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# Comparative structural analysis of cytoplasmic and chloroplastic 5S rRNA from spinach*

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ABSTRACT

5S rRNAs from Spinacea oleracea cytoplasmic and chloroplastic ribosomes have been subjected to digestion with the single strand specific nuclease $S_1$ and to chemical modification of cytidines by sodium bisulphite in order to probe the RNA structure. According to these data, cytoplasmic 5S rRNA can be folded as proposed in the general eukaryotic 5S rRNA structure (1) and 5S rRNA from chloroplastides is shown to be more related to the general eubacterial structure (2).

## INTRODUCTION

Ribosomal 5S rRNA, a unique component of the protein synthesizing complex, is ideally suited as a model system for phylogenetic studies by comparative structural analysis. Heterologous reconstitution experiments (3) using 5S rRNAs of eukaryotic, eubacterial and archaebacterial origin for the incorporation into B. stearothermophilus 50S ribosomal subunits and protein binding studies (4,5) gave rise to the idea of at least two functionally distinct classes of 5S rRNA. Woese and Fox (6) introduced a general secondary structure for eukaryotic as well as for eubacterial 5S rRNAs on the basis of comparative sequence analyses. This model is considered to represent the minimal number of base pairs in any 5S rRNA structure. Recently, a general eukaryotic (1) and a general eubacterial (2,7) 5S rRNA model have been proposed. By analyzing the structure of cytoplasmic and chloroplastic 5S RNA from spinach using the single strand specific nuclease $S_1$ we would like to answer the question as to whether the chloroplast species is of eukaryotic character or of eubacterial origin, as proposed by the endosymbiotic hypothesis.

5S rRNA molecules are supposed to be able to undergo confor-

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mational changes. The precise localization as well as the structural characteristics of such conformational changes in RNA have to be determined. Comparison of the experimental results from sodium bisulphite modification of cytidines and mapping of the single stranded regions of spinach chloroplastic 5S rRNA by nuclease $S_1$ provides us with useful information on this aspect.

## MATERIALS AND METHODS

### Reagents

Chemical sequencing reagents were dimethylsulfate from EGA-Chemie, Steinheim, diethylpyrocarbonate and hydrazine from Eastman, Rochester, USA, and aniline from Merck, Darmstadt. All other chemicals were from Merck, Darmstadt. Biochemical reagents were purchased from Boehringer, Mannheim. $T_4$-RNA ligase, $T_4$ polynucleotide kinase and ribonucleases $T_1$, $U_2$, Phy.M and *B. cereus* for sequence determination were from PL Biochemicals Inc., Milwaukee, USA. Acrylamide and N,N'-methylene-bis-acrylamide were from Bio-Rad, USA. Adenosine 5'-[$\gamma$-$^{32}$P] triphosphate (>2 000 Ci/mmol) and Cytidine 3', 5'-[5'-$^{32}$P] bisphosphate (2 000-3 000 Ci/mmol) were from Amersham Buchler, Braunschweig.

### Isolation of spinach 5S rRNA

Spinach (*Spinacea oleracea*) leaves were purchased at the local market. Chloroplasts and chloroplastic ribosomes were obtained by the large scale procedure described in (8). For the preparation of 80S cytoplasmic ribosomes the post chloroplastic supernatant was centrifuged (26 000 x g, 30 min, 4°C) to remove mitochondria, proplastides and chloroplastic fragments. After pelleting ribosomes (120 000 x g, 3 hrs., 4°C) 80S and 70S ribosomes (from fragmental chloroplasts) were separated by zonal centrifugation (Beckman Type 15 rotor, 17 000 r.p.m., 19 hrs., 4°C) in a 10-14% sucrose gradient (in 10mM Tris.HCl, pH 7.8, KCl 50mM, $MgCl_2$ 10mM). 80S ribosomes were then pelleted by centrifugation (120 000 x g, 17 hrs., 4°C).

After extraction of r-proteins with acetic acid (9) 5S rRNA was purified by gel filtration on a Sephadex G100 column as described (10).

