Synthesis of (+) and (-) RNA molecules of potato spindle tuber viroid (PSTV) in isolated nuclei and its impairment by transcription inhibitors

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Transcription studies with highly purified potato cell nuclei in combination with a 'transcription-hybridization analysis' unequivocally demonstrate that the nucleus is the subcellular site where the entire process of PSTV replication takes place. Inhibition experiments with actinomycin D and α-amanitin furthermore suggest that the nuclear DNA-dependent RNA polymerases I and II are involved in the synthesis of PSTV (+) and (-) RNA, respectively.

Viroids are subviral agents, which cause several economically important diseases in higher plants. They have an exceptional position among all presently known pathogens because they exist as small circular RNA molecules not encapsidated into a protein coat and their potential genetic information is most probably not translated into polypeptides (for review see Sänger, 1982, 1984; Diener, 1983, 1984). Therefore it is generally assumed that the replication of all viroids is completely dependent on the RNA-synthesizing and -processing machinery preexisting in the host cell. On the basis of sequence homologies the presently known viroids can be allocated into four groups, represented by the potato spindle tuber viroid (PSTV), the hop stunt viroid (HSV), the avocado sunblotch viroid (ASBV) and the coconut cadang cadang viroid (CCCV). The following lines of indirect evidence suggest that the replication of the viroids of the PSTV and HSV group proceeds with the aid of host enzymes in the cell nucleus. 1. The bulk of their infectivity was found in the nuclear fraction (Diener, 1971; Sänger, 1972; Semancik et al., 1976; Takahashi et al., 1982), and it could be demonstrated recently that the PSTV RNA is predominantly associated with the nucleolus (Schumacher et al., 1983). 2. Oligomeric PSTV RNA molecules of (+) and (-) polarity, the putative intermediates of replication, were detected by molecular hybridization in nuclei isolated from PSTV-infected potato cells (Spiesmacher et al., 1983). 3. α-Amanitin was found to inhibit the replication of the HSV-related cucumber pale fruit viroid (CPFV) in inoculated protoplasts in concentrations which are known to inhibit specifically the nuclear DNA-dependent RNA polymerase II (Mühlbach and Sänger, 1979). 4. Purified DNA-dependent RNA polymerase II of

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plant origin was found to be capable of transcribing PSTV (+) RNA into (-) RNA copies in vitro (Rackwitz et al., 1981) and to specifically recognize and bind PSTV RNA molecules stronger than expected for a non-specific protein-nucleic acid interaction (Goodman et al., 1984).

Previous attempts to demonstrate the biosynthesis of viroid RNA in nuclei from plant tissue infected with PSTV (Takahashi & Diener, 1975) and with citrus exocortis viroid (CEV) (Flores & Semancik, 1982; Semancik & Harper, 1984) suffered from several shortcomings; (i) only preparations enriched in nuclei but no highly purified nuclei were used in all these studies; (ii) the incorporation of radioactivity into the presumed viroid RNA was analysed by gel electrophoresis under conditions which were not fully denaturing so that the existence of complexes between unlabelled viroid RNA and radioactively labelled non-viroid-specific nuclear transcription products cannot be excluded; (iii) the conclusions drawn with regard to the presumed viroid synthesis were thus only based on the comigration of radioactivity with the band of unlabelled viroid RNA and no other evidence was presented to verify the actual viroid-specificity of the newly-synthesized and labelled RNA, and (iv) no attempts were made to differentiate between the synthesis of viroid (+) and (-) RNA molecules.

To investigate the synthesis of PSTV (+) and (-) RNA we used highly purified nuclei as obtained by two steps of Percoll centrifugation. They were isolated from a cell suspension culture established about four years ago from PSTV-infected potato cells (Mühlbach and Sänger, 1981), which have been capable of continuously producing PSTV ever since. After incubation with radioactive RNA precursors the labelled nuclear transcription products were hybridized to purified membrane-bound unlabelled PSTV (+) RNA and to unlabelled single-stranded PSTV DNA of (+) and (-) polarity as obtained by molecular cloning in bacteriophage M13 (Tabler and Sänger, 1984). By using this highly specific and sensitive 'transcription-hybridization analysis' and appropriate transcription inhibitors we could clearly demonstrate that the entire process of PSTV replication takes place in the nucleus of the host cell and that the nuclear DNA-dependent RNA polymerases I and II are involved in the synthesis of PSTV (+) and (-) RNA molecules, respectively.

