The 7S RNA from tomato leaf tissue resembles a signal recognition particle RNA and exhibits a remarkable sequence complementarity to viroids

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From tomato leaf tissue we sequenced and characterized a 7S RNA which consists of 299 nucleotides with either two or three additional uridine nucleotides at its 3'-terminus. About 56% of the nucleotides of this higher plant 7S RNA are in nearly identical positions as those of the human 7SL RNA which is an integral component of the signal recognition particle (SRP) that mediates protein translocation. Computer modelling and digestion studies with nucleases led to a secondary structure model for tomato 7S RNA, the overall shape of which is very similar to that of the human 7SL (SRP) RNA. This structural similarity strongly suggests that tomato 7S RNA is actually an SRP RNA and an integral part of the plant SRP, and that the protein translocation system of higher plants is very similar to the one operating in mammalian cells. Tomato SRP RNA contains a stretch of 36–53 nucleotides which exhibit a high degree of sequence complementarity to five viroid 'species' that cause disease in tomato. In the case of potato spindle tuber viroid and citrus exocortis viroid this complementarity spans the lower strand of the region, the nucleotides of which are known to modulate virulence. This extensive sequence complementarity could lead to a thermodynamically favoured base-pairing *in vivo* which renders the tomato SRP RNA a possible host target with which viroids could interact and thus incite disease.

Key words: plant RNA/potato spindle tuber viroid/protein translocation/SRP RNA/viroid pathogenesis

Introduction

Upon electrophoresis in 5% polyacrylamide (PAA) gels of 2 M LiCl-soluble nucleic acids from tissue of higher plants (Sänger *et al.*, 1976), four small RNA-species namely tRNA, 4.5 S RNA, 5S RNA and 7S RNA are well separated and appear as prominent bands when visualized by staining (Figure 1). All these RNAs are also found in other organisms, and a wealth of information is available on the structure and biological function of most of these RNAs. However, the nucleotide sequence and biological function of 7S RNA from higher plants have not yet been elucidated. In view of a possible involvement of plant 7S RNA in viroid pathogenesis, we determined the primary and secondary structure of the 7S RNA from tomato which is a widely used host plant for various viroid species (reviewed by Diener, 1979, 1987).

Mammalian 7SL RNA and six proteins (Walter and Blobel, 1982) are the components of the so-called signal recognition particle (SRP). SRPs play an essential role in the programming of ribosomes which are synthesizing proteins destined either for translocation across the membrane of the endoplasmic reticulum (ER) or for their insertion into this membrane (Walter *et al.*, 1981, 1984; Meyer *et al.*, 1982). 7S RNA-containing SRP-like particles also operate in protein-synthesizing and -accumulating tissue of higher plants such as wheat germ (Prehn *et al.*, 1987) and maize endosperm (Campos *et al.*, 1988).

At present, only the sequences of 7SL (SRP) RNA from human cells (Balmain *et al.*, 1982; Li *et al.*, 1982; Ullu *et al.*, 1982; Ullu and Tschudi, 1984), dog cells (Siegel and Walter, 1986), and cells from *Drosophila melanogaster* (Ullu and Tschudi, 1984; Gundelfinger *et al.*, 1984), *Xenopus laevis* (Ullu and Tschudi, 1984), *Halobacterium halobium* (Moritz *et al.*, 1985), and *Schizosaccharomyces pombe* (Ribes *et al.*, 1988) are known. On the basis of nuclease digestion experiments (Gundelfinger *et al.*, 1984), by the compensatory base change approach (Zwieb, 1985) and by mere comparison and computer modelling (Moritz and

Fig. 1. Separation of 2 M NaCl-soluble RNAs from viroid (PSTV)-infected tomato leaf tissue in a 5% PAA slab gel under non-denaturing conditions. RNAs were visualized by staining with methylene blue and the prominent species are indicated. The multiple banding of the 7S (SRP) RNA is most probably due to its existence in different structural configurations as it has also been reported for the human 7SL (SRP) RNA (Zwieb, 1985; Zwieb and Ullu, 1986).

Fig. 2. Sequence analysis of the 5'- and 3'-terminal region of tomato SRP RNA. Autoradiogram from the mobility shift analysis (A and C) and from the RNA sequencing according to the method of Donis-Keller (B and D). The position of certain nucleotides in the tomato SRP RNA sequence is indicated. The pattern of radioactive spots in the autoradiogram shown in (A) can be attributed to two sequence variants of tomato SRP RNA that differ in the nucleotide in the second position which is a guanosine or an adenosine in the major and in the minor species, respectively. From the autoradiogram shown in (C), it becomes evident that the tomato SRP RNA species contain either two or three uridine nucleotides at their 3'-end. The 3'-terminal sequence concluded from the autoradiogram shown in (D) contains some ambiguities in certain pyrimidine nucleotides which are marked by an asterisk, respectively. These nucleotides could be determined by cDNA sequencing.

Goebel, 1985), secondary structure models for these 7SL (SRP) RNAs have been proposed and refined. All those 7SL (SRP) RNAs which are ~300 nt long exhibit 50–99% similarity in their sequence. In addition, the overall shape of their secondary structure models is nearly identical. The 7SL (SRP) RNA from *S. pombe* also fits into this pattern although it consists of only 256 nt due to a lacking domain at its 5'-terminus.

Here we present the nucleotide sequence and a secondary structure model for the 7S RNA from tomato leaf tissue which exhibits a striking overall similarity with all the non-plant 7SL (SRP) RNAs presently known. Our data strongly suggest that the plant 7S RNA is an integral part of the SRPs of higher plants, and that it has similar biological functions as those demonstrated for these other 7SL (SRP) RNAs (reviewed by Siegel and Walter, 1988a). Sequence comparison furthermore shows that this plant SRP RNA could also be the cellular target with which viroids base-pair thus causing disease.

