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Imaging of viroids in nuclei from tomato leaf tissue by *in situ* hybridization and confocal laser scanning microscopy

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The intracellular localization of viroids has been investigated by viroid-specific *in situ* hybridization and analysis by digital microscopy of the distribution of the fluorescent hybridization signals. Isolated nuclei from green leaf tissue of tomato plants infected with potato spindle tuber viroid (PSTVd) were bound to microscope slides, fixed with formaldehyde and hybridized with biotinylated transcripts of cloned PSTVd cDNA. The bound probe was detected with lissamine-rhodamine conjugated streptavidin. Nucleoli were identified by immunofluorescence using the monoclonal antibody Bv96 and a secondary FITC-conjugated antibody. In plants infected with either a lethal or an intermediate PSTVd strain, the highest intensity of fluorescence that arose from hybridization with the probe specific for the viroid (+)strand was found in the nucleoli, confirming results of previous fractionation studies. A similar distribution was found for (−)strand replication intermediates of PSTVd using specific (+)strand transcripts as hybridization probes. In order to determine if viroids are located at the surface or in the interior of the nucleoli, the distribution of the fluorescence hybridization signals was studied with a confocal laser scanning microscope (CLSM). It was shown by three-dimensional reconstruction that viroids are neither restricted to the surface of the nucleoli nor to a peripheral zone, but are instead homogeneously distributed throughout the nucleolus. The functional implications of the intranucleolar location of viroids and their replication intermediates are discussed with respect to proposed mechanisms of viroid replication and pathogenesis.

Key words: nucleoli/fluorescence microscopy/replication intermediates/digital imaging/three-dimensional reconstruction

Introduction

Viroids are non-encapsidated, single-stranded, circular RNA molecules which cause systemic infections in higher plants. They are the smallest known infectious agents; their best studied example, the potato spindle tuber viroid (PSTVd), is composed of only 359 nucleotides. The sequences of more than a dozen viroid 'species' and series of isolates thereof are known (for reviews see Riesner and Gross, 1985; Diener, 1987). On the basis of sequence similarities and of biochemical and biophysical studies, it has been shown that viroids exhibit a general principle with respect to the structure and dynamics of the RNA (for review see Riesner, 1987a). 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. Upon thermal denaturation, the latter undergoes a transition to a branched structure composed of stable hairpins not present in the original conformation.

Although viroids do not code for a translation product and are infectious as naked RNA molecules, inside the host cell they probably exist in specific complexes with cellular components. The latter may be required for replication, the expression of disease symptoms, the spreading of the systemic infection, and for protection against degradation.

We have previously studied the *in vivo* structure of viroids in two ways. In one series of studies, complexes of PSTVd with plant nuclear proteins were investigated by *in vitro* reconstitution as well as by *in situ* cross-linking with UV radiation (Riesner *et al.*, 1987; Klaff *et al.*, 1989). A 43 kd nuclear protein was identified as a major component of cellular viroid-protein complexes. Other studies were concerned with the subcellular location of viroids. Infectivity tests had led to the suggestion that viroids are associated with nuclei (Sänger, 1972; Takahashi and Diener, 1975; Takahashi *et al.*, 1982) and/or membranes (Semancik *et al.*, 1976). Applying improved fractionation techniques and a quantitative analysis of viroid concentration, we showed subsequently (Schumacher *et al.*, 1983b) that in PSTVd-infected tomato plants nearly the same number of viroids, i.e. up to 10^4 molecules, are found upon examination of whole cells or isolated nuclei. In the latter case, 90% of all viroid molecules are present in the nucleolar fraction. It was also demonstrated that viroids are absent from chloroplasts.

In the present work, the localization of viroids was investigated further using more accurate methods of analysis, that is by applying *in situ* hybridization and immunological labelling techniques and by imaging the viroid distribution with fluorescence microscopy. Whereas the earlier fractionation studies yielded only average copy numbers of viroids per nucleus or nuclear component (Schumacher *et al.*, 1983b), we have now determined the viroid distribution among and within individual nuclei by the *in situ* hybridization technique. Prior biochemical analysis and microscopy of nucleolar fractions had shown that isolated nucleoli were associated with a considerable amount of perinucleolar chromatin (Gruner, 1987). Thus, it was not clear whether viroids are directly associated with the nucleolus or with the perinucleolar chromatin. This uncertainty has also been resolved by exploiting the optical sectioning capability of the confocal laser scanning microscope to examine stained nucleoli in infected cells. In addition, the localization of replication intermediates has been determined by use of viroid-specific (−)strand sequences for hybridization.

Results

The subnuclear distribution of viroid (+)strand and (−)strand sequences was analysed by *in situ* hybridization using biotinylated transcripts as probes. Hybridization conditions had to be selected carefully for several reasons. Firstly, viroids form a highly self-complementary secondary structure (reviewed by Riesner and Gross, 1985) which has to be denatured, at least partially, for hybridization. However, this condition cannot be achieved merely by heating to a high temperature because of the potential destruction of sensitive target nuclear structures. Secondly, since strand-specific hybridization was required, the stringency had to be high in order to avoid homologous hybridization of the partially self-complementary viroid sequences. The detection of (−)strand sequences was particularly critical because of the inherent large excess of (+)strands in the form of mature viroids. Therefore, a high stringency of hybridization was combined with the high detection sensitivity provided by the biotin moiety. The degree of biotinylation in the transcript was varied systematically. It was determined that 100% biotinylated UTP led to probes yielding the most intensive fluorescence signals, which could be enhanced further by the use of a sandwich staining protocol. In some experiments, the hybridization and labelling procedures were carried out in solution and nuclei were fixed on the slide according to Pinkel *et al.* (1986). This method led to the loss or aggregation of many nuclei. Therefore, we selected for routine use an alternative procedure in which the nuclei are fixed to the slide prior to cytochemical processing.