**Materials and Methods**

**Isolation of nuclei and RNA polymerase assay**

Nuclei were isolated from suspension cultures of healthy and PSTV-infected cells of the wild type potato *Solanum demissum* L. (Mühlbach et al., 1983) as previously described by Spiesmacher et al. (1983). RNA polymerase activity was assayed by incubating 2 x 10⁶ nuclei in 60 mM Tris/HCl buffer (pH 8.0), containing 40 mM (NH₄)₂SO₄, 5 mM MgCl₂, 1 mM EDTA, 250 mM sucrose, 0.5% bovine serum albumin (BRL), 15% glycerol, 0.3 mM each of ATP, CTP, GTP, and UTP and 25 μCi 5,6-³H-UTP (specific activity 43.5 Ci/mmol, Amersham) in a total vol. of 250 μl at 37°C. ³H-UMP incorporation into polynucleotides was determined in 25 μl aliquots after removal of
unincorporated $^3$H-UTP by paper chromatography according to the method of Rackwitz et al. (1981). In order to follow the synthesis of PSTV-specific products, $2 \times 10^6$ nuclei were incubated for 30 min at 29°C in a total vol. of 200 μl of the assay buffer described above, but with 0.02 mM instead of 0.3 mM UTP, with 250 μCi of α-$^32$P-UTP (specific activity 410 Ci/mmol, Amersham) and with 40 mM vanadyl ribonucleoside complex (BRL) as nuclease inhibitor. The transcription inhibitors α-amanitin and actinomycin D (Boehringer, Mannheim) were added to the incubation mixture in a final concentration of $10^{-6}$ mol/l and 10 μg/ml, respectively. The transcription reaction was terminated by phenolization and the unincorporated $^32$P-UTP was removed by gel filtration on Biogel A-0.5 m (Bio-Rad) in 0.1 M triethylammonium-bicarbonate buffer pH 7.6. The fractions containing the transcription products were pooled, lyophilized and redissolved either in water (for gel electrophoretic analysis) or in hybridization buffer, when used as hybridization probes.

**Gel electrophoresis and autoradiography**

All nucleic acid preparations were denatured by glyoxalation (McMaster & Carmichael, 1977) and separated on 3.5% polyacrylamide, 0.09% bisacrylamide slab gels (20 x 20 x 0.15 cm) in 10 mM sodium phosphate buffer pH 6.5. Gels were run with 5 V/cm until the bromophenol blue dye marker had reached the bottom of the gel. Gels run with $^32$P-labelled RNA were dried and autoradiographed onto Fuji RX X-ray films at -80°C, using DuPont Cronex intensifying screens.

**Blotting of PSTV-specific nucleic acids and hybridization with $^32$P-labelled nuclear transcription products**

For the detection of PSTV synthesis in isolated nuclei the $^32$P-labelled nuclear transcription products were hybridized to unlabelled filter-bound RNA and single-stranded DNA. PSTV-specific single-stranded DNA of (+) polarity [PSTV (+) DNA] and of (-) polarity [PSTV (-) DNA] was obtained by subcloning a 359 bp fragment representing the entire PSTV sequence (Tabler et al., 1985) into the bacteriophage M13 mp 11 in both possible orientations. Further subcloning of PSTV (+) DNA allowed the construction of head-to-tail connected dimers [PSTV (+++) DNA] and tetramers [PSTV (++++) DNA] (Tabler and Sänger, 1984). To optimize the conditions of hybridization, Northern blots were prepared from 3.5% polyacrylamide gels run with preparations of purified PSTV and of 2 M LiCl-soluble RNA from healthy and PSTV infected potato cells. The nucleic acids were blotted onto GeneScreen Transfer Membranes (New England Nuclear) by electrotransfer (Stellwag & Dahlberg, 1980) in 25 mM sodium phosphate buffer pH 6.5. For dot-blotting the BRL 'Hybr-Dot' system was used. Gene Screen transfer membranes were presoaked first with water and then with 20 x SSC (3 M NaCl/0.3 M trisodium citrate). 700 ng of M13-cloned single-stranded DNA containing (+) and (-) PSTV sequences, respectively, and 10 ng of glyoxalated PSTV were dissolved in 200 μl of 3 M lithium acetate and spotted onto the membrane. To guarantee optimal absorption of the samples 300 μl of
20 x SSC were then sucked through the dotted spots. Northern blots and dot blots were baked in vacuo for 4 h at 80°C. The nuclear transcription products used as probes were denatured by heating them in hybridization buffer to 100°C for 2 min and then quenched in ice. Hybridization, washing of the blots and autoradiography followed the previously described protocols (Mühlbach et al., 1983).