Results

Direct RNA sequencing

The sequence of the 5'- and 3'-terminal region of tomato

SRP RNA was determined by direct RNA sequencing. Pilot experiments had revealed that its 5'-terminus contains several phosphate groups (data not shown) which required enzymatic dephosphorylation prior to radioactive end-labelling by T4 polynucleotide kinase (T4 PNK) using [γ - 32 P]ATP. The 5'-terminal guanosine nucleotide of tomato SRP RNA was determined by PEI-cellulose thin layer chromatography (Randerath and Randerath, 1967; Volkaert and Fiers, 1977) of the total nuclease P1 digest (data not shown).

Mobility shift analysis (Sanger *et al.*, 1965; Brownlee and Sanger, 1965; Frank and Blöcker, 1982) was applied to determine the 5'-terminal region of tomato SRP RNA. From the autoradiographic pattern (Figure 2A) of the separated fragments generated by partial digestion with nuclease P1 (Silberklang *et al.*, 1977) it becomes evident that in the RNA preparation at least two SRP RNA species are present that differ in the second nucleotide which is either G or A. The 5'-terminal region was also sequenced (Figure 2B) by the method of Donis-Keller (1977) through partial digestion of the 5'-end-labelled RNA with base-specific RNases (Randerath and Randerath, 1983).

To determine the sequence of the 3'-terminal region of tomato SRP RNA, radioactive end-labelling was performed with [$5'$ - 32 P]pCp using T4 RNA ligase (England *et al.*,

1980). After its purification on an 8.4% PAA sequencing gel, the labelled RNA was totally digested with RNase T2 and the 3'-terminal uridine nucleotide was then identified by PEI-cellulose chromatography (data not shown). In order to obtain the sequence of the nucleotides adjacent to the 3'-terminal nucleotide, the SRP RNA was 3'-end-labelled with [5'-³²P]pCp, treated with alkaline phosphatase from calf intestine (CAPase) to remove terminal phosphate groups and then partially digested with nuclease P1. The resulting fragments were submitted to mobility shift analysis (data not shown) which demonstrated the absence of guanosine residues in the last twenty nucleotides of the 3'-terminal region. To substantiate this finding the mobility shift analysis was also carried out with an SRP RNA sample that had been treated with RNase T1 before partial digestion with nuclease P1. Figure 2C shows that the spots on the corresponding autoradiogram of the final DEAE cellulose thin layer plate can be allotted to two main and nearly parallel sequence tracks. From this pattern it becomes evident, that tomato SRP RNA carries two or three uridine nucleotides at its 3'-terminus. However, the sequence of the 3'-terminal region could not be determined by mobility shift analysis alone, because the spots in the lower part of the autoradiogram can be arranged in several ways. In order to obtain improved sequence information on the 3'-terminal region, the RNase T1-generated fragments were electrophoretically separated on a 20% PAA sequencing gel. We isolated four radioactively labelled fragments from the gel and sequenced them according to the method of Donis-Keller (1977). From two of these fragments a reliable sequence could not be obtained. The 3'-terminus of the two other and most abundant fragments contained either two or three uridine nucleotides. Except for the uncertain nature of five pyrimidine nucleotides (Y) the sequence of these two fragments proved to be 5' YYYCAAYAAAYAACCACCUU(U) 3' (Figure 2D).

cDNA sequencing

Direct RNA sequencing of the 5'- and 3'-terminus had shown that different species of tomato SRP RNA exist. Since these species cannot be separated, it is impossible, as yet, to determine their complete primary structure by direct RNA sequencing. Therefore, sequence analysis of the SRP RNA was performed at the level of its *in vitro* synthesized cDNA as obtained by primer-initiated reverse transcription. We chose a synthetic 14mer DNA primer complementary to the ultimate section of the partly known 3'-terminal region from two fragments of those SRP RNA species which are predominantly present in the preparation (Figure 2D). We anticipated that for reverse transcription this primer would select from the total population of the SRP RNA only one species that can be chemically sequenced by the method of Maxam and Gilbert (1980). As shown in Figure 3, the autoradiogram obtained from the corresponding sequencing gel reveals that the sequence of the SRP cDNA can be read without any signs of heterogeneity close to its 3'-terminus which resembles the 5'-terminus of the SRP RNA. The total sequence of tomato SRP RNA was established by combining the short 5'- and 3'-terminal sequences as obtained by direct RNA sequencing and the long internal sequence as derived from cDNA sequencing (Figure 4). Thus, the sequenced SRP RNA from tomato leaf tissue is characterized by a chain length of 299 nt consisting of 73 (24.5%) adenosine, 64 (21.4%) cytosine, 89 (29.8%) guanosine, and 73 (24.4%)

Fig. 3. Autoradiogram from chemical cDNA sequencing of tomato SRP cDNA. The [5'-³²P]-labelled cDNA was partially cleaved at guanines (G), adenines and guanines (A/G), thymines and cytosines (T/C) and cytosines (C) using reactions R5–R8 (Maxam and Gilbert, 1980), respectively, and then electrophoresed on a 0.18 × 299 × 400 mm 8.4% sequencing gel. The cleavage pattern represents the 3'-terminal region of tomato SRP cDNA. The position of distinct cytosine nucleotides in tomato SRP RNA is indicated.

uridine residues plus two or three uridine nucleotides at its 3'-terminus.

Sequence comparison

From its size and from its abundance in plant tissue, we inferred that tomato SRP RNA might be structurally and functionally related to the mammalian 7SL (SRP) RNA. Therefore the sequence of both RNAs was aligned for comparison (Figure 4). It was found that an overall sequence similarity of 56% exists between the plant SRP RNA and the human 7SL (SRP) RNA. The sequence similarity between the tomato SRP RNA and the 7SL (SRP) RNA from *Drosophila*, *Xenopus*, *H. halobium* and *S. pombe* is ~31, 35, 39 and 45%, respectively. Since such sequence similarities must not necessarily reflect a similar function of these RNAs, a statistical treatment of the sequence comparison was performed according to the method of Smith *et al.* (1985) which allows the evaluation of the biological significance of sequence relationships. When the sequence of tomato SRP

Fig. 4. The primary structure of tomato SRP RNA and its alignment with human 7SL (SRP) RNA. Capital letters indicate matching nucleotides, lower case letters denote mismatching nucleotides. Nucleotide gaps which have been introduced into the sequences to allow optimal matching are marked by an asterisk. Boxed-in sequence motifs indicate matching of three or more consecutive nucleotides. The Alu-like portions of the human 7SL (SRP) RNA are underlined. The regions of the sequence of tomato SRP RNA and human 7SL (SRP) RNA which are included in brackets exhibit the highest similarity score of 25.4 when both RNAs are compared by the UWGCG alignment program BESTFIT using a gap weight of zero and a gap length weight of 2.

