Plant Cell Suspension Cultures Sustain Long-Term Replication of Potato Spindle Tuber Viroid

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Cell suspension cultures were established from tomato plants (Lycopersicum esculentum cv. "Rutgers") infected with either a severe (TPS cell line) or a mild (TPM cell line) strain of potato spindle tuber viroid (PSTV) and from uninfected plants (TH cell line). Based on measurements of packed cell volume, the TPS line exhibited a slower growth rate than the TH line, and reached lower levels of total cell volume during the stationary phase. The TPM line was intermediate between the other two. All the lines were highly polyploid (over 72 chromosomes). PSTV was detected consistently by electrophoretic analysis in both the TPS and TPM lines after more than 1 1/2 years of subculturing. Newly synthesized PSTV in actively dividing cells was detectable after 1 hr of [3H]uridine labeling (ca. 0.1% of the radioactivity incorporated into total soluble RNA) and its proportion increased in subsequent samplings up to a steady state level of 0.24–0.28 or 0.31–0.39% for TPM and TPS, respectively. Analysis on two-dimensional gels of proteins synthesized in the TH and TPS cell lines following incorporation of radiolabeled amino acids detected neither quantitative nor qualitative changes resulting from maintenance of viroid in the cell line.

INTRODUCTION

Potato spindle tuber viroid (PSTV) is one of a group of the smallest known self-replicating pathogenic agents; it is a covalently closed single-stranded RNA ring of 359 nucleotides (Gross et al., 1978) which can cause disease of economic significance in the potato and can be transmitted to a number of other plant species (Diener, 1979). The mechanism of replication of viroids is poorly understood. Undoubtedly, cultured cell or tissue systems should be useful in replication investigations. To date, PSTV replication has been demonstrated in isolated nuclei (Takahashi and Diener, 1975) and in leaf protoplasts (Mühlbach et al., 1977; Mühlbach and Sänger, 1979), as judged from incorporation of radiolabeled precursor into viroid RNA. In the latter case, protoplasts prepared from healthy plants could be infected with viroid; replication in both protoplasts and isolated nuclei was studied only on a short-time basis in systems which were not perpetuated. In this report we demonstrate that viroids can replicate in actively dividing cells of suspension cultures derived from infected plants. The cultures can be subcultured and maintained over long periods of time without apparent loss of the cells' capacity to replicate viroid. In addition, we report our findings of the lack of viroid-specific protein synthesis in these cells, giving further support to the observations of others that suggest that viroids don't seem to code for proteins, either in plant tissues or in in vitro translational systems.

MATERIALS AND METHODS

Plant material and PSTV strains. Lycopersicum esculentum cv. "Rutgers" tomato seedlings were mechanically inoculated 1 week after their emergence. Homogenized tissue infected with either of two different strains of PSTV were used as inoculum: the PM strain, producing relatively mild sympt-
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Symptoms under greenhouse conditions, and the PS strain, producing very severe symptoms and overall stunting of the plants (Niblett et al., 1980).

The primary sequences of the RNAs of these strains differ in only a few bases, based on fingerprints of their ribonuclease A or T₁-fragments (Dickson et al., 1979). Their distinctive symptoms were clearly recognizable 15–20 days or 10–14 days after inoculation, for PM and PS, respectively.

Establishment and maintenance of cell suspensions. Young stem segments, 1 to 2 cm long, from noninoculated or infected plants (4 weeks after inoculation) were surface sterilized for 30 sec in 70% ethanol followed by 5 min in 20% Chlorox (a commercial sodium hypochlorite solution). After extensive rinsing in sterile water, both ends of each segment were cut off and discarded. The stem was divided into 1- to 2-mm slices; 5–10 slices were cultured in 10-cm petri dishes containing 15 ml of agar-solidified SH medium (Schenk and Hildebrandt, 1972). The newly developed callus tissue was subcultured several times and the resulting friable calli were subsequently transferred to liquid medium and incubated on an orbital shaker at 100 rpm. Each culture was derived from several callus pieces, all derived from a single stem. The resulting cell suspensions were routinely maintained as 25- or 45-ml batch-cultures contained in 125- or 250-ml capacity Delong flasks, respectively. Transfers were made regularly at approximately 2-week intervals by diluting stationary phase suspensions 10- to 20-fold into new medium. Calli and cell suspensions were cultured in SH medium unless otherwise stated, at 28.5°C and under continuous light (mixed fluorescent and incandescent) at an intensity of ca. 1900 lux.