Results

RNA polymerase activity of isolated nuclei

The transcriptional activity of purified nuclei was determined by $^3$H-UMP incorporation into high molecular weight products, which were chromatographically separated from unincorporated $^3$H-UTP. In order to optimize the conditions for RNA synthesis the influence of different MgCl$_2$ and (NH$_4$)$_2$SO$_4$ concentrations was investigated. $^3$H-UMP incorporation was found to be optimal at a combination of 5 mM MgCl$_2$ with 40 mM (NH$_4$)$_2$SO$_4$ (data not shown). In most experiments the incorporation of UMP by PSTV-infected nuclei was twice as high as in healthy nuclei. When ATP, CTP, and GTP were omitted from the reaction mixture no incorporation was observed, indicating that the $^3$H-labeling of the products resulted from genuine transcription and not from endlabeling due to terminal transferase activity (Table 1). A kinetic study of $^3$H-UMP incorporation revealed that RNA synthesis was linear for the first 30 min and then slowed down but continued for a further 90 min (Fig. 1). After 1 h of incubation, $10^6$ nuclei incorporated approx. 90 pmol of UMP. RNA synthesis was inhibited up to nearly 90% by $10^{-6}$ mol/α-amanitin (Fig. 1 and Table 1), implying that this proportion of the total activity could be attributed to the highly α-amanitin-sensitive RNA polymerase II. Actinomycin D at a concentration of 10 μg/ml inhibited RNA synthesis by approx. 60%. Preincubation of nuclei with 10 μg/ml RNase-free DNase I for 30 min at 29°C reduced the incorporation of UMP to 0.1%, whereas in control experiments preincubation of nuclei under identical conditions without DNase had no effect on RNA synthesis.

Table 1. RNA polymerase activity in nuclei isolated from suspension culture cells of the wild type potato Solanum demissum L

| Source of nuclei | Additions to or deletions from the assay medium* | pMol UMP incorporated by $10^6$ nuclei | Percent of control
<table>
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<tr>
<td>PSTV-infected potato cells</td>
<td>None</td>
<td>459.1</td>
<td>100</td>
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<tr>
<td></td>
<td>10-6 mol/l α-amanitin</td>
<td>27.5</td>
<td>6.0</td>
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<tr>
<td></td>
<td>10 μg/ml actinomycin D</td>
<td>174.5</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>Nuclei preincubated with DNase I (10 μg/ml)</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>- ATP, - CTP, - GTP</td>
<td>0.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Uninfected potato cells</td>
<td>None</td>
<td>257.1</td>
<td>56.0</td>
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*The conditions of the assay are described in Materials and Methods.
Size of the newly synthesized nuclear transcription products

In order to determine the size of the newly synthesized RNA, transcription experiments were performed in the presence of $^{32}$P-UTP. To ensure their full denaturation the transcription products were glyoxalated and then separated on a 3.5% polyacrylamide gel. This analysis revealed a polydisperse size distribution, where the majority of the newly synthesized and labelled RNA species migrated in the size range of molecules with chain length between 800 and 2000 nucleotides (Fig. 2). No differences could be detected between the gel patterns of the transcription products from uninfected and PSTV-infected nuclei and no distinct band of radioactivity comigrating with PSTV marker RNA was found in transcripts of nuclei from PSTV-infected cells (Fig. 2, lanes 2 and 6). The addition of $\alpha$-amanitin to a final concentration of at least $10^{-6}$ mol/l (Fig. 2, lanes 4 and 5) inhibited RNA transcription almost completely.