Distribution of PSTVd (+)strand sequences in nuclei

The distribution of PSTVd (+)strand sequences in infected nuclei was analysed in a series of experiments. These RNAs are either mature circular viroid molecules or (+)strand

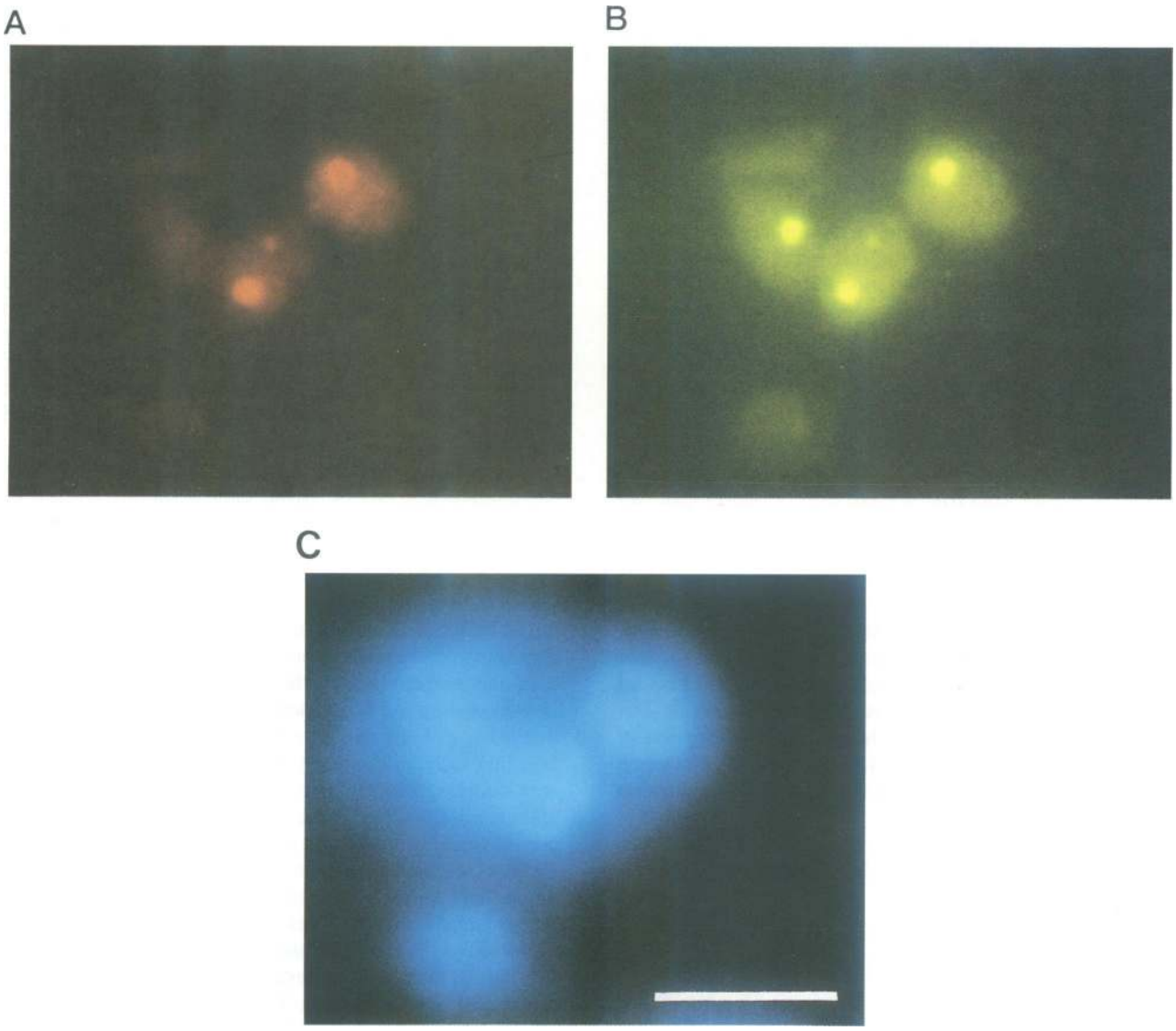

Fig. 1. Location of viroids in nuclei from PSTVd-infected tomato plants by viroid-specific *in situ* hybridization. Nuclei were isolated from tomato leaf tissue infected with PSTVd intermediate strain, bound to adhesion slides, fixed with formaldehyde and hybridized with biotinylated PSTVd (−)strand RNA under stringency condition B (Materials and methods). The bound probe was visualized with lissamine−rhodamine labelled steptavidin. The nucleoli were identified with the nucleolus specific monoclonal antibody Bv96 and a FITC-labelled secondary antibody. (A) Lissamine−rhodamine fluorescence corresponding to viroid specific *in situ* hybridization; (B) fluorescein fluorescence in Bv96-stained nucleoli; (C) DAPI fluorescence from nuclear DNA. In two nuclei shown in the micrographs viroids are concentrated in distinct areas congruent with the immunological labelling of the nucleoli. The bar represents 10 μm.

replication intermediates, the former being present in large excess (Hecker *et al.*, 1988). Isolated nuclei from PSTVd-infected and uninfected tomato leaf tissue were incubated first with a probe consisting of a biotinylated PSTVd (−) strand sequence and subsequently with lissamine−rhodamine labelled streptavidin. The results of *in situ* hybridization according to this protocol are shown in Figures 1A and 2A. Two of the nuclei from infected tissue showed a strong rhodamine fluorescence in a limited circular area (Figure 1A). This localized fluorescence signal was absent in nuclei from non-infected tissues (Figure 2A). In order to identify the regions exhibiting high fluorescence intensity, all samples were double-labelled after *in situ* hybridization by incubation with the nucleolus-specific monoclonal antibody Bv96 (Frasch, 1985) followed by a secondary FITC-conjugated goat-anti-mouse antibody. The locations of the fluorescein and rhodamine fluorescence signals were compared. The viroid-containing areas in Figure 1A were unequivocally stained by the antibody (Figure 1B), demonstrating that they corresponded to nucleoli. This result is in agreement with the earlier fractionation data (Schumacher *et al.*, 1983b).

The ability to identify nucleoli allowed us to perform unambiguous negative controls. If the above procedure was carried out with nuclei from non-infected tissue, the nucleoli stained strongly with the Bv96 antibody (Figure 2B) but not at all with the viroid-specific hybridization probe (Figure 2A). We conclude that the hybridization signal in nucleoli is derived exclusively from the presence of viroids and not from nonspecific binding of the biotinylated RNA probe or of labelled streptavidin.

