific RNA's. The two major forms studied here, unit length circular and linear plus strands, separate by a wide margin during electrophoresis in gels containing urea (Fig. 1A, lanes a and h). In fact, in 5 percent polyacrylamide gels containing 7*M* urea, the circular RNA comigrates with linear RNA's that are larger than 1100 nucleotides in length (data not shown), although it is actually only 359 bases long (*12*). The strikingly slow mobility of viroid circular molecules (circles) provides the basis for an assay of PSTV ligation (see below), and permits highly purified samples of both circular and linear PSTV to be prepared after elution of gel bands. For further study, the viroid RNA's can be labeled in vitro with $^{125}I$ (*22–24*). The linear molecules purified from plant ex-

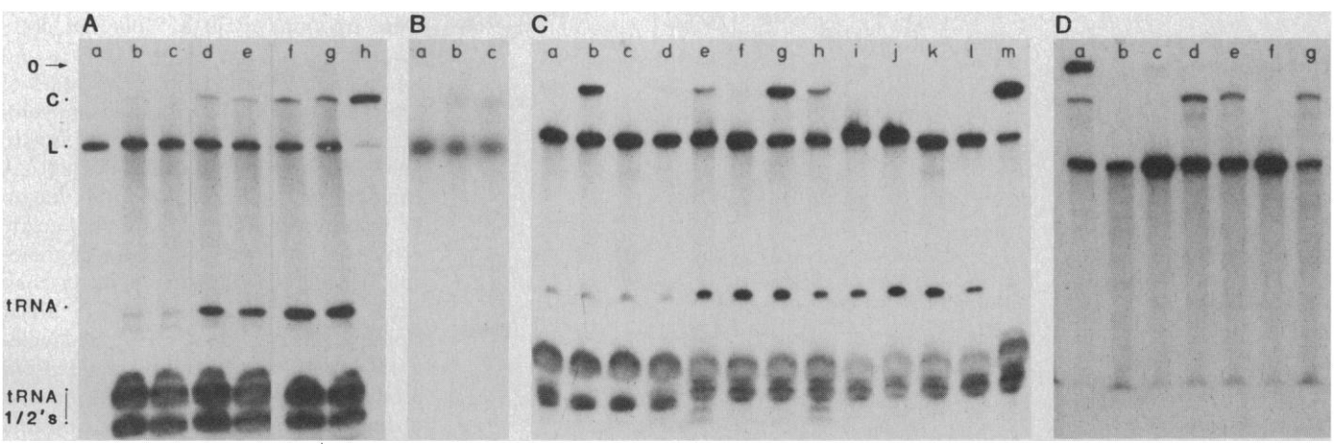


Fig. 1. Viroid circularization by the RNA ligase from wheat germ. (A) $^{125}I$-Labeled PSTV natural linears and $^{32}P$-labeled tRNA half-molecules incubated with increasing concentrations of wheat germ RNA ligase. Lanes a and h show PSTV linear and circular size markers, respectively. Lanes b to g show reaction mixtures including ligase, diluted 1:50 (b and c); 1:25 (d and e); 1:12.5 (f and g). Incubations were for 20 minutes (lanes b, d, and f) or 40 minutes (lanes c, e, and g) at 30°C under conditions used before (*4–6*). The positions of the tRNA half-molecules and the mature sequence tRNA are indicated. (B) $^{125}I$-Labeled PSTV natural linears incubated with wheat germ ligase for various times. Reactions were either not incubated (lane a) or incubated at 30°C for either 1 minute (lane b) or 2 minutes (lane c). (C) Linear PSTV incubated with each of three different RNA ligase activities. Reactions analyzed in lanes a to d contained phage T4–induced RNA ligase (P-L Biochemicals), those in lanes e to h contained RNA ligase from wheat germ, while those in lanes i to l contained RNA ligase from yeast. $^{125}I$-Labeled breakdown linears were substrates in the reactions shown in lanes a, e, and i; nuclease S1-derived linears in lanes b, f, and j; ribonuclease T1-derived linear molecules in lanes c, g, and k; and natural linears in lanes d, h, and l. Circular and linear size markers appear in lane m. Transfer RNA half-molecules were included in all reactions. (D) Effect of prior treatment with calf alkaline phosphatase (CAPase) on the circularization of PSTV linear RNA. Nuclease S1-derived linears were either incubated with CAPase buffer alone (0.25*M* NaCl, 0.001*M* EDTA, 0.035*M* (a) or with CAPase (b) and then were treated with phage T4-induced RNA ligase. Natural linears enzyme treatment (c); or pre-treatment with either CAPase buffer alone (d) or CAPase itself (e) followed by incubation with RNA ligase from wheat germ. Lanes f and g contain linear and circular (plus linear) size markers, respectively. "O" marks the origin of electrophoresis.