Detection of newly synthesized PSTV (-) RNA

From the polydispersity of the nuclear transcription products it becomes evident that the only reliable method to detect newly synthesized PSTV sequences in such material is molecular hybridization. For the detection of PSTV (-) RNA we utilized the $^{32}$P-labelled total nuclear transcription products as probes which were hybridized to Northern blots prepared by electrotransfer from 3.5% polyacrylamide gels on which purified but unlabelled and glyoxylated PSTV (+) RNA had been run (Fig. 3 a–d, lanes 3). In order to detect any non-specific hybridization, unlabelled and glyoxylated 2 M LiCl-soluble RNA preparations from PSTV-infected (Fig. 3, lane 1) and from healthy (Fig. 3, lane 2) potato cells were run as controls on the same gel. They are known to contain the different cellular RNA species such as tRNA, 5S RNA, 7S RNA, and, in the case of PSTV-infected cells, also
Fig. 2. Autoradiograph of the pattern of glyoxalated and hence fully denatured $^{32}$P-labelled transcription products of isolated potato cell nuclei after separation on a 3.5% polyacrylamide gel. In lane 1, $^{125}$I-labelled PSTV. In lanes 2-5, transcription products of nuclei isolated from PSTV-infected potato cell suspensions. The transcription assays contained different concentrations of $\alpha$-amanitin, namely in lane 2, none; in lane 3, $10^{-8}$ mol/l; in lane 4, $10^{-6}$ mol/l; in lane 5, $10^{-4}$ mol/l. Lane 6, transcription products of nuclei isolated from uninfected potato cell suspensions. Lane 7, $^{32}$P-labelled Hinf I restriction fragments of pBR 322 DNA. C: Circular PSTV, L: Linear PSTV.

PSTV. The blots were hybridized in the presence of 50% formamide and at different temperatures with equal amounts of heat-denatured $^{32}$P-labelled transcription products of healthy and PSTV-infected nuclei. The hybridization patterns in Fig. 3 show that at 65°C the radioactive transcripts from healthy and PSTV-infected nuclei hybridized to practically all cellular RNA species and also to PSTV, whereas PSTV-specific signals were found only after hybridization at 75 and 80°C. Under these stringent conditions hybrids can only be formed between the unlabelled PSTV (+) RNA and the fully complementary and radioactively labelled PSTV (-) RNA molecules present in the nuclear transcripts and not between the partially self-complementary PSTV (+) RNA molecules themselves (Mühlbach et al., 1983). Hybridization with alkali treated transcription products gave no signal at all, indicating that the newly synthesized and hence labelled products consist exclusively of RNA. From this finding it can be inferred that in nuclei isolated from PSTV-infected potato cells PSTV(-) RNA is newly synthesized within 30 min of incubation.
Fig. 3. Detection of newly synthesized $^{32}$P-labelled PSTV (-) RNA present in the transcription products of nuclei isolated from a PSTV-infected potato cell suspension culture by Northern blot hybridization. Unlabelled 2 M LiCl-soluble RNA from PSTV-infected (lanes 1 and 1') and uninfected (lanes 2 and 2') potato cells, and purified PSTV (lanes 3 and 3') were separated on 3.5% polyacrylamide gels, electro-blotted to Gene Screen filters and probed with the heat-denatured $^{32}$P-labelled nuclear transcription products. The blots with lanes 1-3 were hybridized with transcripts of nuclei from uninfected cells, whereas the blots with lanes 1'-3' were hybridized with transcripts of nuclei from PSTV-infected cells. Hybridization was performed in the presence of 50% formamide at the temperatures indicated.

