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Subcellular localization of viroids in highly purified nuclei from tomato leaf tissue

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Approximately 95% of the viroid RNA which is present in potato spindle tuber viroid (PSTV)-infected tomato plant leaf tissue, is associated with the nucleolar fraction obtained from purified nuclei. Viroids were released from the nucleolar fraction by increasing the ionic strength of the medium to 0.66 suggesting that viroid RNA is present in these subnuclear components in a protein-nucleic acid complex. A purification procedure for nuclei from leaf tissue had to be newly developed; it involves two Percoll density centrifugations as final steps. The nuclei were sonicated and the sonicate fractionated into fractions either highly enriched in nucleoli or in broken chromatin and ribonucleoprotein particles. The viroid content in the different samples was determined by gel electrophoresis. Depending upon the progress of the disease, viroid copy numbers between 200 and 10 000 per cell were observed in homogenized tissue, purified nuclei and in the nucleolar fraction. In chloroplasts, practically no viroids were detected. The results are discussed in the light of current hypotheses about the replication, pathogenicity and origin of viroids.

Key words: purification of nuclei/nucleolar association/ viroids

Introduction

Viroids are the smallest known agents of infectious diseases. They have been identified as pathogens only in higher plants. Viroids differ from typical plant viruses, however, in that they are devoid of a protein coat and consist of only a small, single-stranded circular RNA molecule. These molecules appear to depend completely on host enzymes for their replication and circularization (Diener, 1979; Sänger, 1982). The sequences of five different viroid 'species' and several viroid 'strains' are known (for review, see Riesner *et al.*, 1983; Sänger, 1982). On the basis of these sequences and the results of biochemical and biophysical studies, it has been shown that viroids follow a single general principle of structure and dynamics (Riesner *et al.*, 1983). Under physiological conditions, intramolecular base pairing leads to a secondary structure in which short double helices and small internal loops form an unbranched, rod-like structure. Thermal denaturation studies have shown that their rod-like structure can switch over into a branched structure composed of stable hairpins not present in the rod-like structure. These and other details of the *in vitro* structure of viroids may be relevant *in vivo*, however, no data are available about the actual structural state of viroids within the cell and their possible complexes with cellular components.

We have approached the problem of the *in vivo* structure of viroids by asking first at which site and in which organelle of the cell viroids are located. Additionally we determined the actual macromolecular complexes into which viroids are integrated within the cell and estimated the average copy numbers of viroid molecules in these complexes. Earlier studies based on infectivity tests suggested that viroids were associated primarily with nuclei (Sänger, 1972; Takahashi and Diener, 1975; Takahashi *et al.*, 1982) and/or membranes (Semancik *et al.*, 1976). At that time, however, there were neither sufficiently stringent purification procedures for subcellular components from plant tissue nor was direct quantitative analysis of viroid concentration possible. Consequently, no quantitative conclusions about relative distribution and copy number of viroids could be drawn. These data are one of the prerequisites to understanding the interaction between viroid molecules and host components. Furthermore, although the term 'viroid' was introduced originally to express that the infectious entity is a coat protein-free nucleic acid, this did not imply that viroids might be present in the cell as completely 'naked' nucleic acids. According to present hypotheses about viroid replication, pathogenicity and origin, one might expect viroids to be associated with different subnuclear components such as chromatin, nucleoli or ribonucleoprotein particles.

Results

Purification of nuclei from tomato leaf tissue

Earlier isolation techniques such as those described by Chen *et al.* (1975) for soybean hypocotyl and by Mennes *et al.* (1977) for cultured tobacco pith explants were not successful in isolating nuclei when applied to tomato leaf tissue. The optimized procedure presented here deviates radically from these earlier techniques. After incubation of the tissue in buffer and homogenization, the fraction containing nuclei is separated from the cell debris by straining through nylon cloth and sieves. The chloroplasts which are also present in the filtrate are lysed by the neutral detergent Nonidet. Subsequent velocity sedimentations concentrate the nuclei by separating them from components of lower mol. wt. In a first centrifugation through a Percoll cushion of a density of 1.13 g/ml (95% Percoll), starch grains and other large particles such as aggregates of fractured cell walls and lysed nuclei were pelleted, whereas the intact nuclei remain floating as a greyish thin pellicle. In a second centrifugation through a Percoll cushion of a density of 1.05 g/ml (36% Percoll), the nuclei are pelleted in a highly purified form.