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tracts (here designated "natural linears") have potential biological significance but are not well defined and may be very heterogeneous. Therefore, we prepared linear molecules with chemically defined termini by cleaving circular viroid with either ribonuclease T1 or nuclease S1 (*25–27*) under conditions for limited digestion and recovering unit length molecules after gel electrophoresis. Linear molecules arising on storage of $^{125}$I-labeled circular RNA were also purified.

Various linear viroid RNA's were incubated with RNA ligase from either bacteriophage T4–infected *Escherichia coli* cells, wheat germ, or yeast, and the reaction products were analyzed by electrophoresis in 5 percent polyacrylamide gels containing 7*M* urea. To permit enzyme activity to be monitored, yeast leucine tRNA half-molecules (*4*) were included in many of these reactions.

The RNA ligase activity from wheat germ circularized natural linear viroid RNA to a level that was comparable to ligation of tRNA half-molecules present in the same reaction (Fig. 1A). Furthermore, the extent of ligation of these two substrates increased proportionally with increasing amounts of enzyme. The kinetics of viroid circularization were very rapid, with ligation occurring within 1 to 2 minutes (Fig. 1B).

The RNA ligase activity from wheat germ was also capable of ligating linear molecules produced by cleavage of circular viroid by ribonuclease T1. Under conditions of limited digestion such as those used here, this enzyme produces linear molecules that have 5′-hydroxyl termini and 3′ termini that contain either a phosphate on the 3′ position or a cyclic 2′,3′-phosphate (*25*). However, the wheat germ activity did not ligate nuclease S1 derived linears [which have 5′-phosphate and 3′-hydroxyl termini, (*26*)]. The nature of the termini of linear molecules derived by nuclease S1 and ribonuclease T1 cleavage were verified by control studies with T4-induced RNA ligase (see Fig. 1C). These experiments suggest that the viroid natural linear RNA's containing either 3′-phosphate or cyclic 2′,3′-phosphate termini are substrates for the wheat germ activity.

No ligation of viroid RNA by a yeast RNA ligase fraction was detected under conditions that produced ligation of tRNA half-molecules (Fig. 1C). This might reflect a more restrictive substrate specificity of the yeast enzyme, or could be due to contaminants in the yeast fraction that alter the substrate.

Recently, Konarska *et al.* (*6*) have reported a novel RNA ligase activity in crude extracts of wheat germ which can circularize a ribonuclease T1–resistant oligonucleotide derived from tobacco mosaic virus RNA. These authors demonstrated that their activity requires a cyclic 2′,3′-phosphate, a phosphatase-resistant moiety (*7*). The viroid circularization studied here is also resistant to prior treatment with calf alkaline phosphatase (see Fig. 1D), strongly suggesting that this reaction also involves a cyclic 2′,3′-phosphate.