Growth-rate determination. The growth rate of cell suspensions was estimated by monitoring its "cell packed volume" (CPV) at different times after transfer. For this purpose, 10-ml aliquots of cell suspension were removed from the culture with a wide-mouthed pipet and centrifuged at 250 g for 5 min (Clinical Centrifuge, swinging-bucket rotor) in sterile graduated tubes. After recording the CPV, the aliquot was returned to the original flask.

Chromosome staining procedure. One-half to one-milliliter aliquots of actively dividing cells were incubated for 3–4 hr with 1 mg/ml colchicine under the usual culture conditions and were then fixed overnight in cold ethanol–acetic acid (3:1). After further treatment with 1 N HCl for 4 hr, cells were stained in carbol–fuchsin stain, squashed, and examined under a light microscope at 1000× magnification.

Labeling and extraction of cell RNA. One to two milliliters of cell suspension were incubated with [¹⁰H]uridine (~50 Ci/mmol, ICN, Irvine, Calif.) at different concentrations and for different lengths of time. The labeling was stopped by removing the medium by aspiration and freezing the drained cells at ~85°C. Frozen cells were homogenized in a Teflon-glass motorized homogenizer in the presence of 2 ml extraction buffer (100 mM NaCl, 100 mM Tris, 10 mM Na₂-EDTA, 1% sodium dodecyl sulfate, pH 8.9) and 2 ml of phenol saturated with 1 mM EDTA. The nucleic acids were precipitated from the aqueous phase with 2 vol of cold ethanol and digested at room temperature with 10 μg/ml DNase (Worthington, Code DPFF) for 45 min in 10 mM Tris, 10 mM KCl, 2 mM MgCl₂, pH 7.4. Following a second ethanol precipitation, the RNA pellet was dispersed in 3–4 ml of 2 M LiCl by vigorous vortexing for 2 min and immediately clarifying at 12,000 g for 10 min. The supernatant phase containing the "soluble RNA" was subsequently precipitated with ethanol and suitable aliquots were electrophoresed in 5% polyacrylamide slab gels in the presence of 40 mM Tris, 20 mM Na-acetate, 1 mM EDTA, pH 7.2, at 50 V. Gels were stained for 5 min with 20 μg/ml ethidium bromide and destained in water, in order to locate the marker species, and processed for fluorography using either the original procedure (Bonner and Laskey, 1974) or a commercially available preparation ('Enhance', New England Nuclear Corporation). To determine the proportion of the label which was incorporated into viroid, the fluorograms were scanned with a densitometer (Ortec, Model 4310). The scans were reproduced by xerography and the peaks cut and weighed. Incorporation into the viroid
band is expressed as a percentage of the total incorporation in the scan.

Labeling and extraction of cell protein. Early log phase cells (4 days after transfer to fresh culture medium) were used for labeling. One milliliter of cell suspension was transferred to a 3-cm plastic petri dish to which 50 μCi of [3H]algal hydrolysate (Schwarz/Mann) was added. After incubation at 28.5°C under continuous light for 24 hr cells were collected, rinsed with fresh medium, drained and frozen at -85°C.

Uptake of radioactive amino acids was about 40% of the total input radioactivity for both TH and TPS cell lines. Roughly half of the label taken up by the cells was incorporated into TCA-precipitable material. Disrupted cells have a high protease activity and the cells are difficult to disrupt. Because cellular disruption often led to protein degradation (loss of high MW proteins) when homogenization procedures were used, labeled cells were disrupted by releasing them quickly from high pressure. Frozen cells were suspended in an equal volume of 0.0625 M Tris–HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol. The cell suspension was equilibrated at 750 psi, 4°C for 10 min in a nitrogen pressure bomb. Disrupted cell suspensions were centrifuged at 5000 rpm, 4°C, 5 min (Sorvall SS-34) and the supernatants were collected and frozen at -85°C.

Two-dimensional electrophoresis. Cellular proteins were prepared for separation by isoelectric focusing by the addition of 10.5 μl NP-40, 3.9 μl Pharmalyte, pH 3–10 (Pharmacia), 69 mg urea to 150 μl protein solution. Isoelectric focusing was performed according to O’Farrell (1975). Separation in the second dimension was on a discontinuous SDS slab gel (Laemmli, 1970) with a total acrylamide concentration of 18%; the bis acrylamide to total acrylamide ratio = 0.0067 in the separating gel. Newly synthesized proteins were detected by fluorography according to Bonner and Laskey (1974).