The following points are of importance. The choice of buffers is critical during the initial incubation of leaf tissue and all the subsequent centrifugation steps. In contrast to earlier reports (Kuehl, 1964), addition of n-octanol and incubation for >30 min decreased the yield drastically. The use of mannitol instead of sucrose resulted in higher yields. When 40% glycerol had to be present to keep the lysed chloroplasts floating as a dark green layer, 600 mM mannitol became insoluble and had to be replaced by sucrose. The minimum concentration of the detergent Nonidet was determined by observing the deterioration of the chloroplasts under the microscope. Pellets containing nuclei could be readily

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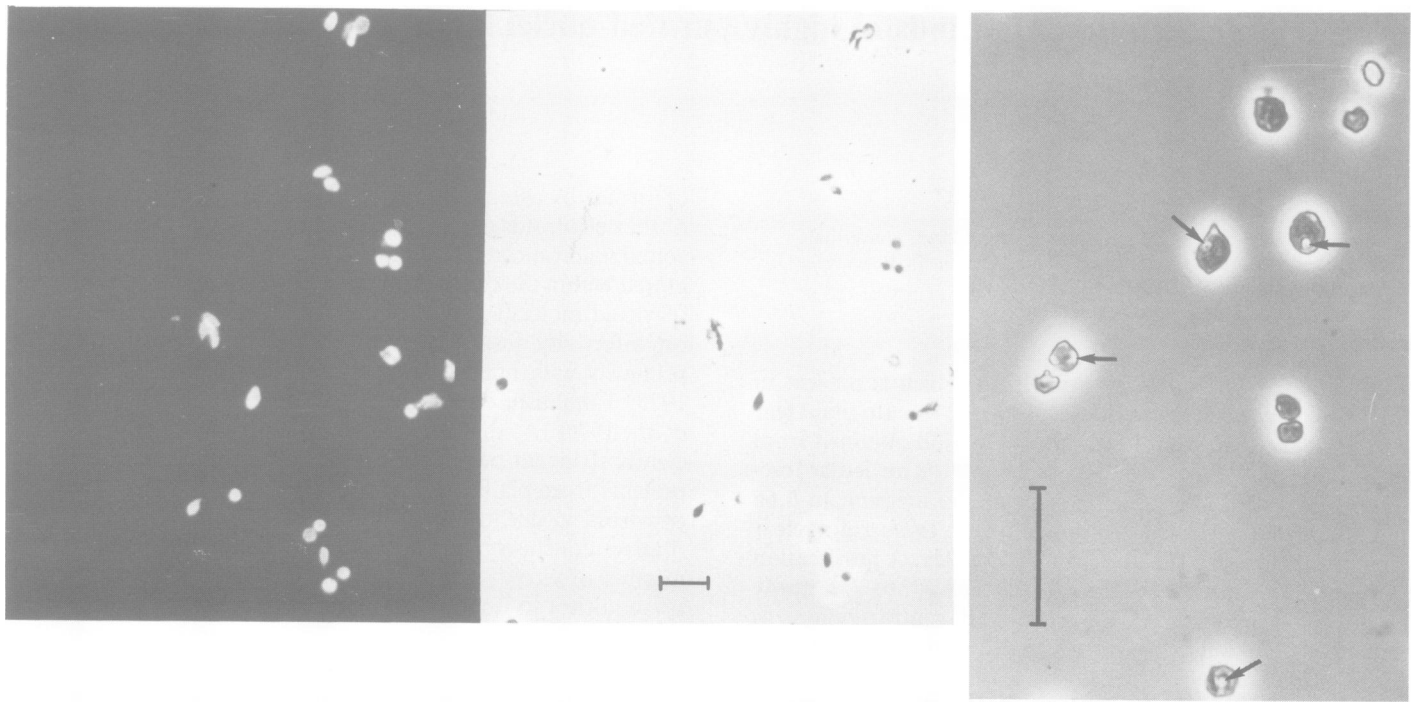

Fig. 1. Micrographs of ethidium-stained nuclei isolated from PSTV-infected tomato leaf tissue under fluorescence **(left)** and under phase contrast **(right)**. Identical areas are shown in both micrographs indicating the purity of the sample. The bar represents 20 μm.

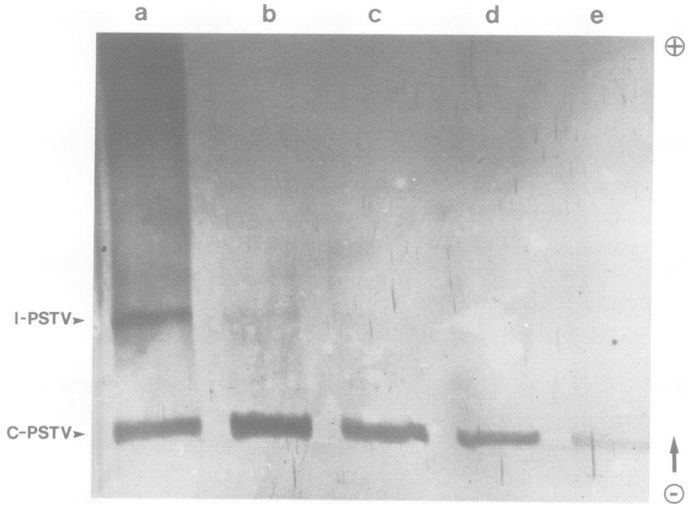

Fig. 2. Estimation of viroid concentration in isolated nuclei by bidirectional gel electrophoresis. The silver-stained bands of the circular and linear viroid RNA obtained from 2 x 10^8 nuclei are visible in **lane a**. The viroid concentration was estimated by comparison of the intensity of the bands with purified viroid preparations in **lane b** (60 ng PSTV), **lane c** (24 ng PSTV), **lane d** (9.6 ng PSTV) and **lane e** (3.8 ng PSTV). From this experiment 2000 viroid molecules were calculated per nucleus. The band of the circular and linear form of PSTV RNA are indicated by c-PSTV and l-PSTV, respectively.

suspended only in the presence of Percoll. Consequently, the Percoll had to be diluted out of the nuclei when subsequent steps required lower density and viscosity.