### Sequencing of 5S rRNA

5S rRNAs were labeled at the 5'-end using $T_4$ polynucleotide

kinase mediated transfer of $^{32}$P-phosphate from 5'-[$\gamma$-$^{32}$P] ATP as described (11) and the 3'-end by addition of [5'-$^{32}$P] pCp by $T_4$-RNA ligase as published (12). Terminally labeled 5SrRNA was purified by preparative gel electrophoresis on 12% polyacrylamide gels in 7M urea (40 x 60 x 20 x 0.05cm at 1.5kv for 20 hours). Chemical sequencing was carried out as described by Peattie (13) and enzymatic sequencing as described by Donis-Keller et al.(14).

$S_1$ digestion

5'- or 3'-end labeled 5S rRNA was mixed with 5µg of E. coli t-RNA and hydrolyzed with 200 U nuclease $S_1$ in 35µl 0.1M NaCl, 5mM $ZnSO_4$, 30mM NaOAc, 5% glycerin, pH 5.0 at 37°C for 30 minutes. The reaction was stopped by ethanol precipitation of the oligonucleotide fragments, and the fragments were analysed on 12% polyacrylamide gels (60 x 20 x 0.05cm).

Sodium bisulphite modification

3'-$^{32}$P labeled 5S rRNA was incubated in 400µl of 1.5M sodium metabisulphite pH 5.8, 10mM $MgCl_2$, together with 10-20µg t-RNA as carrier at 20°C for 24 hours. Excess reagent was removed by gel filtration on Sephadex G-50 in 20mM Tris-base pH 9.9, 200mM NaCl, 3mM EDTA and the modified rRNA was incubated at pH 9.9 for 12 hours at 25°C followed by ethanol precipitation. Aliquots of the modified 5S rRNA were used as a control and in the uridine specific reaction of Peattie's chemical sequencing procedure (13). The positions of modified cytidines were established by comparing the 'U' lanes of modified and unmodified 5S rRNA on sequencing gels.

## RESULTS

### Nuclease $S_1$ digestion

Nuclease $S_1$ has been shown to be useful in the secondary structure analysis of small RNA molecules (2, 15, 16). The resulting oligonucleotides carry a 5'-terminal phosphate group. In the sequencing analysis, the $S_1$ fragments migrate slower than those fragments obtained by $T_1$ digestion or alkali hydrolysis, lacking the 5'-terminal phosphate group (16). Digestion of 5'- and 3'-end labeled chloroplastic 5S rRNA and of 3'-end labeled cytoplasmic 5S rRNA from spinach with subsequent sequencing gel analysis of the resulting labeled oligonucleotides (Figures 1 and