In addition to the strong hybridization signals in nucleoli from PSTVd-infected cells a weak rhodamine fluorescence was also seen in the nucleoplasm (Figure 1A). In some nuclei, the fluorescence was distinctly higher than the background levels seen in uninfected cells (compare Figures 1A and 2A), indicating that the viroids were not confined to the nucleolus but were also present in the nucleoplasm, albeit at a much lower concentration.

The fraction of nuclei infected by PSTVd was determined by *in situ* hybridization (Figure 1A) and by counterstaining with the fluorescent dye 4′,6-diamidino-2-phenylindole-hydrochloride (DAPI) (Figure 1C). DAPI is used routinely for visualizing nuclear DNA (Kapuściński and Yanagi, 1979); the nucleoli appear as dark spots in the strongly fluorescent nucleoplasm (Figures 1C and 2C). Only some of the DAPI stained nuclei exhibited positive viroid-specific hybridization signals (Figure 1A and C; Table I). A different result was obtained with the anti-nucleolar monoclonal antibody Bv96 (Figures 1B and 2B). In this case, the correlation with DAPI staining was quite high, i.e. 35−98% of the nuclei stained with DAPI were also positive for Bv96 (Table I).

The statistical analysis of viroid distribution as summarized in Table I was carried out with two different viroid strains, the 'intermediate' and the 'lethal', which induce different disease symptoms. Two independent preparations of nuclei were analysed after infection with each of the two strains and for the non-infected control. The joint occurrence of positive signals for viroid-specific *in situ* hybridization and for labelling of nucleoli with the Bv96 antibody was also established. It should be emphasized that such data could be derived only by counting the double labelled nuclei and not by calculations from individual distributions.

Although the results of the duplicate experiments deviate considerably (Table I), a few general conclusions may be derived. (i) The frequency of viroid staining by *in situ* hybridization was less than 20%—6 to 18% of DAPI stained nuclei were positive—with both viroid strains. (ii) Most nucleoli (35−98% from all experiments) were positive for the Bv96 antibody. We assume that all nucleoli are in principle stainable by the antibody, as seen from the maximum observed frequency of 98% and in immunofluorescence experiments without prior *in situ* hybridization (results not shown). Taking into consideration the many steps of the double-labelling procedure, it is conceivable that the preservation of structural integrity of the nuclei varies considerably between and within preparations leading to a low labelling efficiency in some cases. (iii) Less than 20% of Bv96 antibody labelled nuclei gave a hybridization signal. That is, more nucleoli were visualized by antibody staining than by viroid labelling. In this case, we presume that some nuclei of infected tissue may be *a priori* negative for the viroid, for example in the event that the infection has not propagated uniformly throughout the cellular population.

Distribution of PSTVd (−)strand sequences in nuclei

The local distribution of PSTVd (−)strand sequences in nuclei was analysed by application of biotinylated PSTVd (+)strand RNA transcripts as hybridization probes. The stringency of the hybridization and washing steps was the same as that for the (+)strand distribution The nucleoli were stained as above with the Bv96 antibody.

Table I. Statistical evaluation of the PSTV (+)strand-specific *in situ* hybridization data

Source of nuclei	Experiment	Total nuclei	Bv96 positive		PSTVd (+)strand positive		Bv96 positive of PSTVd (+)strand positive	
			no.	%	no.	%	no.	%
PSTVd(I)-infected leaves	1	395	293	74	40	10	39	95
	2	881	ND	—	89	10	47	53
PSTVd(L)-infected leaves	1	509	273	54	32	6	31	97
	2	1080	378	35	196	18	70	36
Non-infected leaves	1	237	136	57	1	0.4	1	—
	2	191	187	98	0	0	0	—

PSTVd (+)strand-specific *in situ* hybridization and labelling with the nucleolus-specific Bv96 antibody was carried out in two independent experiments on isolated nuclei from tomato leaves infected with PSTVd(I) or PSTVd(L) viroids and from uninfected plants. [The PSTVd(I) strain causes intermediate disease symptoms, whereas infection with the PSTVd(L) strain may ultimately be lethal to the plant.] ND, not determined.