The true circular form of the viroid species produced by the action of the wheat germ ligase was established through the tests shown in Fig. 2. To obtain material for these studies, we performed a preparative scale ligation with highly purified $^{125}$I-labeled natural linears (70,000 dis/min) and RNA ligase from wheat germ. The reaction products were fractionated in a 5 percent polyacrylamide gel containing 7*M* urea and the material migrating in the position of PSTV circular RNA was eluted and compared to circular RNA from tomato plants (naturally occurring circles). A characteristic quality of unit length PSTV circular RNA is its ability to be converted into unit length linear molecules (which migrate much faster than circles in certain gel systems) by RNA cleavage. As is evident in Fig. 2A, nuclease S1 treatment of in vitro ligated viroid produces RNA that migrates at the rate of linear PSTV (lanes h to l). Controls show that the behavior of in vitro ligated circles is indistinguishable from that of authentic PSTV circles (lanes c to g). Furthermore, in a fully denaturing system involving glyoxalation of the RNA's and electrophoresis in 1.6 percent agarose gels (*27*), the in vitro ligated circles ran as unit length viroid RNA, and not at the rate expected for a 718-nucleotide viroid dimer (Fig. 2B), indicating that circularization (rather than intermolecular dimerization) had occurred. In addition, natural and ligated circles had the

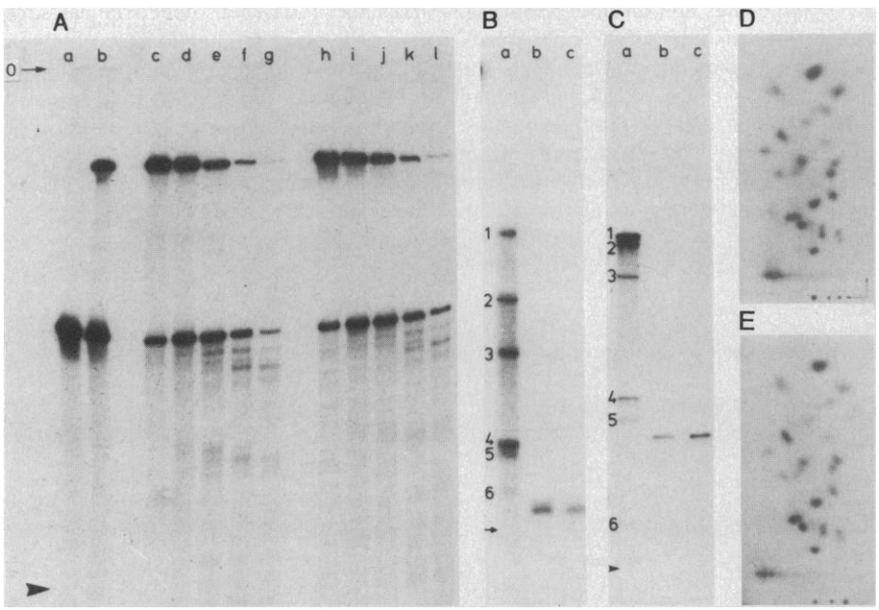


Fig. 2. Conversion of linear PSTV into a circular molecule by an RNA ligase from wheat germ. (A) Nuclease nicking of natural and in vitro ligated PSTV. Circular PSTV (2000 dis/min) was resuspended in 5 μl of solution (*26*) containing nuclease S1 (1 U/μl) and incubated at 37°C for various times. (Lane a) Natural linears used in the preparative scale ligation; (lane b) portion of the preparative scale ligation mixture; (lane c) natural circles in nuclease S1 buffer alone; lanes d to g: natural circles digested for 1 minute (d), 5 minutes (e), 15 minutes (f), or 40 minutes (g); (lane h) in vitro ligated circles in buffer alone; lanes i to l: in vitro ligated circles digested for 1 minute (i), 5 minutes (j), 15 minutes (k), or 40 minutes (l). (B) Electrophoresis of natural and in vitro ligated circles under denaturing conditions. Natural (b) or in vitro ligated circles (c) (500 dis/min) were treated with 1*M* glyoxal, 50 percent dimethyl sulfoxide at 50°C for 1 hour and subjected to electrophoresis along with phage T7 mRNA's whose nucleotide lengths are (1) 2749, (2) 1670, (3) 1140, (4) 578, (5) 561, (6) 394 (line a). (C) Electrophoresis in a 3 percent polyacrylamide gel (0.3 mm thick) containing 7*M* urea. Lane a shows RNA size markers (see above); (b) in vitro ligated circles; (c) natural circles. "O" marks the origin of electrophoresis; arrowheads and arrows indicate positions of xylene cyanol and bromphenol blue dye markers, respectively. (D and E) Either natural (D) or in vitro ligated (E) circular RNA (1500 dis/min) was digested by ribonuclease T1 and subjected to two-dimensional oligonucleotide analysis (*11*). The horizontal and vertical arrows in panel D indicate the directions of first- and second-dimension separations.