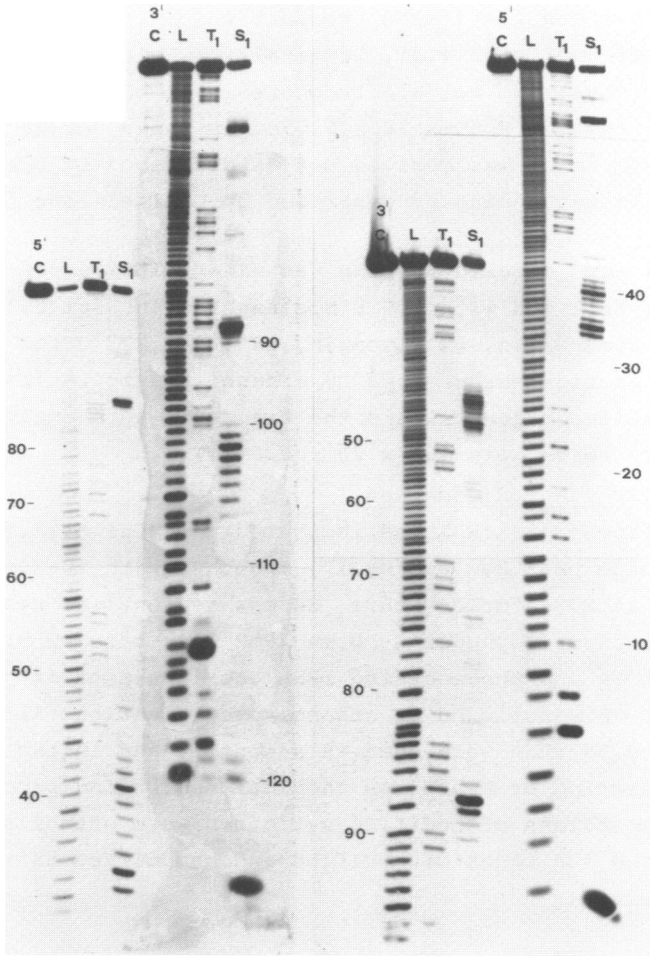


Figure 1

Limited digestion of 3'- and 5'-$^{32}$P labeled 5S rRNA from spinach chloroplastides. Lane C: control, L: alkali ladder, $T_1$: G specific enzymatic sequencing reaction and $S_1$: limited digestion with the single strand specific nuclease $S_1$ as described in Materials and Methods. Electrophoresis was carried out on a 12% polyacrylamide gel (60 x 20 x 0.05cm) under denaturing conditions for six hours at 1.5kv revealing the terminal nucleotides and for 14 hours at 1.2kv followed by six hours at 1.5kv in the other lanes.

2) reveals cleavage sites of varying intensity as indicated in Figures 3 and 4.

Chemical modification of cytidine residues in spinach chloroplastic 5S rRNA

The conversion of easily accessible cytidines to uridines through reaction with sodium bisulphite was used to probe the

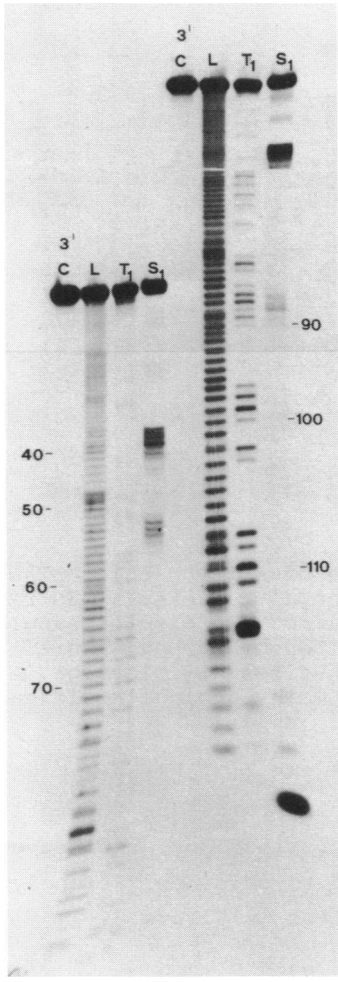


Figure 2

Limited digestion of 3'-end labeled 5S rRNA spinach cytoplasmic ribosomes. The experimental procedure was as described in the legend to Figure 1.