RNA is compared with a randomized RNA of the same size and base composition as the human 7SL (SRP) RNA, a mean best similarity score of 12.46 is obtained. The actual comparison of tomato 7S RNA with the human 7SL (SRP) RNA revealed that the highest similarity score between both RNAs is 25.4 and refers to the region between nucleotides 62 and 237 of tomato 7S RNA and nucleotides 62–241 of human 7SL (SRP) RNA, respectively (Figure 4). This high similarity score arises from numerous clusters of three, four and six consecutive identical nucleotides which are found in this particular part of both RNAs. The difference between the highest similarity score of 25.4 and the mean best similarity score of 12.46 is 13.94 and therefore 7.27 times greater than the estimate of the standard deviation of 1.78 for this type of comparison (Smith *et al.*, 1985). This result indicates the existence of a high statistical significance for a biological and hence functional relationship between these two RNA species. In contrast, the pairwise comparison of tomato SRP RNA with the SRP (7SL) RNAs from *Drosophila*, *Xenopus*, *H. halobium* and *S. pombe* reveals no such statistical significance (data not shown).

Secondary structure modelling

We assumed that a functional similarity between tomato SRP RNA and human 7SL (SRP) RNA should be reflected in a more obvious way in the secondary structure of these RNAs than in the statistical evaluation of their sequence similarity. Therefore, we performed a computer-aided secondary structure modelling with tomato SRP RNA. We anticipated that in this model thermodynamically stable domains appear which are similar to the ones proposed for the human 7SL (SRP) RNA. For the latter, a plausible model had been developed by Gundelfinger *et al.* (1984) which was further refined by Zwieb (1985) by comparing the sequence of 7SL (SRP) RNA from human cells and from cells of *Xenopus* and *Drosophila*.

Although their sequence similarity is only 56%, the visual comparison of the corresponding planar secondary structure shape of tomato SRP RNA (Figure 5A) and human 7SL (SRP) RNA (Figure 5B) shows that a very close overall structural similarity exists between both RNAs in that all

characteristic structural domains are present in both of them (see also Table 1). The free enthalpy value for each of the two structures in solution at 1 M NaCl, neutral pH and 37°C was calculated according to the thermodynamic parameters for structure formation published by Freier *et al.* (1986) in which energy parameters for bifurcated loop structures are not considered. The corresponding free enthalpy values are –96.4 and –86.8 kcal/mol for tomato SRP RNA and human 7SL (SRP) RNA, respectively. In our secondary structure model of tomato SRP RNA 33 AU, 54 GC, and 15 GU base-pairs are present so that altogether 74% of the total number of the nucleotides are involved in base-pairing.

Secondary structure analysis with nucleases

In order to substantiate the computer-generated secondary structure model of tomato SRP RNA, additional enzymatic studies were undertaken to determine whether or not this structure actually occurs in solution. In one set of experiments, 5'- and 3'-end-labelled tomato SRP RNA was partially digested with RNase T1, nuclease S1 and RNase V1 under non-denaturing conditions, respectively, and the corresponding cleavage sites were determined by separating the fragments on 8.4% and 20% PAA sequencing gels (Figure 6). RNase T1 cleaves at the 3'-side of guanosine nucleotides provided they are present in unpaired regions of the RNA, nuclease S1 also cleaves phosphodiester bonds in unpaired regions of the RNA and RNase V1 cleaves next to nucleotides which are base-paired in the RNA molecule.

These partial digestion experiments revealed that virtually all of the RNase T1 cleavage sites were found in those unpaired (loop) regions for which cleavage was expected from the computed secondary structure model (Figure 8). Some of these sites were also sensitive to nuclease S1 which had cleaved, with high preference, all the phosphodiester bonds in unpaired (loop) regions of the tomato SRP RNA in accordance with the computed model. Nuclease S1 cleavage was also observed in base-paired regions within the domain formed by nucleotides 178–210. This was most probably due to the thermal melting of the three short, and therefore less stable, helices of this domain. RNase V1

A Tomato SRP RNA

B Human 7SL (SRP) RNA

Fig. 5. The secondary structure model of tomato SRP RNA (A) as compared with that of human 7SL (SRP) RNA (B) and an alternative configuration of the bifurcated loop structure in the S-part (C) of the latter, both as proposed by Zwieb (1985). To avoid undue disruption of the loop regions, the computer generated planary shape of the tomato SRP RNA has not been adjusted to the one of the human 7SL (SRP) RNA as presented by Siegel and Walter (1986). (D) and (E) show configurations of the S-part which are derived from the structures depicted in (B) and (C), respectively, by introducing possible base-pairing in loop regions in addition. In this way the structural similarity between the S-part of the human 7SL (SRP) RNA in (D) and the large bifurcated structure of tomato SRP RNA shown in (A) becomes more evident. Tomato SRP RNA cannot form an alternative structural configuration similar to that of the human 7SL (SRP) RNA shown in (E). The Alu- and S-part and the two functional domains of human 7SL (SRP) RNA are indicated. The formation of the structural alternatives of human 7SL (SRP) RNA is based on the possible base-pairing of region a with region b or c which are marked by heavy lines.

cleavage in the tomato SRP RNA was observed predominantly at locations next to the 3'-terminal nucleotides of base-paired regions which is also in accordance with our computed secondary structure model (Figure 8).