Detection of viroid in cell lines. In order to detect the presence of PSTV in different cell lines, a simplified extraction procedure was found suitable (Pflümer et al., 1980). Briefly, 0.5 g of drained cells was homogenized with 0.5 ml distilled water, 0.2 ml 4 M NH₄OH, 0.2 ml 0.1 M ethylene glycol tetraacetic acid adjusted to pH 7.0 with Tris, 0.6 ml 10 M LiCl, and 2 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline. Following the precipitation of nucleic acids from the aqueous phase, samples were electrophoresed and stained as described above.

RESULTS

Characteristics of the Cell Lines

Different standard media were evaluated during the first stages of the work. B₅ (Gamborg and Wetter, 1975) and MS (Murashige and Skoog, 1962) media containing different hormone concentrations, and SH medium (Schenk and Hilbrandt, 1972). The latter medium was adopted both for callus induction and maintenance of cell suspensions, as it promoted faster growth and better tissue friability. Friable cell suspensions were obtained after transfer to liquid medium of second- or third-passage callus tissue.

Two to three independent lines were established for each of the treatments (TH, TPM, and TPS, corresponding to healthy, PM-infected and PS-infected tomato plants, respectively). It was evident from the first passages that some differences could be seen between cell lines corresponding to different treatments but that the several isolates of each line seemed similar; for the sake of convenience one isolate of each line was selected for future studies, and the TH and TPS lines were studied more closely.

Figure 1 represents the growth rate of both these lines, based on the changes in CPV at different times after transfer to fresh medium. Following a lag period of 3–4 days for both, TH exhibited a faster rate of increase in CPV, reaching levels of over 75% in stationary phase. On the other hand, the TPS cell line showed a relatively longer doubling time during the linear growth phase and usually did not increase in CPV beyond 50–55% even upon prolonged culture. Both lines exhibited distinct microscopic features. TPS contained compact clumps of relatively small cells with very conspicuous starch granules either dispersed in the cytoplasm or concentrated in
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The growth rate of healthy and viroid-infected cell suspensions. The "cell packed volume" of TH and TPS cell lines was determined, as described under Materials and Methods, at different times after transfer to new medium. The experiment shown was conducted 6 months after the establishment of cell suspensions.

In order to monitor the appearance of newly synthesized PSTV in the cell lines, aliquots of the suspensions were labeled with [3H]uridine to measure the incorporation of that label into PSTV. In a typical

**Fig. 1.** Growth rate of healthy and viroid-infected cell suspensions. The "cell packed volume" of TH and TPS cell lines was determined, as described under Materials and Methods, at different times after transfer to new medium. The experiment shown was conducted 6 months after the establishment of cell suspensions.

the perinuclear region. On the other hand, TH cells were bigger, lacked conspicuous starch granules and the clumps broke apart into substantially single cells during late stationary phase. Although the TPS suspension had a distinct yellowish to orange color during the linear phase of growth, microscopic observations have shown that the viability of both cell lines was higher than 95%. At late stationary phase, the TPS cultures became dark brown.

The TPM line was not included in the above described systematic studies, but preliminary observations suggested that it exhibits intermediate characteristics between TPS and TH in terms of growth rate and microscopic features.

All three lines are highly polyploid. Although the precise chromosome number and its deviations were not determined for each line, in no instances were mitotic figures observed containing less than 72 chromosomes (2N for tomato = 24; Rick and Butler, 1956).

**Viroid Production by Cell Suspensions**

The electrophoretic profile of partially purified LiCl soluble nucleic acid fractions from the different cell lines was determined after separation in 5% polyacrylamide gels. The presence of an additional band corresponding to the known mobility of PSTV was consistently detected in both infected lines (TPM and TPS) over 1 1/2 years after their establishment but not in the healthy line (Fig. 2). Extracts from both infected lines were highly infectious. Mechanical inoculation to Rutgers tomato showed that the typical symptomatology of each PSTV strain was faithfully conserved after long periods of continuous culture (results not shown).