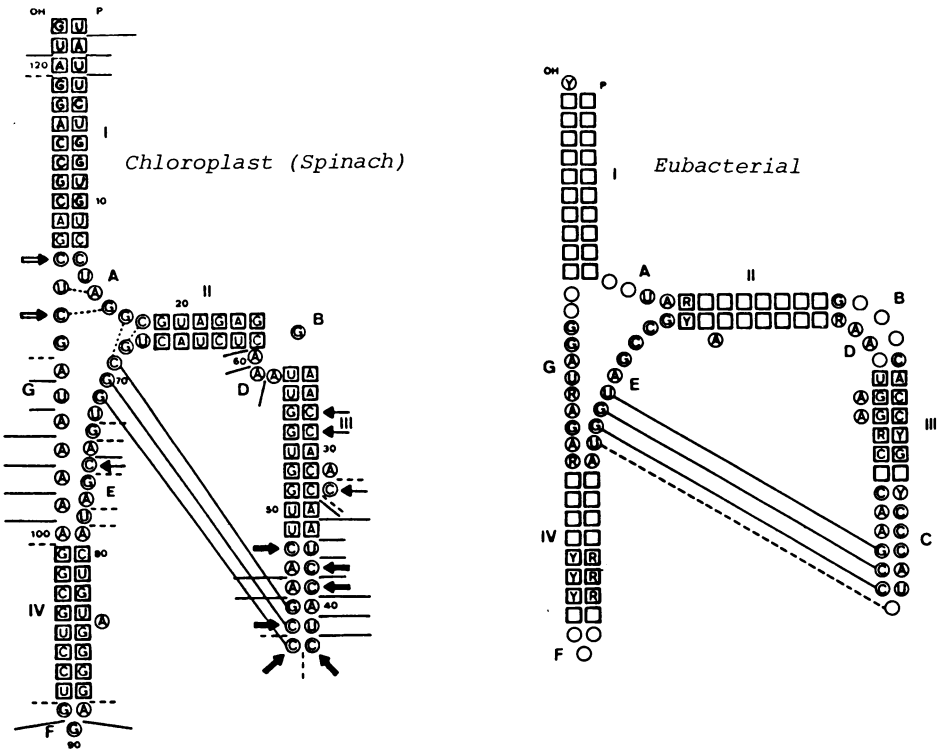


Figure 3

Secondary structure of spinach chloroplastic 5S rRNA [sequence from (20)] and the general eubacterial 5S rRNA structure (2). Boxes indicate base paired and circles single stranded regions. Long lines indicate sites of strong, short lines of medium and broken lines of weak nuclease $S_1$ cleavage. Arrows indicate sites of strong, medium and weak chemical modification.

secondary structure of RNA (17). Figure 5 shows the uridine specific chemical sequencing reactions for native and modified 5S rRNA from spinach chloroplastides, thus revealing the positions of cytidines converted to uridines. Strongly and weakly modified nucleotides are indicated in Figure 3.

## DISCUSSION

Evaluation of the $S_1$ nuclease digestion studies is in support of the secondary structure for cytoplasmic and chloroplastic 5S rRNA from spinach as shown in Figures 3 and 4. Cytoplasmic 5S

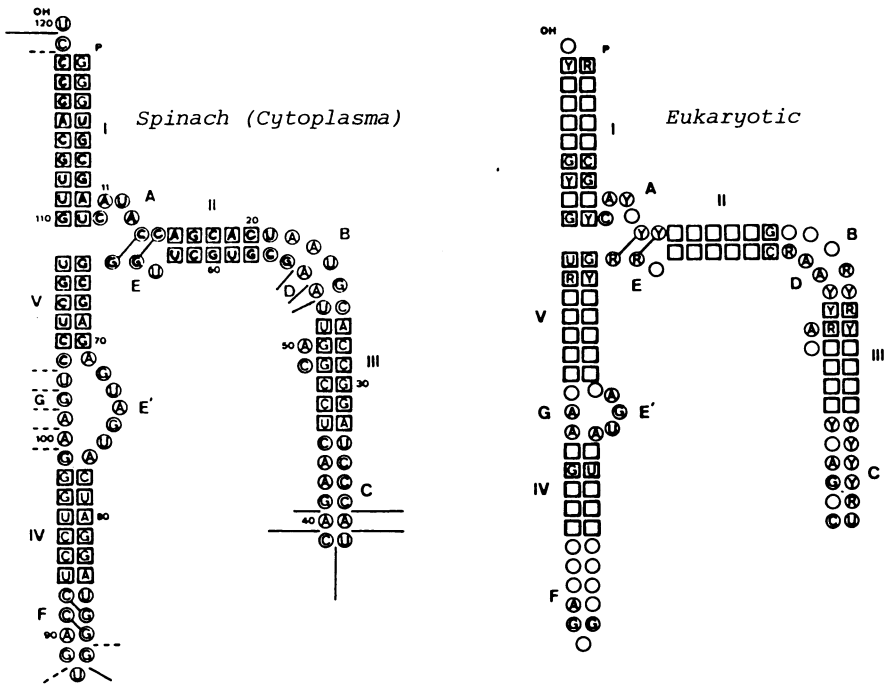


Figure 4

Secondary structure of spinach cytoplasmic 5S rRNA [sequence from (21)] and the general eukaryotic 5S rRNA structure (1). Presentation and experimental results as described in the legend to Figure 3.

rRNA represents the general eukaryotic type (1), whereas the chloroplastic 5S rRNA is more related to the general eubacterial structure (2). The main arguments for this classification of chloroplastic 5S rRNA are as follows:

Cytoplasmic 5S rRNA contains the additional helical region V (Figure 4) of considerable stability and an enlarged loop F. Helix IV might be extended as indicated, taking into account a single uridine bulge loop.