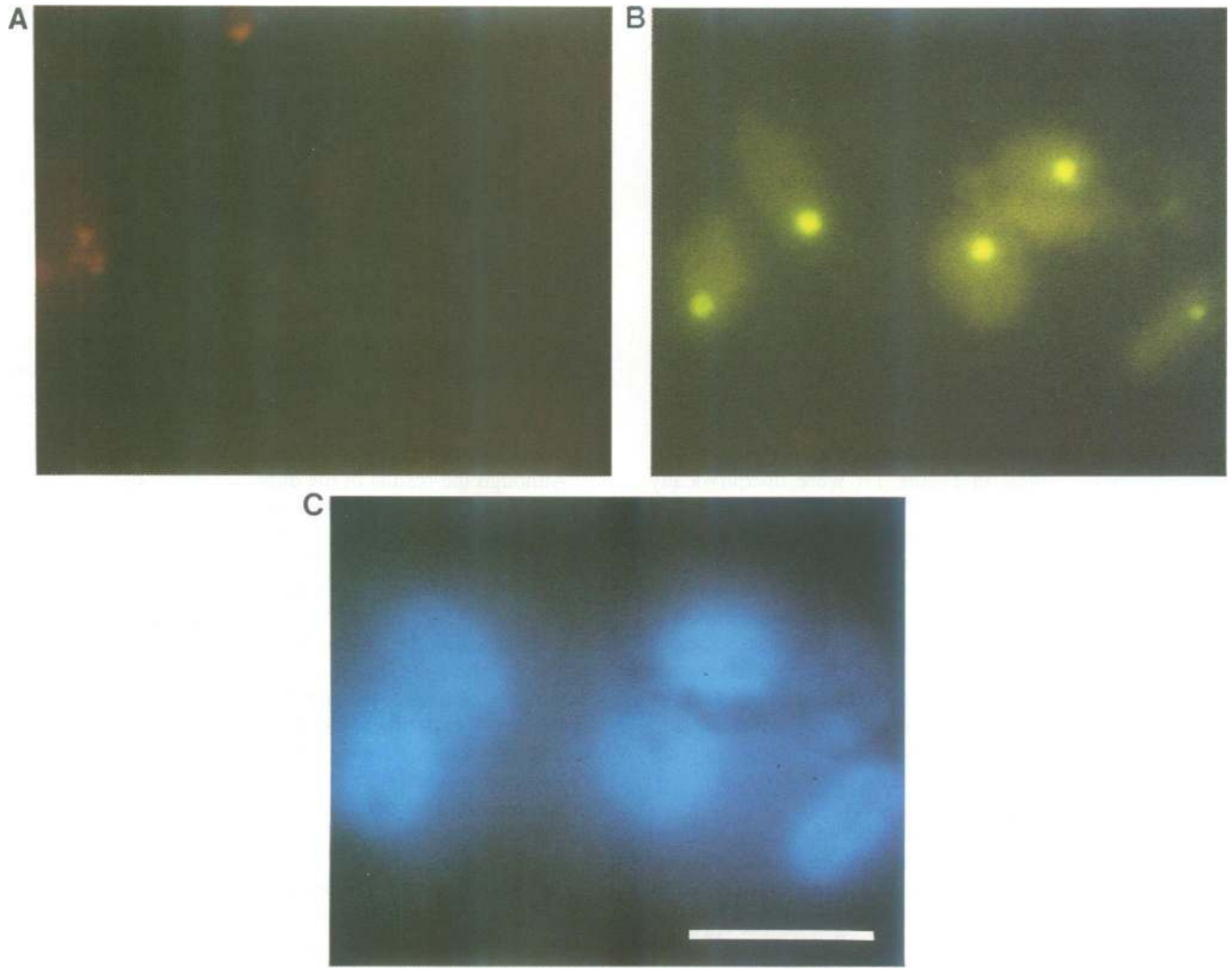

Fig. 2. Negative control for *in situ* hybridization in nuclei from healthy viroid-free tomato plants. Nuclei were bound to adhesion slides and subjected to exactly the same procedures as in Figure 1. (**A**) Lissamine–rhodamine fluorescence corresponding to viroid specific *in situ* hybridization; (**B**) fluorescein fluorescence in Bv96-stained nucleoli; (**C**) DAPI fluorescence from nuclear DNA. The absence of positive hybridization signals in healthy nuclei (rhodamine fluorescence in A) demonstrates the specificity of the viroid-specific *in situ* hybridization in Figure 1. The fuzzy spots of rhodamine fluorescence in (A) arise from non-specific binding of the dye to cell debris. They do not overlap with the positions of nucleoli as established in (B). Same magnification as in Figure 1.

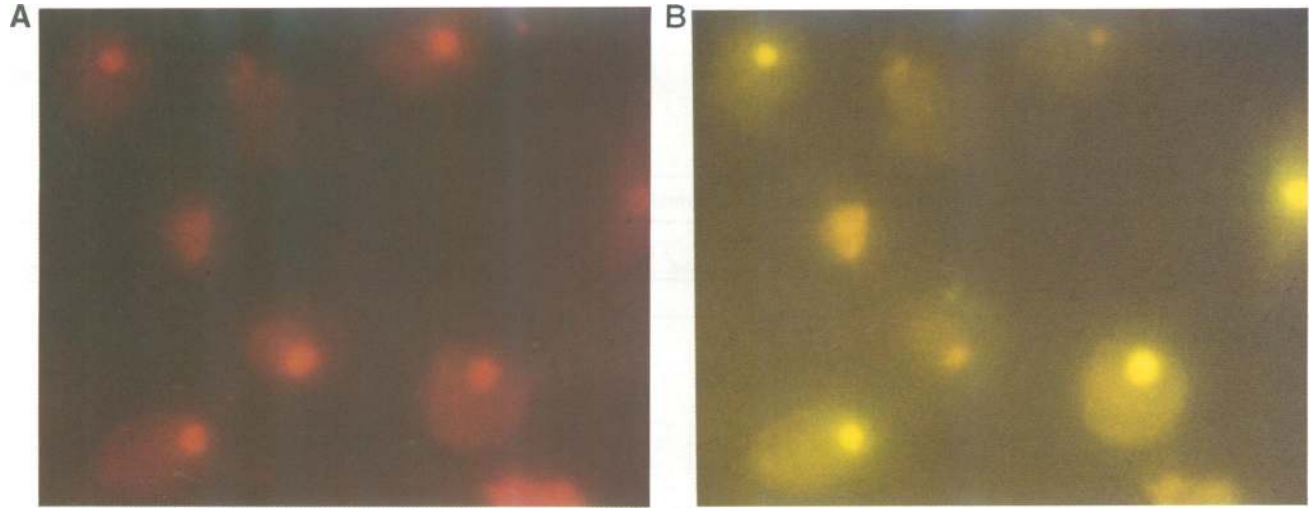

Fig. 3. Location of PSTVd (−)strand replication intermediates in nuclei from infected tomato plants. Nuclei were isolated from leaf tissue of 13 week old tomato plants infected with the lethal strain of PSTVd. Biotinylated PSTVd (+)strand RNA was used as a probe for *in situ* hybridization under the high stringency condition B. Detection of hybrids between (−)strand replication intermediates and biotinylated (+)strand RNA with lissamine–rhodamine labelled streptavidin (**A**) and immunological identification of the nucleoli by fluorescein fluorescence (**B**) was performed as in Figure 1. Same magnification as in Figure 1.