The purification steps using Percoll cushions are similar to those developed by Willmitzer and Wagner (1981) for the purification of nuclei from tobacco cell suspension cultures or by Guilfoyle (1980) from turnip leaves. The other steps described by these authors, however, particularly the enzymatic treatment with cellulase and pectinase or the centrifugation through 1.8 M sucrose, could not be applied successfully for the isolation of nuclei from tomato leaf tissue.

Purity

The purity of the nuclei was determined microscopically by observing the same specimen under phase contrast and fluorescence conditions. An example is shown in Figure 1. When ethidium bromide is added to the samples, particles visible under phase contrast show up also as highly fluorescent objects. This brilliant yellow-red fluorescence is typical for nuclei and demonstrates that the sample consists only of nuclei. Most of the nuclei are observed to be intact with diameters in the range of 3 – 7 μm. Damaged or aggregated nuclei are hardly detectable. In most nuclei a nucleolus is clearly visible. The purity of chloroplast preparations was analyzed in a similar way. The fluorescence of chloroplasts is dark red and much lower in intensity than that of nuclei.

Yield

The yield of purified nuclei was determined by counting under the microscope. Under optimum conditions, 5 x 10^8 nuclei were isolated from 100 g of healthy plant leaves; 2 – 3 x 10^8 nuclei were typically obtained from viroid-infected plants. This corresponds to a yield of 0.5 – 1% of the total nuclei present in the leaves. Using leaves from plants older than 6 weeks resulted in much lower yields.

Presence and copy number of viroid molecules in tomato leaf cells, nuclei and chloroplasts

Isolated nuclei were analyzed electrophoretically for the presence of viroids as described in Materials and methods. Figure 2 shows a bidirectional gel in which the band of the circular and the linear viroid RNA is clearly visible. The concentration of viroid in the nucleus was estimated by comparison