A tertiary interaction involving $CCG_{45}$ and $CGG_{71}$ in chloroplastic 5S rRNA similar to the one proposed for E. coli 5S rRNA (2) and typical for the eubacterial 5S rRNA structure can be proposed on the basis of the $S_1$ cleavage data. There is a typical

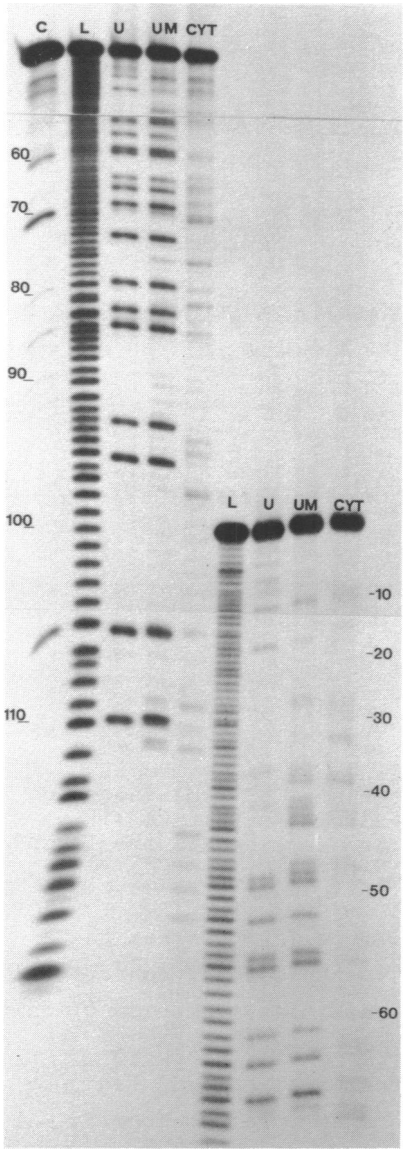


Figure 5

Autoradiograph of the sequencing gel analysis of chemically modified 5S rRNA from spinach chloroplastides. Lane C: control, L: alkali ladder, U: U specific chemical sequencing reaction, UM: U specific chemical sequencing reaction of the 5S rRNA modified with sodium bisulphite and CYT: C specific chemical sequencing reaction. Electrophoresis was carried out on a polyacrylamide gel under denaturing conditions as described in Figure 1.

gap in the $S_1$ cleavage pattern (Figure 1) of spinach chloroplastic 5S rRNA in those positions thought to be involved in this interaction, which cannot be detected for cytoplasmic 5S rRNA (Figure 2).

Suggesting an E. coli 5S rRNA-like tertiary structure (Figure

6) for spinach chloroplastic 5S rRNA, we can conclude that the order of preference for nuclease $S_1$ cleavage is as follows: hairpin loops (regions C and F in Figure 3) > single stranded regions on the surface of the tertiary structure (regions D and G)>single strands located in the inner part of the molecule and possibly involved in tertiary interactions (region E).

Aligning homologous structural elements of chloroplastic 5S rRNA and of the general eubacterial type reveals 75% structural homology, whereas in the same alignment with the general eukaryotic type only 60% homology can be found. The homology between the eubacterial and the eukaryotic type is 46% (Figure 7). A similar relation can be found by aligning homologous structural elements of spinach chloroplastic 5S rRNA with spinach cytoplasmic 5S rRNA (38% homology) and with E. coli 5S rRNA (52% homology) (Figure 8). 5S rRNA from Anacystis nidulans has been shown