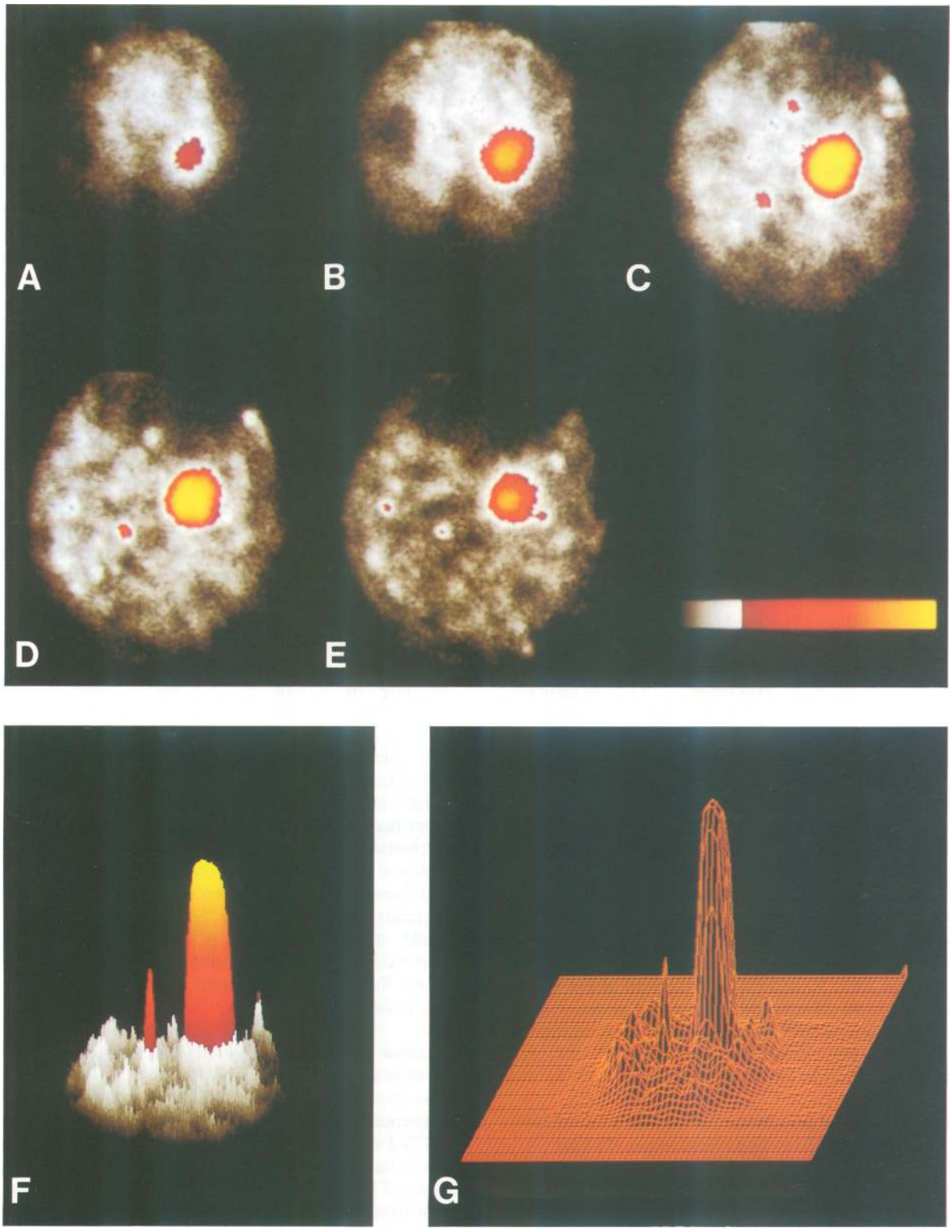

Fig. 4. Three-dimensional distribution of viroids in PSTVd infected tomato nuclei by viroid-specific *in situ* hybridization and imaging with the CLSM. Nuclei were isolated from tomato leaf tissue infected with the intermediate strain of PSTVd, bound to adhesion slides, fixed with formaldehyde and hybridized with biotinylated PSTVd (−)strand RNA under stringency condition A. (The somewhat less stringent condition A was used instead of condition B in order to achieve higher fluorescence signals.) Hybrids between PSTVd and the biotinylated probe were visualized with lissamine−rhodamine labelled streptavidin . **(A−E)** A set of sequential CLSM fluroescence images of one nucleus encompassing a nucleolus. The increment between optical sections was 0.8 μm. The intensity of fluorescence from the rhodamine dye was measured at >570 mm and displayed in pseudocolour increasing in the order grey, red, orange and yellow, as shown in the reference bar. **(F)** Intensity distribution in the optical section (D) displayed as a three-dimensional isometric projection, with pixel greyvalues represented both by pseudocolour and by vertical displacement. **(G)** Same distribution as in (F) but in wireframe representation. The uniform central intensity in this and other examples demonstrates that viroids are not restricted to the surface of the nucleolus. The bar represents 2 μm. Objective: plan-apochromat 63×/NA 1.4, zoom setting 90.

It was anticipated that the signal from the *in situ* hybridization of (−)strands would be much lower than that of (+)strands, because only replication intermediates are targets for the former whereas the vast excess of mature viroids account for most of the signal with the (+)strands as probes. Indeed, much lower hybridization signals were found in these experiments. Although direct inspection of the samples in the microscope permitted the assignment of the fluorescence to the nucleoli, the intensity in most cases was too low for satisfactory colour photography. An exceptionally high intensity was obtained with nuclei from 13 week old tomato plants infected with the lethal PSTVd strain (Figure 3). The distribution of the PSTVd (−)strand sequences in nuclei was similar to that of the (+)strands, i.e. was predominantly restricted to the nucleoli.

In view of the generally very low intensity of the (−) strand hybridization signal, its significance had to be considered carefully. For example, a critical control was to rule out the possibility that weak signals are derived from partial base pairing of the (+)strand probe to the vast excess of the PSTVd (+)sequences. Due to the highly self-complementary secondary structure of viroids (reviewed by Riesner and Gross, 1985) partial homologous base pairing of (+)strands may arise under low stringency conditions. At an ionic strength of 1 M, (+)strand/(+)strand hybrids dissociate completely at 90°C (Steger *et al.*, 1986). Under the same ionic conditions, it has been calculated that fully paired double helices of (+) and (−)strands maintain stability up to 110°C (Steger *et al.*, 1980). This difference of 20° had to be re-evaluated under the conditions of stringency used in the present experiments. The denaturation temperature increases with ionic strength by 13.2°/log[Na^+] (Steger *et al.*, 1980) and decreases with formamide concentration by 0.47°/% formamide (Riesner *et al.*, 1987a). Extrapolation to the hybridization condition B (0.1 × SSC, 50% formamide) leads to an estimate of the denaturation temperature of 43°C for (+)strand/(+)strand hybrids and 63°C for (+)strand/(−)strand hybrids. Thus, the washing temperature of 50°C was well above the denaturation temperature for 'wrong' (+)strand/(+)strand hybrids, implying that the latter should not have been present. Experimental support for the accuracy of the above estimate is provided by the finding that an increase in the washing temperature from 50 to 55°C or extension of the washing time from 15 to 25 min led to complete abolition of the hyridization signal corresponding to the (−)strand probe. We conclude that hybridization condition B was highly stringent, i.e. specific for the (+)strand/(−)strand hybrids.