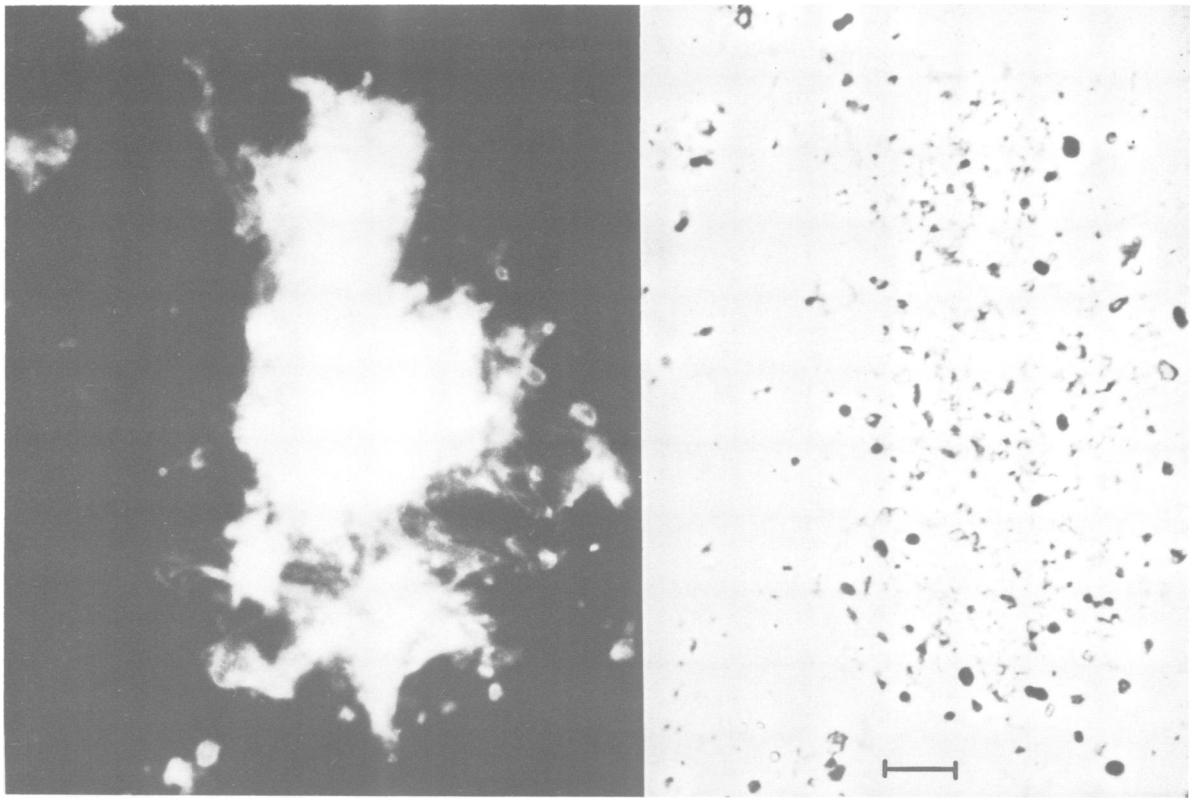

Fig. 3. Micrograph of a chromatin-rich fraction of lysed nuclei from PSTV-infected tomato leaf tissue obtained during the chloroplast purification after staining with ethidium bromide. The identical area is shown under fluorescence **(left)** and phase contrast **(right)**. The chromatin network is visible under fluorescence only. The bar represents 20 μm.

with a dilution series of purified viroid of known concentration. Depending upon the progress of the systemic infection and the corresponding increase in the intensity of the disease symptoms, between 200 and 10 000 viroid molecules per nucleus were observed. These values represent average numbers, and the actual copy number may vary, not only in individual cells, but also between different cell types.

During the isolation of chloroplasts, a sucrose gradient step was applied in which chloroplasts formed two bands whereas the nuclei were lysed and sedimented into the pellet in the form of an aggregated network of chromatin and other nuclear components. Figure 3 shows such an ethidium-stained network with the intensive yellow-red fluorescence characteristic for chromatin. One to two viroid molecules were observed per chloroplast organelle. This can be attributed to contamination. In marked contrast, the chromatin-rich networks contained up to 10 000 viroid molecules per nucleus.

If viroids were isolated on a preparative scale (Colpan *et al.*, 1983) from leaf tissue without prior isolation of nuclei, 1 mg purified viroid (corresponding to 5 x 10^{15} molecules) was obtained from 1 kg highly diseased tissue. Taking into consideration that ~5 x 10^{8} cells/g tissue were counted from leaf sections, it follows that, on average, ~10 000 viroid molecules are present per cell.

Localization of viroids in subnuclear fractions

Nuclei were sonicated and fractionated according to Pederson (1974). This procedure had been developed for rat liver nuclei and was reported to quantitatively pellet the nucleoli leaving the fractured chromatin and the ribonucleoprotein particles on top of the 30% sucrose cushion. According to this technique, four fractions were harvested after the sedimentation run: the pellet, the lower third of the sucrose cushion, the upper two thirds of the cushion and the top frac-

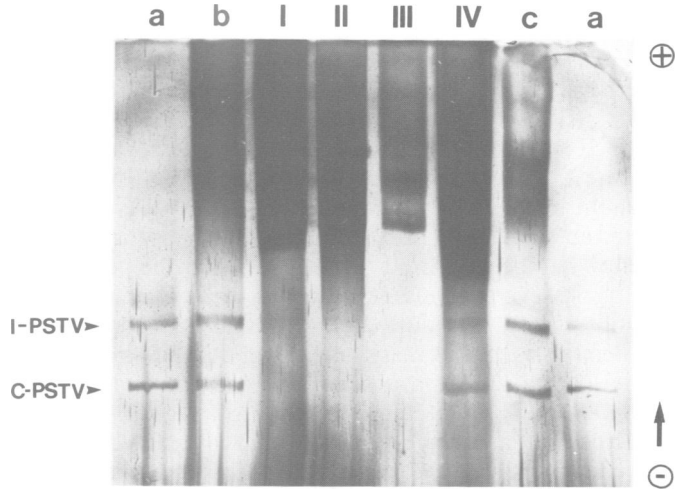

Fig. 4. Analysis of the distribution of viroid RNA in subnuclear fractions by bidirectional gel electrophoresis. The lanes in this silver-stained second-direction gel correspond to the following samples. **Lane a**: 16 ng purified linear and circular forms of viroid as in Figure 2; **lanes b** and **c**: nucleic acid extract from nuclei; **lane I**: subnuclear top fraction; **lane II**: upper two thirds of the sucrose cushion; **lane III**: lower third of the sucrose cushion; **lane IV**: nucleolar pellet fraction. 40% of the total nucleic acid extract from the nuclear and subnuclear fractions was loaded onto the gel. From the comparison of the viroid bands in **lane IV** and the very faint bands in **lane I** and **II** it becomes evident that >95% of the viroid RNA is associated with the nucleolar pellet fraction. The band of the circular and linear form of PSTV RNA are indicated by c-PSTV and l-PSTV, respectively.