Synthesis of PSTV (+) and (-) RNA in isolated nuclei

Since filter-bound PSTV does not allow the detection of newly synthesized PSTV (+) RNA, we used M13-cloned unlabelled single-stranded DNA, containing PSTV-complementary sequences of unit length, denoted PSTV (-) DNA, for this purpose. This PSTV (-) DNA was spotted on Gene Screen filters and dot-blot hybridization was carried out with $^{32}$P-labelled nuclear transcription products. In order to be able to simultaneously detect the PSTV (+) RNA and PSTV (-) RNA sequences in the labelled nuclear transcripts the suitability of dot-spotted PSTV (+) RNA and of M13-cloned single-stranded PSTV (+) DNA of unit length [PSTV (+) DNA], two times unit length [PSTV (++) DNA], and four times unit length [PSTV (+++) DNA], respectively was tested. A strong hybridization signal was only observed (Fig. 4) when PSTV (-) DNA was probed with the transcripts of PSTV-infected nuclei, clearly substantiating that isolated nuclei are capable of quite efficiently synthesizing PSTV (+) RNA (Fig. 4a).
Fig. 4. Analysis of PSTV (+) and (-) RNA synthesis in isolated nuclei by dot blot hybridization. 10 ng PSTV (+) RNA and 700 ng of M13-cloned single-stranded DNA containing a PSTV-complementary sequence of unit length PSTV (-) DNA, PSTV sequences of unit length PSTV (+) DNA, twice [PSTV(++)DNA] and four times [PSTV(++++)DNA] unit length, respectively, were dotted onto Gene Screen membranes. The heat-denatured 32P-labelled nuclear transcription products were used for hybridization at 75°C and in the presence of 50% formamide. In panel a, hybridization with transcripts of nuclei from PSTV-infected (1), and from uninfected (2) potato cells. In panel b, hybridization with transcripts of nuclei from PSTV-infected potato cells preincubated for 30 min without DNase (1); preincubated for 30 min with 10 μg/ml of DNase I (2); incubated without ATP, CTP, and GTP (3). In panel c, hybridization with transcripts of nuclei from PSTV-infected potato cells incubated without inhibitors (1); with 10−6 mol/l α-amanitin (2); with 10 μg/ml actinomycin D (3).
contrast, the amount of newly synthesized PSTV (-) RNA appeared to be much lower than that of PSTV (+) RNA. With the quantities employed, it was only detectable by its hybridization to the filter-bound PSTV (+++) DNA but not with either PSTV(+), RNA, PSTV (+) DNA or PSTV (+++) DNA (Fig. 4a, lane 1). The control experiments with the transcription products of healthy nuclei revealed no hybridization signal at all (Fig. 4a, lane 2). Since the filter bound PSTV (+++) DNA proved to be the most suitable material to detect the minute quantities of newly synthesized PSTV (-) RNA, this DNA was utilized in additional hybridization experiments.

PSTV (+) and (-) RNA synthesis was drastically reduced when the nuclei were preincubated with DNase I (Fig. 4b, panel 2), or when the unlabelled nucleoside triphosphates ATP, CTP, and GTP were omitted from the reaction mixture (Fig. 4b, panel 3). These results correspond to the data on the total incorporation of UMP (Table 1) and show that genuine de novo synthesis of viroid RNA molecules takes place and that the incorporation of 32P-UMP into PSTV cannot be attributed to possible end-labeling by the action of terminal transferases.

Effect of transcription inhibitors on PSTV synthesis

The different sensitivity of the RNA polymerases I, II, and III to α-amanitin and actinomycin D was expected to yield experimental evidence on the possible involvement of these host enzymes in PSTV replication. Therefore, we analysed the effects of these two drugs on the synthesis of PSTV (+) and PSTV (-) RNA. α-Amanitin was added to the incubation mixture to give a final concentration of 10⁻⁵ mol/l, and actinomycin D to 10 µg/ml. As shown in Fig. 4c, α-amanitin inhibited the synthesis of both PSTV (+) and (-) RNA more efficiently than actinomycin D. The densitometric analysis of the original autoradiographs revealed that α-amanitin (Fig. 4c, panel 2) inhibited the synthesis of PSTV (+) and (-) RNA by about 80%, which strongly suggests that the DNA-dependent RNA polymerase II is involved in PSTV replication. On the other hand, actinomycin D (Fig. 4c, panel 3) inhibited PSTV (+) RNA synthesis by 65%, but PSTV (-) RNA synthesis only by 10%, which indicates that the latter is largely resistant to this drug and that two RNA polymerases which differ in their sensitivity to actinomycin D are involved in PSTV replication. From the reported sensitivity of the RNA polymerase I-mediated ribosomal RNA synthesis to actinomycin D (Reich & Goldberg, 1964) we conclude that it is the nucleolar DNA-dependent RNA polymerase I which synthesizes PSTV (+) RNA. It cannot be decided from this inhibition experiment which of the lesser sensitive RNA polymerases II or III mediates the synthesis of the PSTV (-) RNA. However, the inhibition of the PSTV synthesis by 10⁻⁶ mol/l α-amanitin, a concentration which is known to inhibit RNA polymerase II and not RNA polymerase III, strongly suggests that DNA-dependent RNA polymerase II rather than polymerase III is involved in the synthesis of PSTV (-) RNA.