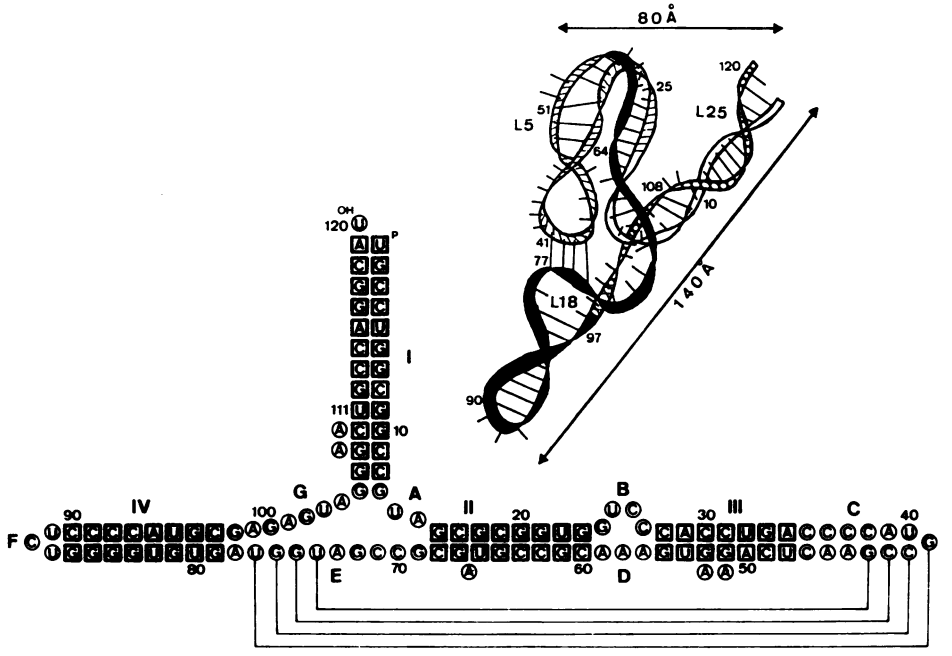


Figure 6

Schematic drawing of a three dimensional molecular model for E. coli 5S rRNA (2). The primary binding regions of the E. coli 5S rRNA binding proteins E-L5, E-L18 and E-L25 (24) are indicated.

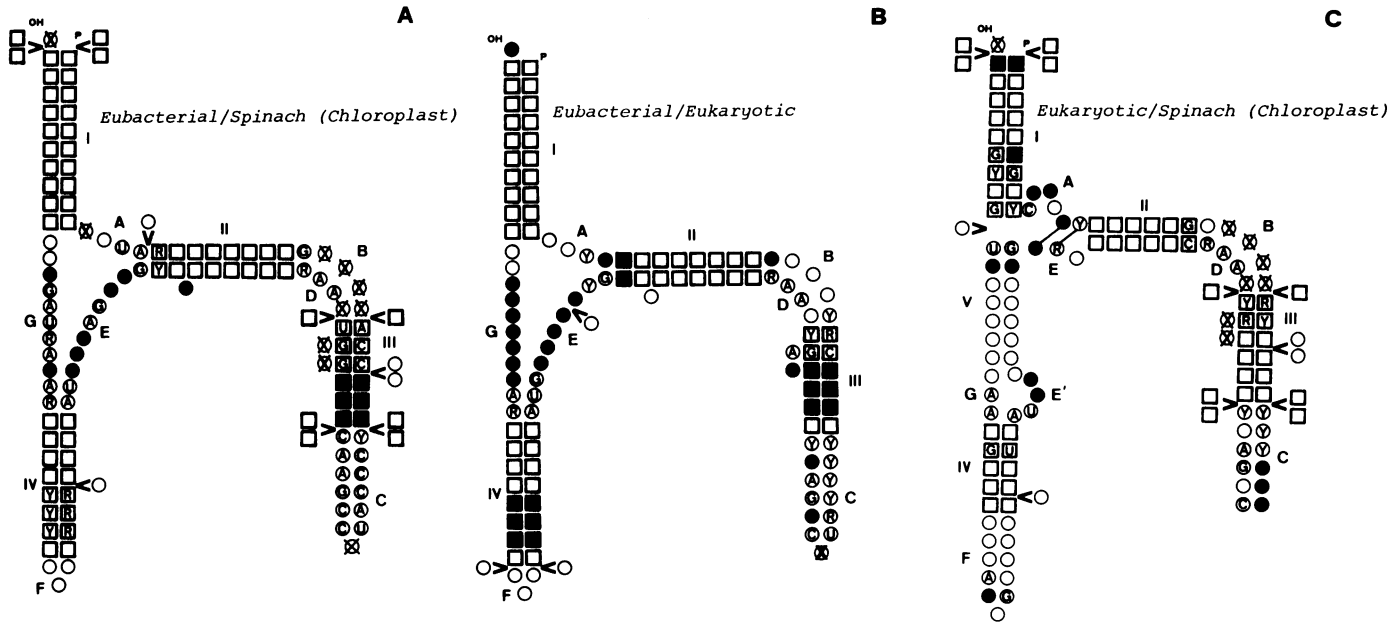


Figure 7 Structural comparison of the general eukaryotic and eubacterial 5S rRNA models (Figures 3 and 4) and spinach chloroplastic 5S rRNA (20).