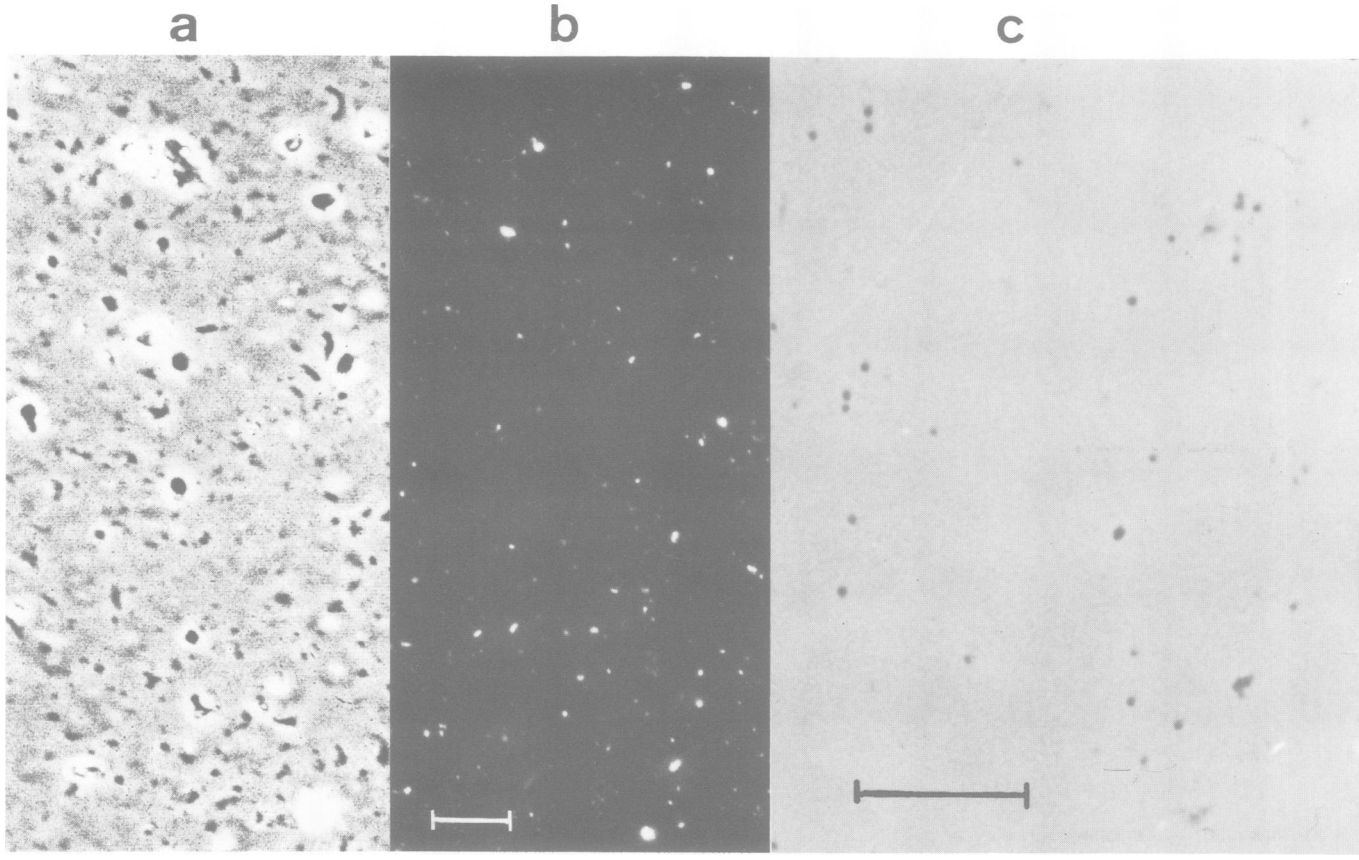

Fig. 5. Micrographs of ethidium-stained subnuclear fractions from PSTV-infected tomato leaf tissue. The sonicate of the nuclei **(a)** is shown under phase contrast in order to visualize all particles present. The top fraction is shown under fluorescence demonstrating the presence of fractured chromatin **(b)**. The nucleoli in the pellet fraction **(c)** are only visible under phase contrast. The bars represent 20 μm.

tion consisting of the layer on top of the cushion together with the interface. The viroid content of each fraction was determined in the corresponding nucleic acid extract as described in Materials and methods. These results are shown in Figure 4. It is evident that >95% of the viroid RNA is present in the nucleolar pellet, whereas viroid molecules are barely detectable in the top fraction and in the sucrose cushion. If the copy number of viroids is determined in the pellet fraction, again several thousand viroid molecules per nucleolus were observed as previously found for intact cells and nuclei. This demonstrates that no significant amount of viroid is lost during the fractionation and assay procedure and that the bulk of viroid RNA is associated with the nucleoli.

Microscopic analysis revealed that, as in the case of rat liver nuclei, the pellet obtained from sonicated plant nuclei contained the nucleoli whereas the fractured chromatin was found in the top fraction. The micrographs in Figure 5 show the sonicate before fractionation (a), the top fraction (b) and the pellet (c). Only the particles in the top fraction are highly fluorescent after ethidium bromide staining as expected for chromatin. The particles in the pellet, however, are visible only in phase contrast which clearly excludes the presence of chromatin in this fraction.