Three-dimensional distribution of viroids studied with the confocal laser scanning microscope (CLSM)

The studies described so far demonstrate that the majority of viroids, as well as their (−)strand replication intermediates, are located in the nucleolus. The more specific question as to whether the viroid molecules are situated in the interior or at the surface of the nucleolus must be addressed by determining the three-dimensional distribution in cytological specimens. This may be achieved in principle by preparing thin sections of the nuclei (and thereby of the nucleoli) and analysing the two-dimensional fluorescence distribution as described above. However, the desired objective can also be met by optical sectioning with a CLSM, an instrument characterized by excellent discrimination against out-of-focus contributions (reviewed by Jovin and Arndt-Jovin, 1989).

Five successive serial scans of a single infected, labelled nucleus with the fluorescence CLSM are shown in Figure 4A−E. The interval between neighbouring focal planes was 0.8 μm. Fluorescence intensities are depicted in pseudocolour. It is seen that the fluorescence intensity increased rapidly from the periphery of the nucleolus, achieved a maximum within a zone of ~0.3 μm, and remained nearly uniform over the central region of the nucleolus. These features are clearly shown in Figure 4F, in which the distribution of fluorescence in the optical section depicted in panel D is displayed as an isometric three-dimensional projection with intensities coded in pseudocolour as well as by their vertical displacement (see also the cover illustration). A corresponding wireframe representation is given in Figure 4G. Several nuclei (some with multiple nucleoli) have been analysed with results similar to those in Figure 4.

From these observations, we conclude that the concentration of viroids is nearly constant throughout the volume of the nucleolus. A nucleolus with lower fluorescence intensity in the central compared to the peripheral region was never observed. The resolution of the CLSM should have sufficed to detect such a phenomenon had it existed, as demonstrated with peripheral markers of the nucleolus in plant and mammalian cells (our unpublished data).

Discussion

Imaging of viroids in the fluorescence microscope demonstrates that the majority of PSTVd (+)strand and (−)strand sequences are located in the nucleoli of infected cell nuclei. These results were obtained by *in situ* hybridization and fluorescence microscopy. The structures exhibiting positive signals for viroid sequences were identified as nucleoli by dual labelling with a specific anti-nucleolar antibody. In addition, images obtained with the confocal laser scanning microscope showed that viroids are distributed homogeneously within the nucleolus, i.e. are not concentrated at the surface, an alternative possibility that could not be excluded prior to these experiments. Methodological aspects of this study have been discussed in the Results, and we restrict the following discussion to the biological implications.

Nuclear distribution of viroids and their replication intermediates

The general finding that viroid (+)strand and (−)strand sequences are located in nucleoli of cells infected with PSTVd is in agreement with results from fractionation studies (Schumacher *et al.*, 1983b; Hecker *et al.*, 1988). The two types of investigation elucidated different details of the local distribution. Whereas the fractionation studies resulted in average values for the cellular and subcellular populations, the combination of *in situ* hybridization and imaging provided information at the level of individual nuclei. The data in Table I show that viroids were detected in only a low percentage (6−18%) of infected nuclei. The absence of detected viroids from certain nucleoli may reflect in part the finite efficiency of hybridization, but we believe it more probable that a significant fraction of the cells may be viroid-free, i.e. uninfected. If the average number of up to 10^4

viroid copies per cell determined previously (Schumacher *et al.*, 1983b) is related to the present upper estimate of ~20% infection (Table I), one must conclude that the copy number in individual cells may be as high as 5×10^4.

The hybridization results show that the partitioning of viroid sequences between nucleolus and nucleoplasm is variable. In the report of Schumacher *et al.* (1983b), 90% of the viroids were found to be associated with the nucleolar fraction; a similar distribution was estimated for replication intermediates by Hecker *et al.* (1988). The contrast between viroid staining in the nucleolus and in the nucleoplasm was qualitatively obvious in all nuclei we examined. However, in some individual nuclei, the apparent viroid concentration in the nucleoplasm achieved a level of ~10% of that in the nucleolus. Taking into consideration the larger volume of the nucleoplasm compared to the nucleolus, one concludes that in such nuclei the viroids may have been approximately equally distributed within and external to the nucleoli.

The particular subcellular localization of viroids clearly differentiates them from plant viruses. The latter generally accumulate and replicate in the cytoplasm and only rarely in the nucleus, as for example the Gemini viruses and Luteoviruses and the pea enation mosaic virus (reviewed by Francki *et al.*, 1985). The aboutilon mosaic virus provides the only known example for localization in chloroplasts (Gröning *et al.*, 1987). No virus, viral RNA or any other form of a pathogen is known to be targeted to the nucleolus, PSTVd being the notable exception. However, since this is the only viroid species and infected tomato leaves the only tissue examined in this connection to date, it remains to be established whether the involvement of the nucleolus in the mechanism of viroid infection is a general phenomenon. An intranuclear localization has been shown for other viroid–host systems: PSTVd in potato cell cultures (Spiesmacher *et al.*, 1983; Mühlbach, 1987), citrus exocortis viroid in *Gynura aurantiaca* (Semancik *et al.*, 1975) and hop stunt viroid in hop tissue (Takahashi *et al.*, 1982). The results of Randles *et al.* (1976) on the cytoplasmic location of coconut cadang-cadang viroid (CCCVd) and Mohamed and Thomas (1980) on the association of avocado sunblotch viroid (ASBVd) with chloroplasts and with the endoplasmic reticulum must still be considered preliminary, inasmuch as only inhomogeneous fractions of cellular components were analysed.