same mobility in 3 percent, 7*M* urea polyacrylamide gels (Fig. 2C). Finally, two-dimensional oligonucleotide analysis showed that no detectable rearrangement of the viroid primary structure accompanied in vitro ligation (Fig. 2, D and E).

Our data show that an RNA ligase activity from wheat germ circularizes viroid RNA to the same extent as it ligates tRNA half-molecules. The wheat germ RNA ligase used in our experiments may well function in vivo in RNA splicing reactions, since it is capable of replacing the yeast tRNA splicing ligase in an in vitro reaction. By several criteria, the viroid circles produced in vitro could not be distinguished from natural progeny viroid RNA circles purified from infected plants.

Several features of the viroid life cycle have been described in detail. Host RNA metabolic enzymes appear to be required throughout. The mechanism of viroid replication appears to involve complementary RNA strands (*27–32*) without DNA intermediates (*33, 34*). Minus strand RNA, opposite in polarity to that of the mature viroid, arises in infected cells, with prevalent size classes on fully denaturing gels which are between two and five times the length of mature viroid RNA (*27*). Branch *et al.* have proposed a rolling circle mechanism to account for these greater than unit length minus strands (*27*).

In addition to multimeric minus strand replication intermediates, recent experiments have revealed several species of greater than unit length plus strands in PSTV-infected tomato plants (*19, 35, 36*). One straightforward way for these in vivo multimeric plus strands to participate in replication would be through cleavage by an endonuclease generating unit length linear RNA molecules with cyclic 2′,3′-phosphate termini. The viroid circularization studied here indicates one mechanism through which such cleavage products could have their ends joined and suggests an approach for determining whether the linear molecules acted on by the wheat germ ligase have specific, nonrandom terminal sequences. Alternatively, the long plus strands may be capable of self-cleavage and ligation similar to that reported for tetrahymena ribosomal RNA species (*37*).

While the mechanism of viroid pathogenesis remains a mystery, it has been proposed that disruption of host cell RNA processing, and in particular of RNA ligation, is involved (*19, 38–41*). Future studies will determine whether PSTV strains differing in the severity of their disease symptoms also differ significantly in their interactions with plant RNA ligase activities.

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## Induction of Melanized Cells from a Goldfish Erythrophoroma: Isolation of Pigment Translocation Variants

Abstract. *Melanization was induced in some cells of a goldfish tumor cell line (GEM-81) by cultivating the cells in autologous serum. The melanized cells continued to proliferate in vitro and several clones were isolated that differed with respect to cell morphology and intracellular distribution of pigment. Some of the clones consisted of cells able to translocate their melanosomes in response to epinephrine, melatonin, or adenosine 3′,5′-monophosphate.*

Although the pigment cells of lower vertebrates have been useful for studies of differentiation and organelle translocation (*1*), their scattered distribution in the skin has hampered biochemical studies. We report here the induction, isolation, and partial characterization of several melanogenic sublines, obtained from a goldfish erythrophoroma line (GEM-81) that may be useful for such studies.

Erythrophoromas arise from xanthophores (erythrophores), which are pigment cells containing pteridines and carotenoids. Despite the retention of small amounts of pteridines, the GEM-81 cells are essentially colorless and have been maintained for over 100 passages without yielding melanogenic sublines (*2*). Previously reported instances of melanization in these cells resulted in terminal differentiation, and pigment translocation was not observed (*3*). However, the melanogenic sublines reported here continue to grow and differentiate in vitro to stages exhibiting a variety of pigment translocation characteristics. These new


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