A: Spinach chloroplastic 5S rRNA superimposed on the general eubacterial 5S rRNA structure. Conserved and semiconserved nucleotides (R = purine and y = pyrimidine) of the general eubacterial 5S rRNA model present at homologous structural positions in spinach chloroplastic 5S rRNA are indicated as well as insertions of single stranded (o>) or base paired residues ( >) and deletions (⊠) in the spinach chloroplastic 5S rRNA sequence. Filled squares and dots represent those conserved nucleotides of the general eubacterial structure not found in spinach chloroplastic 5S rRNA.

B: The general eukaryotic 5S rRNA structure superimposed on the general eubacterial model.

C: The spinach chloroplastic 5S rRNA structure superimposed on the general eukaryotic model.

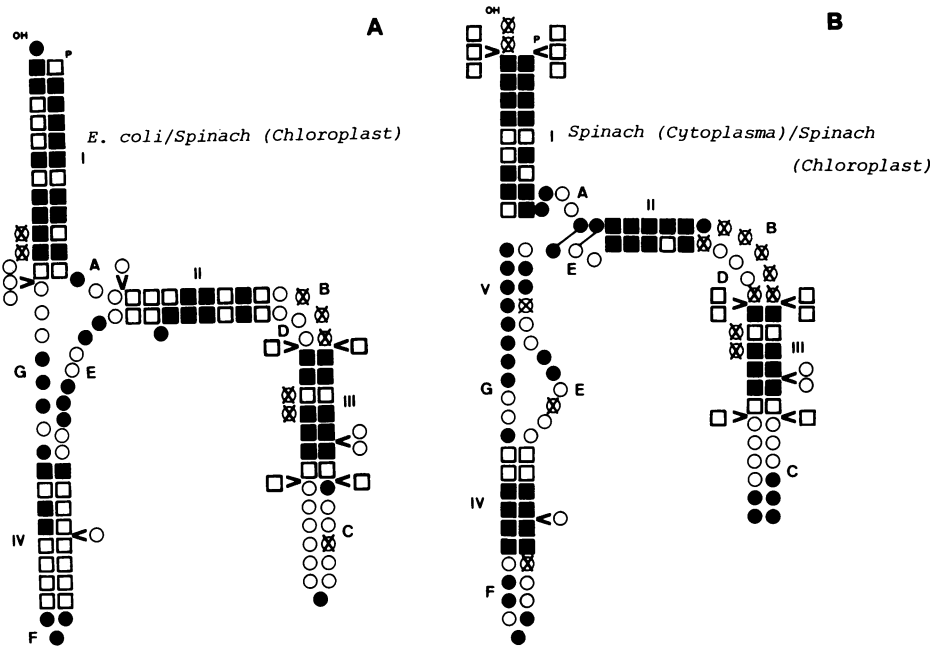


Figure 8

Structural comparison of spinach chloroplastic 5S rRNA (20) with E. coli 5S rRNA (22) and spinach cytoplasmic 5S rRNA (21).
A: Spinach chloroplastic 5S rRNA superimposed on the E. coli 5S rRNA structure. Filled squares and dots represent those E. coli 5S rRNA nucleotides not found in spinach chloroplastic 5S rRNA. Insertions and deletions are indicated as in Figure 7.

B: Spinach chloroplastic 5S rRNA superimposed on the spinach cytoplasmic 5S rRNA.

to be most related to Lemna minor chloroplastic 5S rRNA (18). In our alignment we obtain 63% homology (not shown in Figure 8). Although we have developed strong evidence for an eubacterial classification of chloroplastic 5S rRNA, it should be noted that this structure contains two remarkable elements, which do not belong to any of the two functional 5S rRNA classes.