The secondary structure of tomato SRP RNA was also analysed according to Gordon and Symons (1983) by total

Table I. Comparison between the structural domains of human and tomato SRP (7SL) RNA on the basis of their nucleotide positions

Structural domains	Nucleotide positions in SRP (7SL) RNA from	
	Human	Tomato
Small bifurcated domain	1 – 47	1 – 47
Rod-like core	48 – 127	45 – 123
	223 – 298	218 – 301
Large bifurcated domain	134 – 163	125 – 164
	177 – 220	172 – 216

digestion with RNase T1. The resulting fragments were 5'-end-labelled at 20°C with the aid of T4 PNK using [γ - 32 P]ATP and separated on a thin 12% PAA gel under non-denaturing conditions (Figure 7A). Individual RNA fragments and complexes of such fragments representing predominant bands on the autoradiogram were separately eluted from nine gel slices. The RNAs were treated with formamide to dissociate base-pairing and then electrophoresed on a denaturing 20% PAA sequencing gel to separate the resulting single-stranded fragments (Figure 7B and C). Twenty prominent fragments were eluted and the sequence of eleven of them could be determined according to the method of Donis-Keller (1977).

From these sequence data (Figure 7D) it becomes evident that most of the cleavage sites of RNase T1 are located in those loop regions for which they had been expected from the computed secondary structure model of tomato SRP RNA (Figure 8). Cleavage was also found on the 3'-side of certain guanosine nucleotides which are located in base-paired regions of the model. These unexpected cleavage sites resulted most probably from a preceding cut which caused such a rearrangement of the RNA, that the corresponding guanosine nucleotides were exposed to the enzyme.

The sequence analysis of the RNA fragments obtained by this method allows one to determine all those base-paired regions which are present in the native thermodynamic configuration of tomato SRP RNA which due to their stability, are not disrupted during the enzymatic reactions and by the electrophoretic separation under native conditions. It was found that from the eleven sequenced RNA fragments only two fragments of complementary sequence (3a and 3c) co-migrated during separation (band 3 in Figure 7A) indicating that they might have existed in a complex. The high thermodynamic stability of this complex strongly suggests that its base-pairing scheme may exist also in the native structure of tomato SRP RNA which agrees with our model (Figure 8). The absence of additional complexed fragments could be based on their insufficient thermodynamic stability. It is conceivable that some of these less stable complexes might have still existed after RNase T1 digestion and that they could have been disrupted by phenol extraction, by drying the ethanol precipitated RNA pellet (Svaren *et al.*, 1987) or by the incubation of the fragments at 20°C as required for their 5'-end-labelling.

In conclusion, the combined results of all these enzymatic experiments show that modelling of the secondary structure of tomato SRP RNA according to the structure of the human SRP (7SL) RNA is justified and that, in all probability, the model reflects the actual structure of the plant SRP RNA in solution.

Fig. 6. Determination of the secondary structure of tomato SRP RNA in solution with the aid of nucleases. Autoradiogram of the fragment pattern obtained after electrophoretic separation on 8.4% sequencing gels of partially digested 5'- (A and B) and 3'-end-labelled RNA (C). Lanes in (A) and (B): V1: limited digestion with 0.1 u (a) and 0.02 u (b) RNase V1; T1: limited digestion with 0.05 u (a) and 0.01 u (b) RNase T1; T1: G-specific sequencing reaction with 0.5 u RNase T1; OH: partial alkaline hydrolysis; S1: limited digestion with 0.005 (a), 0.05 (b) and 0.5 u (c) nuclease S1, respectively. Lanes in (C): S1: limited digestion with 0.005 (a), 0.05 (b) and 0.5 u (c) nuclease S1; T1: limited digestion with 0.01 u (a) and 0.005 u (b) RNase T1; T1: G-specific sequencing reaction with 0.5 u RNase T1; OH: partial alkaline hydrolysis; V1: limited digestion with 0.005 u (a), 0.02 u (b) and 0.1 u (c) RNase V1, respectively. The position of certain nucleotides in the sequence of tomato SRP RNA is indicated.

Sequence similarity between SRP RNA and 5S rRNA of tomato

An extensive sequence similarity (16 out of 20 nt being identical) has been reported to exist between nt 236–255 of the human 7SL (SRP) RNA and nt 84–103 of the human ribosomal 5S RNA (Zwieb, 1985). In addition, a stretch of six nucleotides (CGGGUG) was found to occur in 7SL (SRP) RNA (nt 104–109) and also in 5S RNA (nt 105–110). More recently Boehm (1987) has reported that an even more extended sequence similarity exists between a stretch of at least 40 nt in the human 7SL (SRP) RNA (nt 222–263) and 5S rRNA (nt 65–111). Based on these data he has proposed a new secondary structure model for parts of the 7SL (SRP) RNA which consists of two stem-and-loop structures, one of which exhibits a striking structural similarity to the ribosomal protein 15 binding site of the eucaryotic 5S rRNA.

When we compared our tomato SRP RNA sequence and the sequence of the 5S rRNA of tomato as published by Vanderberghe *et al.* (1984) various stretches of similar sequence motifs comprising up to 10 nt were found but they are outside the proposed functionally important regions (data

not shown). Moreover, in the tomato SRP RNA a branched structure according to the model of Boehm (1987) could be constructed but it exhibits a much lower thermodynamic stability as compared to the human 7SL (SRP) RNA. Therefore, no structural preconditions exist that tomato SRP RNA has a binding site for the ribosomal protein L5 as one could assume for tomato 5S rRNA.