The content of DNA and high mol. wt. RNA (>30 000) was determined from Cs_2SO_4 density gradients in the analytical ultracentrifuge. In the pellet fraction the ratio of DNA/RNA was ~6, whereas in the top fraction no significant band of RNA was visible and the ratio DNA/RNA was clearly >25. The much higher portion of RNA in the pellet as compared with the top fraction is in agreement with the results from Pederson with rat liver nuclei. This strongly indicates, together with the microscopic uniformity and extremely fast sedimentation, that the particles in the pellet are in fact nucleoli.

The association of viroid RNA with the fraction of nucleoli prompted us to study the ionic strength dependence of this presumed complex. For this purpose the nucleolar pellet was suspended in 'high salt buffer' which contained 0.5 M NaCl and 50 mM $MgCl_2$ in 10 mM Tris HCl corresponding to an ionic strength of 0.66. After a high speed centrifugation of the suspension, viroid was detected only in the supernatant, showing that it had been released from its association with the nucleolar fraction. Because such high salt treatment has been shown to abolish most of the nuclear protein-nucleic acid interactions (Zieve and Penman, 1981) it is justifiable to conclude that viroid RNA is present in the nucleoli in a protein-nucleic acid complex.

Comparison of potato spindle tuber viroid (PSTV) RNA and nucleolar RNA U3

The finding that the bulk of viroid RNA is associated with the nucleoli suggested the comparison between PSTV RNA and other RNA species present in the nucleolus. The most prominent nucleolar RNA of comparable size is RNA U3 which is capped and consists of 214 nucleotides. Although RNA U3 is always found to be bound to the 28S RNA present in the nucleolus, it is transcribed most probably outside the nucleolus by DNA-dependent RNA polymerase II (Busch *et al.*, 1982). Therefore, it cannot be excluded that a similar difference between the site of synthesis and accumulation may also hold for viroids. An additional feature of interest became

Fig. 6. Sequence homology between PSTV RNA (Gross *et al.*, 1978) and nucleolar RNA U3 from Novikoff hepatoma (Reddy *et al.*, 1979).

apparent when the sequences of PSTV RNA (Gross *et al.*, 1978) and RNA U3 from Novikoff hepatoma (Reddy *et al.*, 1979) were compared. Figure 6 shows that these two RNAs share several areas of sequence homology. In addition, when the two sequences are aligned in a slightly different co-linear manner, a different set of homologies becomes apparent (data not shown). The implications of these sequence homologies with respect to viroid origin and function remain to be established.

Discussion

Quantitation of viroids in nuclei

This work clearly shows that the bulk of the viroid molecules in infected tomato plants is present in the nuclei. This conclusion could safely be drawn because the analysis was carried out with highly purified nuclei. Earlier experiments with less well purified nuclear preparations indicated a similar situation, but they did not allow a quantitative evaluation (Sänger, 1972; Takahashi and Diener, 1975; Takahashi *et al.*, 1982; Flores and Semancik, 1982). The highly sensitive gel electrophoretic detection method for viroids used in the present study allowed an estimation that the average number of viroid molecules per nucleus ranges between 200 and 10 000 copies depending upon the progress of the disease. Cells of increased nucleic acid synthesizing activity might produce even higher numbers. Although the majority of viroid molecules is present inside the nucleus, one cannot conclude that the cytoplasm or other cell organelles are completely devoid of viroids. However, the few viroid molecules which were found per chloroplast, are most probably due to contamination. *In vivo* their complete absence in this organelle can be assumed. Relatively high concentrations of viroids have been found in stems and roots of infected plants (Colpan *et al.*, 1983). It is still uncertain, however, whether viroids also accumulate in the nuclei of these cells.

Microscopic studies show that the average diameter of the tomato nuclei is $4-5\ \mu m$ from which an intranuclear concentration of viroids of $\sim 10^{-7}$ M can be calculated. The dissociation constants of various protein-nucleic acid complexes such as the ones between nucleic acids and polymerases (e.g., de Haseth *et al.*, 1978) or DNA and histones (e.g., Stein, 1979) would guarantee complex formation in this or even lower concentration ranges. Therefore, it is not unreasonable that viroids exist in protein-nucleic acid complexes.

Viroids are complexed in the nucleoli fraction

The fractionation of sonicated nuclei showed that viroids sediment nearly quantitatively together with the fraction of the nucleoli. The most reasonable conclusion from this result is that *in vivo* viroids are associated with the nucleolus. This association has not been observed before, and it excludes the earlier suggestions that viroids are associated with the chromatin. The association of viroids with the nucleolus can be abolished by raising the ionic strength of the buffer to 0.66. Because this is the typical ionic strength dependence of a protein-nucleic acid interaction, it strongly indicates that viroids are complexed in the nucleolus *via* protein-nucleic acid interactions. In contrast, nucleic acid-nucleic acid interactions are favoured by increasing ionic strength and can therefore most probably be excluded as being responsible for the complex.

Functional implications

The fact that viroids are associated with the nucleolus and that their copy number per nucleus is as high as 10 000 can be related to current hypotheses on replication, pathogenicity and origin of viroids.