a) Helix II can be extended pairing $GC_{18}$ with $GC_{69}$ involving a single base bulge loop thought to be important for protein/RNA interaction (19). Sequence comparison of five different chloroplastic 5S rRNAs revealed that this structural feature is not

conserved (Figure 9). Although there is extremely strong homology (95% to 100%), (18), between the individual chloroplastic 5S rRNAs, Dwarf bean has a deletion in the position corresponding to $G_{68}$ in the other chloroplastic 5S rRNAs. Led by this observation in combination with the $S_1$ cleavage data we conclude that $CGG_{71}$ is involved in the proposed tertiary interaction rather than in extension of helix II.

b) Helix III contains two palindromic sequences (AACCA C $ACCAA_{36}$ and UUGG U $GGUU_{57}$). Judging from the $S_1$ cleavage data this region might also be folded in an alternative, 5S rRNA untypical fashion, as shown in Figure 10. De Wachter et al. (23) have proposed a dynamic equilibrium involving a migration of the bulge in helix III for Lemna minor chloroplastic 5S rRNA.

Chemical modification of cytidines preferentially hits those nucleotides located in single stranded regions, and, due to the long reaction times (24 hours at 20°C), cytidines in weakly base paired regions as a result of sequence dependent conformational

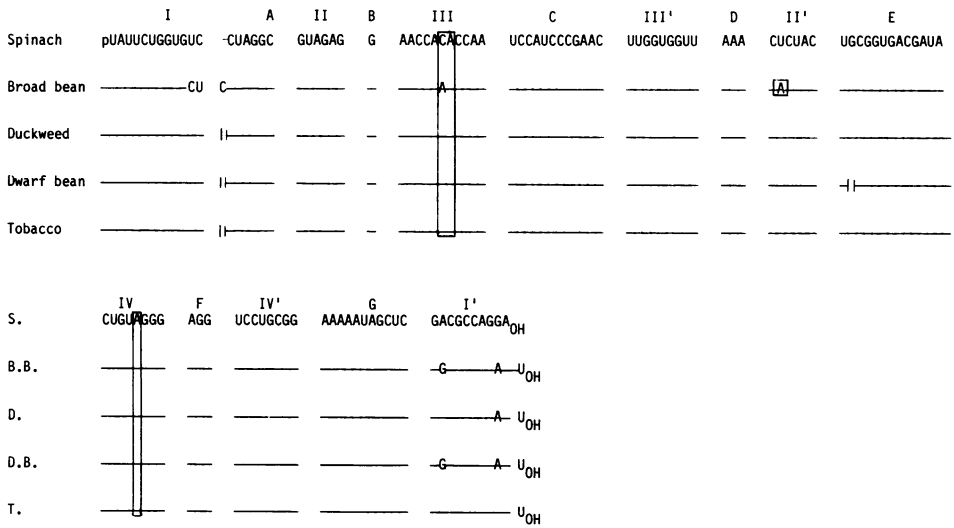


Figure 9

Sequences comparison of homologous structural elements of various chloroplastic 5S rRNAs. Only those positions differing from spinach chloroplastic 5S rRNA are indicated. All sequences from (22).

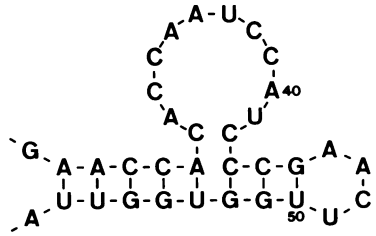


Figure 10

Alternative base pairing for spinach chloroplastic 5S rRNA palindromic sequences, as discussed in the text.

changes. Cytidines accessible for chemical modification and nuclease $S_1$ cleavage and thus located in single stranded regions are $C_{33}$, $C_{38}$, $C_{39}$, $C_{42}$, $C_{48}$ and $C_{78}$. Those nucleotides exclusively accessible for chemical modification and thus located in weakly base paired regions possibly undergoing conformational changes, or in parts of the molecule which are inaccessible for the comparably large enzyme due to sterical hindrance, are $C_{28}$, $C_{29}$, $C_{43}$, $C_{44}$, $C_{108}$ and $C_{110}$ (Figure 5). It is of particular interest to point out that those cytidines thought to be involved in the tertiary interaction are inaccessible for nucleolytic, enzymatic cleavage but strongly hit by sodium bisulphite.

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*On the occasion of his 60th birthday, we would like to dedicate this paper to Professor Friedrich Cramer

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