Functional implications

The nucleolar localization of viroids has to be considered in the context of their replication and pathogenic action. It is assumed that the synthesis of (−)strand oligomers (Mühlbach and Sänger, 1979; Rackwitz *et al.*, 1981), as well as that of (+)strand oligomers (Mühlbach, 1987; I.Schindler and H.P.Mühlbach, personal communication), is carried out by the DNA dependent RNA polymerase II. In the non-infected cell this enzyme is responsible for mRNA transcription in the nucleoplasm and it is partly associated with chromatin. At first glance, the localization of mature viroids and (−)strand and (+)strand replication intermediates in the nucleolus seems to contradict the presumed involvement of RNA polymerase II in the synthesis of these RNAs, particularly since the present work has shown that the viroid RNA is in the interior and not on the surface of the nucleolus. There are two logical possibilities for reconciling this apparent contradiction: either the RNA polymerase II or the viroid, including its replication intermediates, migrate between the nucleoplasm and the nucleolus. We are not aware of any experimental evidence for such a redistribution of RNA polymerase II. However, at least for one RNA species, the small nuclear RNA U3, migration from the nucleoplasm to the nucleolus has been demonstrated. The snRNA U3 is transcribed by RNA polymerase II in the nucleoplasm and is then transported to the nucleolus (for a review see Busch *et al.*, 1982). A similar behaviour may be postulated for the viroids, a model which would be in concordance with our results inasmuch as circular viroid RNA as well as its replication intermediates are found in the nucleoplasms, although at much lower concentrations than in the nucleolus. One may also consider the possibility that the synthesis by polymerase II occurs in the nucleoplasm but that the (+) strand oligomers are processed to the mature circular viroids only in the nucleolus. The identification of the latter organelle as the site of processing would agree with the finding that viroids enter isolated nuclei with which they are incubated but do not subsequently associate with the nucleoli (Rienser, 1987b). Thus, the high concentration of mature viroids observed in infected tissue may represent a reservoir or storage form of the products arising from a maturation mechanism topologically restricted to the nucleolus.

While viroid replication may well occur at sites of low steady state concentration of the template, i.e. in the nucleoplasm, it is tempting to speculate that the pathogenic action of the viroids may be exerted at the locus of highest concentration, namely the nucleolus. If so, it would appear that the mere presence of the viroids might not in itself be pathogenic inasmuch as the same distribution and copy number were found for the RNAs of different PSTVd strains (see also Gruner, 1987). That is, the difference in pathogenicity among viroid strains may originate from fundamental differences in the molecular interactions between viroid and host and not from differences in copy number or subcellular localization. Unfortunately, only hypotheses exist as yet regarding the mechanisms of viroid pathogenesis (for review see Owens and Hammond, 1987). Most of these cannot be related to the nucleolar location. For example, it was proposed recently that viroids interact by base pairing with the RNA from signal recognition particles (SRP), and thereby impair SRP assembly and SRP-mediated translocation and integration of membrane proteins into the endoplasmic reticulum (Haas *et al.*, 1988). There was only one proposal which clearly pointed to the nucleolus as the site of pathogenic action. As pointed out by Schumacher *et al.* (1983b) and Kiss *et al.* (1983), PSTVd exhibits sequence homologies to snRNA U3. This RNA is thought to be involved in preprocessing for pre-rRNA inside the nucleolus (Busch *et al.*, 1982; Crouch *et al.*, 1983). Different mechanisms for viroid interference with rRNA processing may be considered. For example, viroids could compete with snRNA U3 for binding to pre-rRNA (Jakab *et al.*, 1986), or viroid (−)strand sequences could form base pairs with U3 RNA itself. It is also conceivable that viroids bind to the proteins of the U3 ribonucleoprotein particle. So far, the latter have been characterized only from human origin (Parker and Steitz, 1987). One might anticipate from the evolutionary conservation of U3 RNA (Kiss *et al.*, 1985) that the structure and function of U3 ribonucleoprotein particles in plants and mammals are similar. However,

elaboration of more sophisticated hypotheses about viroid interference with rRNA processing requires additional knowledge about the relevant biochemistry in the healthy plant cell.

Materials and methods

Buffers

SSC: 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.6. Buffer 1: 2 × SSC, 0.1% (w/v) sodium dodecyl sulphate (SDS). Buffer 2: 0.1 × SSC, 0.1% (w/v) SDS. Hybridization buffer: 5 × SSC, 50 mM Na_2HPO_4/NaH_2PO_4, pH 6.5, 50% (v/v) deionized formamide, 0.02% (w/v) each of bovine serum albumin (BSA), Ficoll 400 (Serva) and polyvinylpyrrolidone (PVP-10, Sigma), 250 μg/ml denatured herring sperm DNA; M/90 buffer: 12 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 7.6, 121 mM NaCl. BSA-M/90 buffer: 2% (w/v) BSA in M/90 buffer; TE buffer: 10 mM Tris−HCl, pH 8.0, 1 mM EDTA. Buffer E: 25 mM 2-(*N*-morpholino)ethane-sulphonic acid-NaOH, pH 6.5, 250 mM mannitol, 0.1% (w/v) BSA, 10 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol.

Plant material

Tomato plants (*Lycopersicon esculetum* L. cv. *Rutgers*) were grown in a greenhouse under conditions described previously (Schumacher *et al.*, 1983a). Eighteen days after sowing they were mechanically inoculated by rubbing in the presence of carborundum either with purified PSTVd-DI ('intermediate') or with PSTVd-KF 440-2 ('lethal') RNA (Schnölzer *et al.*, 1985). In most experiments completely differentiated leaves of 8−9 week old plants were used for isolation of nuclei. Control experiments were carried out with healthy plants of the same age grown under identical conditions.

Isolation of nuclei

Nuclei of tomato leaf tissue were extracted according to the isolation scheme described previously (Schumacher *et al.*, 1983b) but with the following simplifications. (i) The plant material (200 g in 80 ml buffer) was homogenized for 5 × 5 s and was not re-extracted. (ii) The isolated nuclei were washed once instead of three times in buffer E.