Sequence complementarity between tomato SRP RNA and viroids

In search of a possible relationship between tomato SRP RNA and the RNA of potato spindle tuber viroid (PSTV), we found that a significant sequence complementarity exists in the region between nt 31 and 72 at the 5'-terminal part of tomato SRP RNA and nt 277–309 of PSTV (Figure 9). Provided that both RNAs interact with each other, one can assume that in the putative complex between SRP RNA and PSTV a rather stable ($\Delta G = 12.2$ kcal/mol) intramolecular stem-and-loop structure between nt 314 and 351 of the viroid molecule is formed. This configuration will allow the additional pairing of 4 nt of PSTV (position 353–356) with

Fig. 7. Secondary structure determination of tomato SRP RNA with the aid of RNase T1. (A) Autoradiogram of RNase T1-generated RNA fragments separated on a 12% PAA gel under non-denaturing conditions. RNase T1 digestion and 5'-labelling were carried out as explained in Materials and methods. (B and C) Autoradiogram of the electrophoretic separation on a 20% 'sequencing' gel, i.e. under denaturing conditions of the individually eluted fragments or complexes of fragments which caused the bands 1–9, respectively, in (A) and (D). Results of the sequence analysis of various, gel-purified RNA fragments, which are indicated in (B) and (C). Sequencing was performed according to the method of Donis-Keller (1977). Position numbers are given to the first and the last nucleotide of the fragments and refer to the nucleotide numbering of the tomato SRP RNA. Fragments marked by an asterisk carried mutation(s) which indicates the existence of more than one SRP RNA species.

4 nt of the SRP RNA (position 26–29) so that the PSTV-complementary stretch comprises altogether nt 26–72. Neglecting the energy parameter essential for intermolecular helix-initiation, and the free enthalpy values for intramolecular distortions of distinct base-paired regions and possible rearrangements in both RNA species, the free enthalpy value for the putative base-pairing between tomato SRP RNA and PSTV depicted in Figure 9C is -27.4 kcal/mol. Similar base-pairing schemes with free enthalpy values up to -36.6 kcal/mol can be constructed between tomato SRP RNA and citrus exocortis viroid (CEV), chrysanthemum stunt viroid (CSV), tomato apical stunt viroid (TASV) and tomato planta macho viroid (TPMV) which also replicate and incite disease in tomato like PSTV.

Discussion

Comparison of SRP RNA structures

The sequence of the 7SL (SRP) RNA from mammals, amphibia and insects has been highly conserved through evolution across the entire length of its molecule (Erikson *et al.*, 1973; Reddy *et al.*, 1981; Ulla and Tschudi, 1984) which indicates that it is derived from a common ancestor. Our finding that the sequence similarity between these RNAs and the tomato SRP RNA ranges only from 31–56% can be related to the fact that plants constitute a separate large branch in evolution. Therefore it is not surprising that the

primary structure of their SRP RNA diverged from that of the SRP RNAs from all non-plant organisms.

The strict conservation of the primary structure of all previously sequenced SRP RNAs has also led to very similar secondary structure models (Gundelfinger *et al.*, 1984; Zwieb, 1985; Boehm, 1987) in which three structural domains can be distinguished (Figure 5B). Our secondary structure analysis demonstrates that despite the gross differences in sequence the tomato SRP RNA nevertheless exhibits a very similar overall shape (Figure 5) in which the borders of these three structural domains can be defined by virtually the same nucleotide positions along its chain (Table I). Moreover, the size of and the relative distances between certain loops are practically identical which indicates that these loops might be of functional relevance.

The 'Alu-part' and the 'S-part' of the 7SL (SRP) RNA

In the secondary structure model of the human 7SL (SRP) RNA two segments denoted the 'Alu-part' and the 'S-part', are distinguished (Figure 5B). The Alu-part comprises approximately the first 100 nt at the 5'-terminus and the last 50 nt at its 3'-terminus (Ullu *et al.*, 1982), and shows >80% sequence similarity to the human Alu-like sequences (Ullu, 1982) which represent the most abundant family of interspersed repetitive sequences in primates and rodents (Schmid and Jelinek, 1982). Nothing is known about the function of the Alu-like elements which contain many features of

Fig. 8. Secondary structure model of tomato SRP RNA as derived from computer-aided modelling and verified by nuclease digestion under non-denaturing conditions. The cleavage sites obtained by partial digestion of 5'- and 3'-end-labelled RNA with nuclease S1, RNase T1 and RNase V1, respectively, are indicated by symbols the meaning of which is explained in *inset a*. Similarly, the 5'- and 3'-end of fragments generated by total RNase T1 digestion of unlabelled RNA are marked by the symbols as explained in *inset b*. These fragments were 5'-end-labelled for their further analysis. The RNase T1 generated fragments are denoted as in Figure 7D. In case these fragments could be attributed to complexes which were obtained by PAGE under non-denaturing conditions (Figure 7B), their denotation is underlined. To facilitate the allocation of the cleavages sites on the SRP RNA molecule some of its nucleotide positions are encircled.

processed genes (Sharp, 1983), suggesting that they were initially generated by reverse transcription of processed RNA transcripts. Evolutionary studies have led to the concept that the parent RNA for the Alu-like elements is the highly conserved 7SL (SRP) RNA with its Alu-like sequences (Walter and Blobel, 1982; Ullu and Tschudi, 1984; Ullu *et al.*, 1982).

Thus, the question arises whether a similar relationship exists between the Alu-like portions of the tomato SRP RNA and putative Alu-like interspersed, repetitive sequences in the tomato genome. In our own preliminary hybridization studies (unpublished) we have failed, so far, to demonstrate the presence of such abundant tomato SRP RNA-related elements in tomato DNA. However, additional and more detailed work is needed before a final conclusion on this matter can be drawn.