There is general agreement that viroids are replicated *in vivo* via oligomeric (−)RNA intermediates (Grill and Semancik, 1978; Branch *et al.*, 1981; Rohde and Sänger, 1981) and that viroid-specific DNA sequences do not exist (Branch and Dickson, 1980; Zaitlin *et al.*, 1980). Therefore, it is not surprising that viroids were not found with the major DNA portion of the cell as represented by the chromatin fraction.

Inhibition studies with alpha-amanitin in viroid-infected tomato protoplasts (Mühlbach and Sänger, 1979) and *in vitro* transcription studies (Rackwitz *et al.*, 1981) indicated that DNA-dependent RNA polymerase II is in some way involved in viroid replication. RNA polymerase II is normally responsible for the synthesis of the small capped nuclear RNAs U1 to U6 (Busch *et al.*, 1982) and of nuclear precursors of the cytoplasmic polysomal mRNA. At first sight, the established involvement of polymerase II in viroid replication and the now discovered association of viroids with the nucleolus do not seem to fit together directly because RNA polymerase II is known to be localized in the nucleoplasm and to be partly associated with the chromatin, as is polymerase III which transcribes precursors to 5S rRNA and tRNA and also certain other small uncapped nuclear RNAs. One would expect RNA polymerase I to be involved in viroid replication because this polymerase (which normally transcribes precursors to high mol. wt. rRNAs and 5.5S rRNA) is also localized within the

nucleolus. However, one should bear in mind that the presence of most viroids in the nucleolus does not necessarily imply that viroids are also synthesized in the nucleolus. One might speculate that, as in the case of nucleolar RNA U3, viroids are synthesized outside the nucleolus and later either diffuse or are transported into this subnuclear component. The intimate viroid-protein contact in the nucleolus may protect viroids against RNase degradation.

The partial sequence homology between U1 RNA and viroid RNAs led to the hypothesis that viroids might have arisen from escaped introns and interfere with mRNA splicing thus causing disease (Dickson, 1981; Diener, 1981; Gross *et al.*, 1982). Splicing with the involvement of U1 RNA does not occur in nucleoli but occurs most probably in nuclear ribonucleoprotein particles (Lerner *et al.*, 1980). Since we could not detect a significant portion of viroids in the fraction of these particles the present results do not support the splicing interference model.

Materials and methods

Buffers

Buffers were freshly prepared from reagent grade water (Milli-Q-system, Millipore) and contained 25 mM MES/NaOH, pH 6.5, 10 mM KCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin. Individual buffers contained in addition: buffer A, 600 mM mannitol, 10 mM mercaptoethanol; buffer B, 600 mM sucrose, 40% glycerol, 1 mM dithioerythritol (DTE); buffer C, buffer B + 0.6% Nonidet P40 (NP-40); buffer D, 250 mM mannitol, 1 mM DTE, 95% Percoll, 0.6% NP-40; buffer E, 250 mM mannitol, 1 mM DTE. RSB buffer is composed of 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl, pH 7.0. The 'high salt buffer' is 0.5 M NaCl, 50 mM $MgCl_2$ in 10 mM Tris HCl, pH 7.0. Sucrose (RNase-free grade) was purchased from BRL (Bethesda, MD), Percoll from Pharmacia; all other chemicals were from commercial sources.

Isolation of nuclei

Tomato plants (cultivar Rutgers) were grown as described recently (Schumacher *et al.*, in preparation). The top and the six youngest leaves (150 g) of 6–8 week-old tomato plants were used immediately after harvesting. They were washed twice with distilled water, once with 70% ethanol (15 s) and twice again with distilled water. They were then immersed in 1.5 l buffer A, and infiltrated by two to three short evacuations until they were thoroughly wetted, before they were incubated for 30 min. The leaflets were removed from buffer A and homogenized in 450 ml buffer B for 4 x 5 s in a Waring Blendor on step 'High'. The resulting suspension was filtered through nylon cloth (250 μm) and three stainless steel sieves with pore sizes of 100, 40 and 25 μm. The residue on the cloth was resuspended in 350 ml buffer B, homogenized for 2 x 10 s, and filtered as described. The combined filtrates were adjusted to 0.6% NP-40 and stirred for 5 min. To sediment the nuclei, the suspension was centrifuged 10 min at 5000 r.p.m. in a JA14 rotor (Beckman) in round-bottom 250 ml polycarbonate tubes. The supernatants were decanted, each (nuclei-containing) pellet was resuspended in 5 ml buffer D, the combined suspensions were mixed with 380 ml buffer C, and centrifuged for 10 min at 5000 r.p.m. in a JA14 rotor. The dark green layer on top of the supernatant was sucked off. The supernatant was decanted, each pellet suspended in 3 ml buffer D, the combined suspensions mixed with 150 ml buffer C and centrifuged for 5 min at 5000 r.p.m. in the Minifuge II (Heraeus-Christ) in glass tubes. Again, the green layer was sucked off, the supernatant decanted, and the nuclei-containing pellets were suspended in 45 ml buffer D. After centrifugation in the Minifuge II at 5000 r.p.m. for 10 min, the cell nuclei floated as a greyish thin pellicle because of the high density of the Percoll-containing buffer D. They could be collected from the meniscus with a Pasteur pipet and were added to 28 ml of buffer E. The pellet was resuspended in the remaining buffer content of the tube and recentrifuged as long as floating nuclei could be detected. They were collected and combined with the suspension of the previous steps. Buffer D was added to the nuclei suspension to a final volume of 45 ml yielding 36% Percoll, and the suspension was centrifuged for 5 min at 2500 r.p.m. in the Minifuge to pellet the nuclei. The supernatant was decanted from the nuclear pellet and recentrifuged four to five times until no nuclei were observed in the pellet. The pellets were resuspended, pooled, and washed by three times centrifugation in buffer E. All procedures described so far were carried out in the cold room at 4°C. The nuclei were stored either in buffer E containing 50% glycerol at −20°C or as pellets at −70°C.