Discussion

It is generally accepted that the infectious monomeric circular viroid RNA arbitrarily denoted as (+) RNA, which finally accumulates
in the viroid-infected host plant, represents the viroid entity proper. There is also general agreement that viroid replication proceeds with the aid of host enzymes via complementary RNA forms as replicative intermediates, denoted as (-) RNA, without any DNA being involved. Consequently, in the process of viroid replication at least two steps of 'RNA-RNA transcription' must exist. In step 1 the monomeric viroid (+) RNA is transcribed, presumably by a rolling-circle-like mechanism, into multimeric viroid (-) RNA molecules. In step 2 these replicative intermediate (-) RNAs are then transcribed into multimeric (+) RNAs from which the monomeric circular viroid RNA progeny molecules are finally processed by specific cutting and circularization.

From our previous investigation on viroid replication in protoplasts (Mühlbach and Sänger, 1979) and especially from the in vitro transcription studies with purified DNA-dependent RNA polymerases and purified PSTV (+) RNA (Rackwitz et al., 1981) we had concluded that the DNA-dependent RNA polymerase II is involved in PSTV replication. However, in view of the involvement of various factors in the complex process of in vivo transcription (Lewis & Burgess, 1992, Heintz & Roeder, 1992) we felt compelled to complement experiments with their system-inherent limitations by analyzing viroid replication in isolated potato nuclei, because they certainly reflect the actual in vivo situation much more closely than the rather artificial in vitro systems.

In the experiments described in this report we provide evidence that in the case of PSTV the two steps of viroid transcription are performed in isolated potato nuclei and that the nuclear DNA-dependent RNA polymerase II is involved in the first transcription step from (+) to (-) PSTV RNA whereas RNA polymerase I performs the second step from (-) to (+) PSTV RNA.

Detection of newly synthesized PSTV (+) and (-) RNA in highly purified nuclei

We found that the simple separation of glyoxalated and hence fully denatured radioactively labelled nuclear transcripts on 3.5% polyacrylamide gels did not allow us to detect on the corresponding autoradiographs these newly synthesized PSTV RNAs as distinct bands because they were totally buried under the large amounts of the heterodisperse endogenous nuclear transcription products. Therefore, we applied an alternative approach by hybridizing these labelled nuclear transcripts which include also the newly synthesized PSTV (+) and (-) RNA to filter-bound unlabelled strand-specific M13-cloned single stranded PSTV DNA. Only with this highly specific and sensitive technique of 'transcription-hybridization analysis' on Northern blots and dot blots we could unequivocally demonstrate that PSTV (+) and (-) RNA molecules are synthesized in isolated nuclei.

In contrast to our results in comparable previous investigations with nuclei-enriched preparations from leaf tissue of PSTV-infected tomato (Takahashi & Diener, 1975) and CEV-infected Gynura aurantiaca (Flores & Semancik, 1982; Semancik & Harper, 1984), only a single radioactive band comigrating with the viroid RNA marker was found. However, the gel electrophoretic analyses on which these findings are based were performed under conditions which are now known to be
only partly denaturing even at 8 M urea. Therefore, one must assume that the radioactivity detected at the position of the circular viroid was based on the binding of radioactively labelled short viroid sequences to the unlabelled viroid molecules already present in the infected nuclei, and not necessarily due to the de novo synthesis of circular viroid molecules of unit length as claimed in these reports.