**Fig. 2.** Electrophoretic detection of PSTV in cell suspensions. The soluble nucleic acid fraction from different cell lines was extracted by a simplified procedure (Pfannestiel et al., 1980) and separated in 5% polyacrylamide gels. The arrow indicates the position of marker PSTV.
FIG. 3. Electrophoretic profile of newly synthesized soluble RNA by cell suspensions. One-milliliter aliquots of the different cell suspensions were labeled for 24 hr with 10 μCi/ml of [3H]uridine. The extracted soluble RNA was run on a 5% gel and the gel was processed for fluorography with Enhance as described under Materials and Methods. Each lane was loaded with 250,000 cpm but the 4 S RNA species was partially run out of the gel. The arrow indicates the position of the migration of a marker PSTV preparation.

experiment, TPS cultures (ca. 30% CPV) incubated for 24 hr in the presence of either 1 or 10 μCi/ml of the radioactive precursor incorporated 33 or 26%, respectively, of total label input into trichloracetic acid precipitable radioactivity. Following extraction and analysis of the labeled soluble RNA in 5% polyacrylamide gels, a newly synthesized species with identical electrophoretic mobility to PSTV was consistently detected.
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Fig. 4. Relative amounts of newly synthesized PSTV following different lengths of labeling with [3H]uridine. Two-milliliter aliquots of TPS or TPM cell suspensions in the linear growth phase were labeled for 1, 3, 6, or 48 hr in the presence of 100, 50, 20, or 10 μCi/ml of [3H]-uridine, respectively. Samples were processed as described in Fig. 3. The radioactivity of different fractions was estimated by analysis of the peak areas of a densitometer scan of the fluorograph.

in TPS and TPM but not in the TH samples (Fig. 3). The relative rate of the accumulation of this newly synthesized PSTV in both lines was monitored following the labeling of actively dividing cell suspensions for different lengths of time. In this case, the amount of label present in the PSTV band was compared with the total radioactivity of the soluble RNA profile (Fig. 4). PSTV synthesis could be detected as early as 1 hr after adding the label, although at a relatively low level for both the TPM and TPS lines. The relative amount of newly synthesized PSTV increases substantially with longer labeling periods and seemed, after 3–6 hr to reach a steady-state value of 0.24–0.28 or 0.31–0.39% of the total radioactivity incorporated into soluble RNA for TPM and TPS, respectively.

Protein Synthesis in Cell Suspensions

Because the cells were so efficient in incorporating precursors into macromolecules, we used them to determine if viroid-specific proteins were being synthesized in the TPS cell line. Further, the use of two-dimensional gels provides an increase in resolving power over the one-dimensional procedures used in previous studies with viroid-infected leaf tissue (Zaitlin and Hariharasubramanian, 1972; Conejero et al., 1977). As seen in Fig. 5, the pattern of newly synthesized proteins in TH and TPS cells is virtually identical as resolved by two-dimensional electrophoresis. The minor quantitative differences seen were not reproducible in subsequent experiments. Further analysis on one- and two-dimensional gels, where proteins as small as 6000 daltons should be resolved, showed no differences between the two cell lines.

DISCUSSION

The actively dividing tomato cell suspensions have sustained an efficient replication of PSTV for over 1 1/2 years. Similarly, citrus exocortis viroid has been shown to reach a high titer in the mitotically active outgrowths of crown-gall diseased tomato plants (Semancik et al., 1978), and PSTV synthesis was observed to be maintained during long-term culture of tomato callus tissue (E. Dickson and L. Pape, personal communication). Thus, in culture, it is tempting to speculate that viroids appear to behave differently from some conventional plant viruses, for which there is evidence of a continuous loss of virus production during long-term culture of cell suspensions or callus tissue (reviewed by Ingram, 1976). There is, however, variation in the behavior of different plant viruses in this regard; with cultured tobacco tissues some viruses are maintained during subculture while others are lost (Reinert, 1966). Moreover, for a given virus, the subculturing of different appearing areas of infected callus influenced whether virus was maintained subsequently (Omura, 1978). Thus, with our limited experience we cannot say that, generically, viroids would be retained in all tissue culture situations. In fact, in recent months there is a suggestion of reduced synthesis of viroid in the TPM line.