Isolation of subnuclear fractions and chloroplasts

The fractionation was carried out according to Pederson (1974). 10^8 nuclei were suspended in 1 ml buffer D, added to 9 ml RSB buffer and disrupted by brief sonication (3 x 10 s, 45 W, Braun Labsonic 1510 with standard probe). About 98% of the nuclei were broken, as monitored by fluorescence microscopy. The sonicate was layered on 25 ml of 30% sucrose (w/v) in RSB buffer, centrifuged at 5000 r.p.m. for 15 min in a SW28 rotor (Beckman), and fractionated as described in Results.

Chloroplasts were isolated according to Herrmann (1982).

Gel electrophoretic detection of viroids

Sample preparation for viroid detection in leaf tissue was as described (Schumacher *et al.*, in preparation).

For the anlysis of viroid concentration in nuclei and chloroplasts, 10^8 nuclei (or 10^{10} choroplasts) were lysed in 2 ml buffer containing 500 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris HCl, pH 7.5, incubated with 1000 units DNase (Worthington) for 5 min at 37°C, and the DNase treatment was stopped by adding 2 ml buffer containing 100 mM NaCl, 10 mM Tris HCl, pH 7.5, 20 mM EDTA, 2% SDS, and 3 mg proteinase K and incubation for 2 h. After phenol/chloroform extraction, nucleic acid was precipitated with three volumes of ethanol for 1 h at −70°C. The precipitate was dissolved in 40 μl gel buffer (see below) and loaded in a single slot of the gel.

For viroid detection in subnuclear fractions, the corresponding fractions of the sonication lysate from 10^8 nuclei (for treatment of the pellet see below) were adjusted to 15 mM $MgCl_2$, treated with 1000 units DNase, and the DNase treatment stopped with 2% SDS and 3 mg proteinase K. Nucleic acids were precipitated by adding three volumes of ethanol for 1 h at −70°C. The precipitate was dissolved, phenolized and the nucleic acid was precipitated again. The precipitate was dissolved in 40 μl gel buffer and loaded in a single slot of the gel. The pellet from the sonicate was dissolved in 2 ml RSB buffer containing 15 mM $MgCl_2$ and treated as described for the other fractions.

Gel electrophoresis (5% polyacrylamide, 0.12% bisacrylamide slab gels (14 x 10 x 0.15 cm) in 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) was carried out in the so-called 'bidirectional' procedure the details of which have been described (Schumacher *et al.*, in preparation). In a first run samples were run from top to bottom under native conditions (absence of urea, 25°C); a 1 cm wide strip orthogonal to the direction of the first run which contains the viroid position in all slots is cut from the gel and set across the bottom of a second gel. The second run was performed from bottom to top under denaturing conditions (8 M urea, 50°C). Because of their circularity, viroids run slower than all other linear nucleic acids of the same mol. wt. As little as 0.5 ng viroid RNA may be detected by this analysis after applying the silver staining procedure of Sammons *et al.* (1981) which had been originally developed for proteins.

Light microscopy

Microscopic studies were carried out in a Zeiss Photomicroscope III with fluorescence attachment which allowed observation of one and the same specimen under phase contrast or fluorescence. For fluorescence, the specimen was stained by adding to one drop of sample suspension one drop of a solution containing 10 μg/ml ethidium bromide in distilled water. For fluorescence excitation a Zeiss F I 546 transmission filter in series with a Zeiss F I 580 reflection filter was employed. Fluorescence emission was observed through a Zeiss 58 cut off filter. Cell nuclei were counted in a Thoma counting chamber of 10^{-4} ml volume.

Analytical ultracentrifugation

The content of DNA and RNA was determined by carrying out Cs_2SO_4 density greadient runs in a Spinco model E analytical ultracentrifuge equipped with a u.v. absorption scanner and an electronic multiplexer. Aliquots of 0.1 A_{260} nucleic acid extract were added to a solution of Cs_2SO_4 1.40 g/ml final density for DNA determination, and to Cs_2SO_4 of 1.61 g/ml final density for RNA determination.

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