Binding and fixation of nuclei on microscope slides

Adhesion slides with 2 × 6 adhesion spots (Adhäsionsobjektträger Typ I, Paul Marienfeld KG, Bad Mergentheim, FRG) were rinsed with methanol as described by the manufacturer and washed with M/90 buffer. Drops containing 10^5 freshly isolated nuclei in 20 μl of buffer E were placed on each adhesion spot and allowed to settle for 10 min at room temperature. Unbound nuclei were removed by briefly rinsing the slides and washing them for 5 min in buffer E. Nuclei were fixed for 10 min with 4% formaldehyde (freshly prepared from paraformaldehyde) or 3.7% formaldehyde/1% methanol (Merck, Darmstadt, FRG) in M/90 buffer and washed 3 × 10 min in autoclaved M/90 buffer. Nuclei bound to the adhesion spots of the slides were covered with drops of M/90 buffer and stored in a moist chamber at 4°C until use.

In situ hybridization

Preparation of hybridization probe. Biotinylated runoff transcripts from the viroid-cDNA containing plasmids pRH713 and pRH714 (Hecker *et al.*, 1988) were synthesized with T7 RNA polymerase according to Theißen *et al.* (1989), except that UTP was completely substituted by 5-[*N*(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]-uridine 5′-triphosphate (Bio-11-UTP; Bethesda Research Labs, Bethesda, MD). Transcripts were precipitated with ethanol and redissolved in TE buffer. The fraction of full length transcripts was estimated by denaturing polyacrylamide gel electrophoresis.

Probe specificity. Transcription of pRH714 results in PSTVd (−)strand RNA of unit length plus nine vector derived nucelotides; this RNA was used as an *in situ* hybridization probe specific for viroid (+)sequences. The RNA derived from pRH713 has the same length but consists of (+)strand sequences; it was used as probe specific for (−)strand replication intermediates.

Hybridization procedure. The M/90 buffer was removed from the adhesion slides with a Pasteur pipette, and the fixed nuclei were overlaid with hybridization buffer and incubated at room temperature for 2−5 min. The buffer was removed and each adhesion spot was covered with 2.5 μl of hybridization buffer containing 2 μg/ml of biotinylated probe. Four neighbouring spots were hybridized with the same probe and sealed under an 18 × 18 mm coverslip using rubber cement.

Hybridization was carried out in a moist chamber at 55°C for 10−16 h and the coverslips were then dislodged by flotation in buffer 1. The whole slide was washed at different stringencies: (i) 20 min at 55°C in buffer 1, 15 min at 55°C in buffer 2 and 15 min in buffer 2 + 25% formamide or (ii) 30 min at 55°C in buffer 1, 20 min at 50°C in buffer 2 and 15 min at 50°C in buffer 2 + 50% formamide. The slides were cooled to room temperature in M/90 buffer and washed twice for 10 min in the same buffer. They were then successively incubated at room temperature with (i) BSA-M/90 buffer for 10 min, (ii) 5 μg/ml lissamine−rhodamine conjugated streptavidin (strep-Rhod; Dianova, Hamburg, FRG) for 2 h, (iii) two 10 min washes in M/90 buffer followed by BSA-M/90 buffer for 10 min, (iv) 16 μg/ml biotinylated alkaline phosphatase (Dianova) for 2 h, (v) 2 washes of M/90 buffer followed by BSA-M/90 for 10 min and (vi) strep-Rhod for 2 h and washed as above. All reagents were diluted in BSA-M/90 buffer.

Immunological labelling of nucleoli

After *in situ* hybridization, nucleoli were labelled immunologically by incubation of the nuclei with the monoclonal antibody Bv96 (Frasch, 1985) at room temperature for 2−4 h followed by a secondary goat-anti-mouse FITC-conjugated antibody (Dianova). Both antibodies were diluted with BSA-M/90 buffer and their application was preceded by two 10 min washes in M/90 buffer and incubation with BSA-M/90 buffer for 10 min. The slides were treated finally with three 10 min washes in M/90 buffer.

DNA staining with DAPI

After *in situ* hybridization and immunological labelling, nuclear dsDNA was counterstained for 5 min with 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole-hydrochloride, Sigma) (Kapuściński and Yanagi, 1979). For examination with the microscope, the slides were washed in M/90 buffer for 5 min, air-dried, overlaid with an antifading buffer containing *p*-phenylene-diamine (slightly modified from Johnson and Aroujo, 1981) and sealed with coverslips.

Fluorescence microscopy

Microscopy was with a Zeiss Photomicroscope III equipped with fluorescence and phase contrast attachments. Fluorescence was excited with a HBO 50 high pressure Hg lamp. The Zeiss filter combinations 01, 09 and 15 were used for excitation and observation of the fluorescence corresponding to the dyes DAPI, fluorescein and lissamine−rhodamine, respectively. Each combination contains a transmission filter (BP) in series with a dichroic filter (FT) and a cutoff filter (LP) as follows: (i) 01: BP365, FT395, LP397; (ii) 09: BP450-490, FT510, LP520; (iii) 15: BP546, FT580, LP590. Photographs were taken with Kodak Ektachrome 400 color transparency film using a Zeiss Neofluar 100×/NA 1.3 objective and Zeiss immerison oil.

Confocal laser scanning microscopy

The Zeiss CLSM has been described previously (Robert-Nicoud *et al.*, 1989). It is equipped with three-laser excitation (UV-visible), confocal optics in fluorescence, and a high-precision mechanical stage (0.25 μm in the *x*,*y* scanning field and 0.05 μm along the orthogonal *z* axis) operated by a Zeiss MPC controller through an IEEE interface. The acquisition of data is with an 8-bit, 512 × 512 frame buffer coupled to central DEC Micro Vax II computer system. Image processing was carried out using TCL-Image (Multihouse, Amsterdam), an integrated analysis system developed at the Delft Center for Image Processing.

Confocal sections were obtained using an oil immersion plan-apochromat 63×/NA 1.4 objective and zoom settings between 60 and 90. Excitation was at 488 and 514 nm for the fluorescein and lissamine−rhodamine labelled probes, respectively. Fluorescence emission was measured with the corresponding dichroic/long-pass filter combinations FT510/LP515 and FT562/LP570.

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