Our dot blot hybridization analysis also allowed estimation of the ratio between the newly synthesized PSTV (+) and (-) RNA molecules present amongst the nuclear transcription products. It revealed that PSTV (+) RNA is about twenty times more efficiently synthesized than PSTV (-) RNA, which substantiates the previously postulated role of PSTV (-) RNA molecules as transient intermediates of replication (Rohde & Sänger, 1981; Branch et al., 1981; Owens & Diener, 1982; Mühlbach et al., 1983; Spiesmacher et al., 1983). At least two interpretations can be offered for the extensive PSTV (+) RNA synthesis observed: (i) Under the assumption that PSTV (+) and (-) RNA are synthesized by the same RNA polymerase, the PSTV (-) RNA molecules seem to be a much more suitable template than PSTV (+) RNA, which would explain the increased synthesis of PSTV (+) RNA. (ii) If different polymerases are involved in the viroid replication cycle, the one catalysing the PSTV (+) RNA synthesis could exhibit a higher transcription efficiency than the one involved in the synthesis of PSTV (-) RNA.

\textit{Discrimination between the nuclear DNA-dependent RNA polymerases involved in PSTV replication}

Inhibition studies using α-amanitin and actinomycin D, which are known to affect the three nuclear DNA-dependent RNA polymerases differently, were carried out in order to discriminate between their presumed involvement in the two steps of PSTV (+) and (-) RNA transcription. This became possible because the two forms of newly synthesized RNA could be clearly differentiated independently from each other in our 'transcription-hybridization assay'. As shown in Fig. 4c, in isolated nuclei α-amanitin inhibits already at a concentration of $10^{-6}$ mol/l both RNA-RNA transcription steps of viroid replication, namely the synthesis of both PSTV (-) and (+) RNA. At the concentration applied α-amanitin is known to inhibit RNA polymerase II activity specifically. Actinomycin D, on the other hand, inhibits at a concentration of 10 μg/ml predominantly the second step of viroid replication, namely the synthesis of PSTV (+) RNA, but has only little effect on the synthesis of PSTV (-) RNA (Fig. 4c, panel 3). Regarding the effect of actinomycin D it has been found that this drug causes structural alterations of the chromatin and that the three nuclear DNA-dependent RNA polymerases differ in their sensitivity to the alterations (Reich & Goldberg, 1964). The activity of RNA polymerase I, which is localized in the nucleolus, is inhibited by low concentrations of actinomycin D, at which the RNA synthesis catalysed by the nucleoplasmic RNA polymerases II and III is not affected (Widnell & Tata, 1966). From the inhibition of the second RNA transcription step of viroid replication by the low concentration of actinomycin D specific for RNA polymerase I we conclude that this polymerase transcribes the PSTV (-) RNA intermediates into oligomeric PSTV (+) RNA molecules. The mechanism of the inhibition of
RNA polymerase I by actinomycin D is not yet fully understood but it is known that this drug induces the segregation of nucleoli. Since it is generally accepted that RNA synthesis by DNA-dependent RNA polymerase I requires the morphological integrity of the nucleolus (for review see Goessens, 1984) the inhibition of PSTV (+) RNA synthesis by actinomycin D is presumably an indirect effect and most probably based on the impairment of the nucleolar structure by this drug. All these assumptions are in accordance with our finding that the PSTV (+) RNA is associated with the nucleolus (Schumacher et al., 1983) and they would also indicate that it is not only accumulating but also being synthesized there. The finding that polymerase I is involved in the second transcription step of viroid replication necessitates the assumption that polymerase II is responsible for the first step and transcribes PSTV (+) RNA into the oligomeric PSTV (-) RNA forms, the intermediates of viroid replication. This assumption is in agreement with the observed α-amanitin-inhibition of the PSTV (-) RNA synthesis. The inhibition of PSTV (+) RNA synthesis by α-amanitin, which does not inhibit DNA-dependent RNA polymerase I in vitro, is most probably also based on an indirect effect like the actinomycin D inhibition, because α-amanitin is also known to induce the segregation of nucleoli (Faulstich, 1980). It is also conceivable that the inhibition of PSTV (+) RNA is due to the α-amanitin-reduced supply of the transient PSTV (-) RNA template molecules from which the PSTV (+) RNA progeny is transcribed. At present, it cannot be decided unequivocally whether RNA polymerase III is also involved in the process of PSTV RNA-RNA transcription, because neither α-amanitin and actinomycin D nor any other substance inhibits this enzyme in a clearly discriminating way.