The 'S-part' of the human 7SL (SRP) RNA (Figure 5B) contains a unique sequence of 149 nt (position 107–255)

Fig. 9. Possible base-pairing between tomato SRP RNA and potato spindle tuber viroid (PSTV). (A) Location of the central conserved (CC) region of PSTV and its virulence-modulating (VM) region which is formed by the region of mutation (RM) and the region of premelting (PM) loop 1. The structure model represents the type-strain PSTV-OI which induces disease symptoms of intermediate severity in tomato plants of the cultivar Rentita and Rutgers (Schnölzer *et al.*, 1985). The heavy black bars indicate those nucleotides which are assumed to base-pair with the corresponding nucleotides of tomato SRP RNA. The black line between the bars indicates the nucleotides which form the intramolecular stem-and-loop structure in the putative complex between both RNAs shown in (C). (B) Secondary structure model of the region of tomato SRP RNA which can potentially interact with PSTV RNA. The nucleotides that are assumed to base-pair are indicated by filled-in circles. (C) Possible base-pairing scheme between PSTV-DI and tomato SRP RNA. The intramolecular stem-and-loop structure which arises in the PSTV RNA in this putative complex is boxed-in. The position number of relevant nucleotides is indicated.

which represent the intrinsic part of this molecule (Ullu *et al.*, 1982; Li *et al.*, 1982; Gundelfinger *et al.*, 1984). Extensive sequence similarities between larger parts of the human 7SL (SRP) RNAs and the corresponding ribosomal 5S RNAs have been detected and it has been assumed that a large portion of the S-part of the 7SL (SRP) RNA may have evolved from 5S ribosomal RNA genes. This assumption gains additional support from the finding that the mammalian 7SL (SRP) RNA genes (Ullu and Weiner, 1984, 1985) and the 5S ribosomal RNA genes (Ciliberto *et al.*, 1983) are transcribed by one and the same polymerase, namely DNA dependent RNA polymerase III. However, when we compared the sequence of the SRP RNA and the 5S rRNA from tomato (Vanderberghe *et al.* (1984) no significant similarity between these two plant RNAs could be detected. This indicates that the S-part of the SRP RNA

from higher plants has either not evolved from 5S rRNA genes or that it has completely diverged from them by extensive mutational events.

Comparison of structural configurations of 7SL (SRP) RNAs

It has been proposed that the S-part of the human 7SL (SRP) RNA can exist in two alternative types of bifurcations (Gundelfinger *et al.*, 1984; Zwieb, 1985; Zwieb and Ullu, 1986). In one of these configurations (Figure 5B), a stretch of 6 nt between positions 122 and 127 (region a) base-pairs with the nucleotides between position 223 and 228 (region b), whereas in the other configuration (Figure 5C), the 4 nt of region a (positions 124–127) base-pair with nt 167–170 (region c). These two possible configurations are characterized in particular by a large bifurcated loop and by a large internal loop within the stem-and-loop structure of the so-called 'loop 200' which contains nucleotide 200 in its hairpin. At first sight these two possible configurations of human 7SL (SRP) RNA seem to differ in the size and number of certain loop regions from the ones we propose for tomato SRP RNA (Figure 5A). If, however, the potentially possible base-pairing is introduced into these large loop regions of the two structural alternatives of human SRP (7SL) RNA (Figure 5D and E), one of these configurations (Figure 5D) becomes very similar to the one of the tomato SRP RNA (Figure 5A) which is the only thermodynamically stable configuration that can be constructed from its sequence.

7SL (SRP) RNA structure and SRP functions

The functions of the mammalian SRP, of the six SRP proteins and of the 7SL (SRP) RNA moiety are well established (see reviews by Walter *et al.*, 1984; Siegel and Walter, 1988a,b). The 7SL (SRP) RNA is required for the assembly and structural integrity of the SRP (Walter and Blobel, 1982) in that the RNA serves as a structural lattice, holding the six SRP protein components with their distinct activities in a specific geometry (Andrews *et al.*, 1987; Siegel and Walter, 1988a). The nascent protein chain elongation arrest-activity of the SRP has been mapped to the Alu-part (Walter *et al.*, 1981; Lipp *et al.*, 1987), whereas the signal recognition activity and the protein translocation activity (Scoulica *et al.*, 1987; Siegel and Walter, 1988a) have been assigned to the S-part of the RNA (Gundelfinger *et al.*, 1984; Siegel and Walter, 1986). These functional regions are therefore called 'elongation arrest domain' and 'signal recognition domain', respectively (Figure 5B). The striking structural similarity between the RNA of the human and of the plant SRP implies that this structure–function relationship is most probably identical in both of them.

Structural differences between the mammalian and the plant SRP (7SL) RNA

In contrast to the comprehensive functional model put forward by Siegel and Walter (1986) Zwieb (1985) and Boehm (1987) have proposed different molecular mechanisms for the elongation arrest of protein synthesis *in vitro*. The latter two models are mainly based on similarities between the sequence and the structure of the human 7SL (SRP) RNA and the human 5S rRNA. In our analysis we could neither detect any significant sequence similarity between the presumed functionally relevant regions of the tomato SRP RNA and the tomato 5S rRNA nor could

we find that the large bifurcated structure of the plant SRP RNA is able to undergo the conformational changes of the human 7SL (SRP) RNA which are required in the model of Zwieb (1985). Finally, computer modelling had shown that tomato SRP RNA cannot form in its central core a stem-and-loop structure which is similar to the corresponding part of tomato 5S rRNA as proposed by Boehm (1987) for the human 7SL (SRP) RNA and human 5S rRNA. Therefore, tomato SRP RNA exhibits no significant signs of a putative binding site for the ribosomal L5 protein like in the human system.

It must be stressed that the elongation arrest function of the SRP is still a matter of controversy. It is neither operating in homologous *in vitro* systems constituted from components of plant (wheat germ and maize endosperm) origin (Prehn *et al.*, 1987; Campos *et al.*, 1988) nor in those from reticulocytes or from HeLa cells (Meyer, 1985; Prehn *et al.*, 1987; Campos *et al.*, 1988) that an elongation arrest of protein synthesis operates *in vivo*. Therefore, elongation arrest is assumed to be an experimental artefact of heterologous *in vitro* systems prepared from distantly related organisms. If so, the model of Andrews *et al.* (1987) and of Siegel and Walter (1988a) would only be applicable for the elongation arrest observed in specific heterologous *in vitro* systems.