In the studies described here, it was not our original intention to establish a number of cell lines to determine if any characteristic traits were associated with cell lines infected with PSTV. Rather, we wished to establish a system which would be useful for studies of viroid replication. Thus, for example, we have not investigated whether the distinctive features seen in the TPS cell line (slow growth, dark color) would also be found in
FIG. 5. Fluorograms of two-dimensional gels of proteins from TH and TPS cell lines. Proteins were prepared for electrophoresis as described under Materials and Methods. Electrophoresis was from left to right in the first dimension and from top to bottom in the second dimension. The pH gradient was determined according to O'Farrell (1975). Molecular weight standards were bovine serum albumin (68,000), carbonic anhydrase (30,000), and TMV coat protein (17,500). 3.5 × 10^6 (TH) or 6.75 × 10^6 (TPS) acid-precipitable dpm were applied to the first-dimension gels. Gels were exposed to X-ray film for 14 (TH) or 11 (TPS) days.
other separately derived lines from PSTV-infected tissues.

The markedly more rapid growth rate of the healthy cell lines would suggest that, in the diseased line, unless all of the cells were viroid infected, upon repeated subculturing the uninfected cells would dominate the culture. This has not happened. There are four possible explanations for the observation: (1) all of the cells in the TPS line are infected; (2) noninfected cells in the line become infected in culture; (3) the diseased cells inhibit the growth of healthy ones in the culture; and (4) the slow growth rate and dark appearance of the TPS line is characteristic of those cells, regardless of whether they are viroid infected or not. Experiments are underway to test these postulates. A preliminary finding, using cultures prepared by subculturing mixtures of TH and TPS indicated that after three subculturings, they still looked like mixtures (judging mainly by the intermediate color of the cultures). These observations would tend to eliminate postulate 3. We are currently extending these observations by establishing new lines and attempting to clone single cells from these lines to see what percentage of cells in the original culture were PSTV infected, to see if the growth rate and appearance of viroid-infected cells is consistently different from healthy cells, and whether we can isolate a line which is outstanding in the synthesis of PSTV.

The reported extent of viroid synthesis, as compared to synthesis of either total RNA, t-RNA, or soluble RNA, varies considerably based on the viroid studied and the tissues and techniques used by different authors (Takahashi and Diener, 1975; Mühlbach et al., 1977; Mühlbach and Sänger, 1979; Grill and Semancik, 1980). Therefore, a comparison with the values reported in the present work would not be valid. However, it should be stressed that we used actively dividing cells in our studies, where a very significant synthesis of PSTV could eventually be masked by a massive synthesis of cellular RNA, such as is assumed to occur in these cells. In fact, preliminary observations of one of us (J.V.A.) have suggested that there is probably a reverse correlation between the amount of PSTV synthesis relative to total soluble RNA synthesis and the mitotic activity of cells during different phases of the growth cycle, i.e., viroid synthesis was relatively higher during the lag phase and the stationary phase of culture, indicating that viroid replication is not closely linked with the synthesis of other species of RNA in the cell.

Although the sequence of PSTV which contains no AUG initiation codon (Gross et al., 1978) makes it unlikely that the viroid could function as an mRNA in host cells, the presence of a complementary RNA sequence in citrus exocortis viroid-infected tissues (Grill and Semancik, 1978) has led Matthews (1978) to propose that in PSTV infections an analogous complement could be translated in host cells. Studies of protein synthesis in CEV-infected plants have detected two low-molecular-weight proteins, but these apparently are of host origin, and their syntheses are stimulated both by viroid infection and tissue senescence (Conejero and Semancik, 1977; Conejero et al., 1979). In our studies, in the TPS cell line viroid is maintained without any detectable stimulation or suppression of specific host cell proteins. We were also unable to detect any protein in the TPS cell line resembling the two viroid coded proteins hypothesized by Matthews (1978) as possibly being coded for by complementary viroid RNA. Although many cellular proteins are resolved on the two-dimensional gel system employed here, this system does not resolve very basic proteins. We did look for synthesis of viroid-specific basic proteins on one-dimensional gels, however, but none were detected. We conclude that the maintenance of PSTV in cell suspension culture is not accompanied by major changes in the pattern of host protein synthesis, lending further support to other studies (Zaitlin and Hariharasubramanian, 1972; Davies et al., 1974; Semancik et al., 1977) suggesting that viroids do not code for specific polypeptides.

Viroid-producing cell suspensions are obviously an excellent choice for biochemical studies concerning the replication mechanism of these pathogenic agents; the cell population is relatively homogeneous and aseptic, and large quantities of cells can be easily produced under strictly controlled environmental conditions.
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