From the unexpected finding that PSTV synthesis is inhibited by preincubation of the nuclei with DNase I it could be inferred that a transcription step involving a viroid-specific DNA is required for PSTV replication. However, despite intensive search no such viroid-specific DNA could be detected in PSTV-infected tissue (Branch & Dickson, 1980; Zaitlin et al., 1980; Hadidi et al., 1981). Thus, the inhibition of PSTV synthesis in isolated nuclei by DNase I could be based on the enzymatic disruption of the chromatin environment the integrity of which is an essential prerequisite for the normal functioning of the RNA polymerases including their participation in viroid replication.

It should be noted here that the possible involvement in viroid replication of the DNA-directed RNA polymerase I has also been inferred from the presence in different viroids of sequences which are homologous to portions of a promoter sequence required for the transcription of a rRNA gene of mouse by the nucleolar RNA polymerase I. The absence in PSTV of the consensus sequence 'TATA' or 'CCAAT' active in RNA-polymerase II transcription was taken as additional evidence for this concept (Palukaitis & Zaitlin, 1983). However, the results from such sequence comparisons should be taken with caution for several reasons: (i) The corresponding sequences for higher plants, in particular for viroid host plants, are still unknown. It has become rather doubtful that the nucleotide sequences involved in the regulation of transcription by the eucaryotic RNA polymerase I are identical in mice and plants because they were found to differ greatly in various organisms investigated so far (Shenk, 1983). (ii) There is
no evidence whatsoever that the same signal sequences operating on
genomic double-stranded DNA are also functioning at the level of
single- or double-stranded RNA. Apart from those objections it should
be noted that according to our present model of PSTV replication such
RNA polymerase I-specific signals should actually be present at the
PSTV (-) RNA and not at the PSTV (+) RNA.

Of particular interest are the recent inhibition studies on viroid
replication with nuclei-rich preparations from CEV-infected Gynura
leaves (Semancik & Harper, 1984). No molecular hybridization
techniques were applied in this work and the authors were, therefore,
unable to discriminate between the newly synthesized CEV (+) and (-)
RNA. Thus, they interpreted their data in terms of a general lack of
specificity of the three nuclear RNA polymerases with respect to
viroid RNA synthesis and took the precaution to propose all possible
choices in advance namely that all three RNA polymerases are
involved in CEV replication. In contrast, our claim that only RNA
polymerase I and II are involved in the synthesis of PSTV (+) and
PSTV (-) RNA, respectively, is based on the use of highly purified
nuclei as transcription system and on the analysis of the nuclear
transcription products with the standardized techniques of stringent
molecular hybridization. We consider this experimental system as the
only appropriate one for studying the effects of different inhibitors on
the synthesis of the different forms of viroid RNA. Nevertheless, we
are fully aware of the fact that additional lines of experimental
evidence are required to clearly delineate the involvement of the three
nuclear RNA polymerases in viroid replication. In particular, the
effects of actinomycin D and α-amanitin on the endogenous RNA
synthesis in isolated potato cell nuclei have to be investigated in
detail, because this would allow direct analysis of the differential
inhibition of the activity of the three nuclear RNA polymerases in
situ.

Our experiments clearly show that the cell nucleus is the site of
PSTV replication. Therefore it is reasonable to assume that the other
viroids allocated into the PSTV group and into the closely related HSV
group are also replicated in the nucleus with the aid of the nuclear
RNA polymerases of their corresponding hosts. However, the mecha-
nism of replication (and pathogenicity) of the coconut cadang cadang
viroid (CCCV) and the avocado sunblotch viroid (ASBV) may be
different from that of the viroids of the PSTV and HSV group.
Although they share a more or less extensive part of the central
region conserved in all presently known viroids, CCCV and ASBV
exhibit little overall sequence homologies between themselves and as
compared to all the other viroids. Moreover, in contrast to the
retardation of plant growth and malformation of leaves incited by
these other viroids, CCCV and ASBV cause a different type of disease
which is characterized by yellowing-type symptoms. Finally, they are
evidently not associated with the cell nucleus as PSTV and the others,
but with the chloroplast (ASBV) and/or the cytoplasm (CCCV) and its
reticulum (ASBV, CCCV) (Randles et al., 1976; Mohamed & Thomas,
1980). From these differences in structure and biology one could
expect that the mechanisms of replication (and pathogenesis) of these
two viroids might differ from the one we propose here for PSTV and
its relatives.
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