Possible interaction between tomato SRP RNA and viroids

The observed sequence complementarity between the 5'-terminus of tomato SRP RNA and the 'lower strand' of the rod-like RNA secondary structure model of PSTV allows the formation of ~30 bp (Figure 9). Interestingly, this region comprises the lower strand of the so-called virulence-modulating (VM) region of PSTV (Schnölzer *et al.*, 1985) from where it extends towards the right hand side of the viroid molecule into its central conserved (CC) region. A similar complex can be constructed for four other viroid 'species' (CEV, CSV, TASV and TPMV) which also replicate and incite disease in tomato plants. Thus, we found an extensive, thermodynamically stable and hence potentially possible base-pairing between five viroid 'species' and a functionally important nucleic acid of a host plant in which these viroids cause pronounced disease symptoms. These features render the tomato SRP RNA a possible primary cellular target with which viroid molecules could interact and thus incite disease.

Regarding the still unresolved mechanism of viroid pathogenicity, one has to keep in mind that viroids are not translated into peptides or proteins (reviewed in Semancik, 1987; Diener, 1987). Consequently these pathogenic RNA molecules can only initiate disease via their direct interaction with a specific yet unknown cellular component of its host plant. Various lines of evidence strongly suggest that the severity of the viroid-induced symptoms of disease is modulated by the so-called 'virulence-modulating (VM) region' (Schnölzer *et al.*, 1985) or 'P domain' (Forster and Symons, 1985; Visvader and Symons, 1985, 1986) of PSTV and CEV, respectively. These regions are located at the left hand part of the rod-shaped secondary structure model of these viroids, as shown for PSTV in Figure 9A. It is exactly this region where the base-pairing between the viroid molecules and the tomato SRP RNA can take place (Figure 9A and C).

Possible mechanisms of SRP RNA-related viroid pathogenesis

On the basis of our findings the following molecular mechanism of viroid pathogenesis can now be envisaged: the viroid molecule interacts with the tomato SRP RNA by base-pairing, and thus disturbs the normal function of this host RNA. As a consequence, SRP assembly and the intracellular SRP-mediated translocation and integration of membrane proteins into the endoplasmic reticulum of the differentiating tissue at the growing apex of the viroid-infected plant is impaired. This results in an abnormal formation of cellular membranes in meristematic cells and initiates the development of disease symptoms like leaf malformation and retardation of growth. Electron microscopical studies have, indeed, revealed that in viroid-infected plant tissue the plasma membranes and membrane-associated plasmalemmasomes are malformed, and that cell walls are extremely distorted (e.g. Semancik and Vanderwoude, 1976; Wahn *et al.*, 1980a,b; Hari, 1980; Momma and Takahashi, 1983; Rosenberg *et al.*, 1985). It has also been reported that the sugar and amino acid composition of isolated cell walls from viroid-infected leaf tissue is markedly altered as compared with the one of non-infected tissue (Wang *et al.*, 1986).

An alternative mechanism of viroid pathogenicity can be postulated, if it turns out that the genomic DNA of higher plants like the mammalian DNA contains highly repetitive interspersed Alu-like sequences which are related to segments of the plant SRP RNA. Although the biological function of such Alu-like repeats is unknown, it has been shown in mammalian cells that transcripts of Alu-like DNA are represented in heterologous nuclear RNAs, cytoplasmic mRNAs and small nuclear RNAs (Calabretta *et al.*, 1981; Schmid and Jelinek, 1982). It is possible that some of these sequences can be folded into secondary structures closely resembling the Alu-segment of the SRP RNA. In analogy to their role in the SRP, such Alu-like transcripts could play some regulatory role in RNA transcription and protein translation. Provided that this is also the case in viroid-susceptible plants, one could propose that those SRP RNA-related transcripts might serve as the cellular targets with which viroid molecules interact. If so, the processing of these transcripts, their transport to the cytoplasm and their translation into proteins could be impaired which would disturb the normal regulation of gene expression and result in the development of disease symptoms.

As an even more remote possibility it is also conceivable that viroids might incite disease in that their RNA or its complementary intermediates of replication interact with SRP proteins. However, this possibility cannot be evaluated at present because the site and the structural requirements for the binding of the SRP proteins to the plant SRP RNA are still unknown. Since two proteins (p9 and p14) are associated with the Alu-part of the human 7SL (SRP) RNA (Siegel and Walter, 1986) and since the corresponding domain of the SRP RNA contains the viroid-complementary region, it could be surmised that a complex consisting of the two corresponding tomato SRP proteins might support in viroid binding.

The proposed mode of interaction between the viroid RNA and the cellular target has to take into account the fact that the mature PSTV molecules accumulate in the nucleolus (Schumacher *et al.*, 1983). Therefore, one could envisage

that this interaction becomes effective at the stage of cell division where the nucleolus and the nuclear membranes are disintegrated so that the mature viroid molecules can be transiently released into the cytoplasm. It is likewise conceivable that it is actually the nascent viroid RNA which initiates disease and not its mature and highly base-paired form. In any case, the interaction of viroid molecules with the putative SRP-related cellular target could primarily interfere with protein translocation during cell division and cell differentiation. This would explain why plant tissue that is fully differentiated upon viroid infection does not exhibit disease symptoms. These only develop in newly growing parts of the plant which differentiate after the viroid infection has invaded the meristematic tissue. These observations support the concept that viroid disease symptoms are evidently induced during differentiation.

Materials and methods

The methods used in this study are only described briefly but a detailed protocol for all of them will be supplied upon request. Most of the methods taken from literature and cited in this report were slightly modified. Enzymatic RNA sequencing according to the method of Donis-Keller (1977) was carried out using the corresponding sequencing kit of Pharmacia/PL Biochemicals.

Isolation of tomato SRP RNA

Total nucleic acids were isolated from leaf tissue of the tomato (*Lycopersicon esculentum* Mill.) cultivar 'Rentita' by direct phenol extraction and further fractionated with 2 M NaCl as previously described (Sänger and Ramm, 1975). The resulting 2 M NaCl-soluble RNA was then subjected to liquid-liquid partition chromatography (Müller, 1985) which allows the isolation of the SRP RNA in a highly purified form. It was also possible, to purify tomato SRP RNA from the 2 M NaCl-soluble RNA fraction by PAGE (Sänger *et al.*, 1976) or by ion-exchange HPLC (Colpan *et al.*, 1983) after Cs₂SO₄ density gradient centrifugation of the phenolized total nucleic acid extract of tomato leaf tissue.

Sequencing of the 5'-terminal region

The 5'-terminus of tomato SRP RNA was radioactively labelled using [γ -³²P]ATP with T4 PNK after terminal phosphate groups had been removed by incubation with CAPase (Gross *et al.*, 1977). The reaction mixture was phenolized, ethanol precipitated, redissolved in formamide containing xylene-cyanole FF (XC) and bromophenol blue (BPB) as dye marker, heated for 1 min at 80°C and then subjected to electrophoresis on an 8.4% PAA sequencing gel (0.3 × 200 × 400 mm) until the XC marker had reached the bottom of the gel. The position of labelled SRP RNA was determined by autoradiography and the corresponding portion of the gel was cut out. The RNA was extracted from the gel piece by incubation overnight at 37°C and in the presence of 0.1% SDS and 0.5 M ammonium acetate, pH 5. To remove gel fines, the supernatant was passed through a sterile 0.45 µm pore size filter. The RNA was recovered from this solution by EtOH precipitation and finally dissolved in water.

To determine the 5'-terminal nucleotide the 5'-labelled SRP RNA was fully digested with nuclease P1 and the four unlabelled 5'-monophosphates pA, pC, pG and pU were added to the incubation mixture prior to its chromatographic analysis on PEI-cellulose thin layer plates which were developed in two different ways, namely with 1 M LiCl as solvent (Randerath and Randerath, 1967) and with the formic acid/lithium formate solvent system (Volckaert and Fiers, 1977).

For sequencing its 5'-terminal region, the SRP RNA was partially digested with nuclease P1 (Silberklang *et al.*, 1977) and the reaction mixture was then subjected to mobility shift analysis (Sanger *et al.*, 1965; Brownlee and Sanger, 1965; Frank and Blöcker, 1982).

Sequencing of the 3'-terminal region

The 3'-terminus of the SRP RNA was labelled with [5'-³²P]pCp using T4 RNA ligase (England *et al.*, 1980). In order to obtain only its 3'-terminal region with the [5'-³²P]pCp-labelled endgroup, the SRP RNA was digested with RNase T1 and the resulting fragments were separated on a 20% PAA sequencing gel. The 3'-labelled fragments were isolated as described in the previous section and sequenced according to the method of Donis-Keller

(1977). In case of mobility shift analysis, terminal phosphate groups were removed with CAPase from the labelled SRP RNA and then the RNA purified by PAGE. The RNA was cleaved with RNase T1 and partially digested with nuclease P1.

Sequencing of tomato SRP cDNA

Tomato SRP cDNA was synthesized using reverse transcriptase from avian myeloblastosis virus and the chemically solid-phase synthesized DNA primer 5'd(AAAGGTGGTTGTTT)3' which is complementary to the sequenced 3'-terminal region of two SRP RNA fragments obtained by RNase T1, cleavage. After alkaline degradation of the RNA template, the cDNA transcripts were separated on an 8.4% PAA sequencing gel. Transcripts of appropriate length were isolated from the gel and sequenced chemically (Maxam and Gilbert, 1980).

Determination of secondary structure with specific nucleases

Tomato SRP RNA, radioactively labelled either at its 5'- or 3'-end and purified on PAA sequencing gels, was partially digested under non-denaturing conditions with varying concentrations of RNase T1 (Gordon and Symons, 1983), nuclease S1 (*ibid.*), and RNase V1 (Lockard and Kumar, 1981), respectively. The digests were analysed on 8.4 and 20% PAA sequencing gels. In order to determine the location of the nuclease-cleaved bonds in the SRP RNA, RNase T1 partial digests prepared under denaturing conditions and the alkaline partial hydrolysate of the SRP RNA were electrophoresed in reference tracks on the gels.

Analysis of the secondary structure with RNase T1

The enzymatic determination of the secondary structure of tomato SRP RNA in solution using RNase T1 followed essentially the strategy as applied by Gordon and Symons (1983) for the structural analysis of the satellite RNA (Sat-RNA) of cucumber mosaic virus (CMV). Unlabelled SRP RNA (0.2 OD₂₆₀) was incubated at 0°C with 1000 U RNase T1 for 30 min, and the digestion was terminated by phenol extraction. The resulting RNA fragments were ethanol precipitated and redissolved in the buffer used for the radioactive 5'-labelling. Incubation with [γ -³²P]ATP by T4 PNK was carried out at 20°C for 1 h. The radioactively labelled fragments were separated on a 0.38 mm thick 12% PAA gel under non-denaturing conditions, i.e. at 4°C and in the absence of urea. Representative fragments were isolated from gel pieces by salt extraction as already described and further separated on 20% PAA sequencing gels under the usual denaturing conditions. A series of fragments was again isolated from the gel and sequenced according to the method of Donis-Keller (1977).

Computer-aided sequence comparison and secondary structure modelling

Similarities between the sequence of SRP RNA from tomato and published sequences from other organisms were evaluated by the UWGCG alignment programs COMPARE and BESTFIT (Devereux, 1984). In addition, a search for sequence complementarities between tomato SRP RNA and PSTV was performed using the UWGCG program STEMLOOP in combination with the dot-matrix program DOTPLOT. Secondary structure models were determined with the folding algorithm of Jacobson *et al.* (1984) using the free enthalpy values as tabulated by Freier *et al.* (1986). The generated secondary structures were then graphically displayed by the DRAW program developed by Shapiro *et al.* (1984). All these computer programs were run on a MicroVAX A (Digital Equipment Corp.).

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