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Cotranscription and processing of 23S, 4.5S and 5S rRNA in chloroplasts from *Zea mays*

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ABSTRACT

The termini of rRNA processing intermediates and of mature rRNA species encoded by the 3' terminal region of 23S rDNA, by 4.5S rDNA, by the 5' terminal region of 5S rDNA and by the 23S/4.5S/5S intergenic regions from Zea mays chloroplast DNA were determined by using total RNA isolated from maize chloroplasts and ^{32}P-labelled rDNA restriction fragments of these regions for nuclease S_1 and primer extension mapping. Several processing sites detectable by both 3' and 5' terminally labelled probes could be identified and correlated to the secondary structure for the 23S/4.5S intergenic region. The complete 4.5S/5S intergenic region can be reverse transcribed and a common processing site for maturation of 4.5S and 5S rRNA close to the 3' end of 4.5S rRNA was detected. It is therefore concluded that 23S, 4.5S and 5S rRNA are cotranscribed.

INTRODUCTION

The 50S ribosomal subunit of chloroplasts from higher plants contains in addition to the large 23S rRNA the two small rRNA species termed 4.5S and 5S rRNA (1). Chloroplast 5S rRNAs show sequence homology with bacterial 5S rRNA species; this structural homology is in accordance with positional homology of the 5S rRNA genes in bacterial and chloroplast rRNA operons as depicted in Fig. 1. Sequences of chloroplast 4.5S rRNAs (rDNAs) reveal homology with the 3' end of bacterial 23S rRNA (rDNA) (2,3,4). From this and again from the positional homology of 4.5S rRNA genes (Fig. 1) 4.5S rRNA appears to originate from a 3' terminal fragmentation of the bacterial 23S rRNA gene caused during evolution by a small insertion (78 bp in maize), which encodes the processing signals leading to 4.5S rRNA and to a slightly shorter 23S rRNA (5). In the case of chloroplast 5S rRNA data obtained from competition hybridisation (6) and Northern hybridisation experi-

ments (7) and the existence of promoter-like sequences in the 4.5S/5S intergenic region (8) support the view of the 5S rRNA gene functioning as a separate transcriptional unit.
In order to determine processing sites of the two intergenic regions and to test whether the 5S rRNA gene represents a separate transcription unit we have mapped the termini of *in vivo* synthesized and processed rRNA species which are encoded in the 23S/4.5S and 4.5S/5S intergenic regions. The data presented in this communication indicate that the entire intergenic regions are expressed in the form of precursor rRNA. This is taken as evidence that both the intergenic regions contain processing sites rather than transcriptional terminators and promoters and that 23S, 4.5S and 5S rRNAs are co-transcribed within the primary transcript.

MATERIALS AND METHODS

Materials

Restriction endonucleases, calf intestinal alkaline phosphatase, T4 polynucleotide kinase and Klenow fragment of DNA polymerase I were purchased from Boehringer Mannheim or Bethesda Research Laboratories. Mung bean nuclease obtained from P-L Biochemicals, USA, was used as single strand specific nuclease; AMV-reverse transcriptase and RNase free DNase were obtained from Life Sciences, USA, and Worthington, USA, respectively. Enzyme reactions were carried out as recommended by the suppliers. [γ-^{32}P]-ATP for 5' terminal labelling and [α-^{32}P]-dNTPs for 3' terminal labelling were purchased from Amersham, England.

Preparation of Terminally Labelled rDNA Fragments

The *E.coli* clone pZmc134, which harbors a plasmid in which one complete maize chloroplast rRNA operon is contained (9), was used as a source of rDNA. The respective restriction fragments containing the 23S, 4.5S and/or 5S rRNA coding regions were either 5' terminally labelled with T4 polynucleotide kinase and 70 μCi [γ-^{32}P]-ATP after treatment with calf intestinal phosphatase and phenol extraction or 3' terminally labelled with Klenow fragment of DNA polymerase I and 50 μCi of a suitable [α-^{32}P]-dNTP and one nonradioactive dNTP. Fragments labelled at one single terminus were obtained from fragments labelled on both ends by secondary cleavage reactions or by strand separation.

RNA Preparation

Total chloroplast RNA (tot ctRNA) was isolated as described previously (10). Essentially, leaves were cut from 10 days old maize seedlings (Inrakorn, EWG-Norm, F.R.G.) grown under an 12 h light (26°C)/12 h dark (18°C) regime; after homogenization of leaves and separation from nuclei by filtration through 4 layers of cheesecloth and a double layer of miracloth chloroplasts were pelleted and washed by short-time, low-speed centrifugation. Total chloroplast RNA was prepared by lysis of isolated chloroplasts with phenol/chloroform/SDS and subsequent ethanol precipitation. For primer extension experiments RNA preparations were treated with RNase free DNase in order to exclude potentially contaminating DNA (11). Mature 23S rRNA was prepared by R. Schuhnicht in this laboratory, mainly as described earlier (10).

S_1-Mapping

S_1-mapping (12) was carried out according to Bach et al. (13) with minor modifications. Approximately 1 pMol of the respective DNA fragment labelled at the 5' or 3' end of the coding strand was coprecipitated with 30 µg tot ctRNA (or, in one case, with 30 µg of chloroplast 23S rRNA) and redissolved in 50 µl of 80% deionized formamide containing 0.4 M NaCl, 1 mM EDTA and 40 mM MOPS, pH 6.7. After denaturation at 65°C for 15 min the mixture was incubated at 50°C for 6 h for RNA/DNA annealing. The hybrids were then rapidly chilled by mixing with 450 µl of ice cold mung bean nuclease buffer (30 mM Na-acetate, pH 4.8, 50 mM NaCl, 1 mM $ZnSO_4$ and 5% glycerol); single stranded regions were then digested by addition of 1000 units of mung bean nuclease and incubation for 1 h at 37°C. After subsequent phenol/chloroform extraction and ethanol precipitation the RNA components of the RNA/DNA duplices were hydrolysed with 50 µl of 0.3 M NaOH, 5 mM EDTA at 65°C for 30 min; the resulting mixture was then neutralized with 60 µl of 1 M Tris/HCl (pH 7.5) and precipitated with ethanol without additional salt but in the presence of 5 µg tRNA. The resulting fragments of the coding strand DNA were analysed on a denaturing (8 M urea) PAA gel alongside with the products of sequencing reactions (14) of the same DNA strand.

Primer Extension Mapping

The conditions used were principally as described in (15). After

careful optimisation with a DNA primer complementary to an inner region of 23S rRNA (E. Natt, this laboratory), the following protocol was applied: Approximately 1 pMol of the respective double stranded DNA fragment labelled only at the 5' end of the coding strand was annealed with 30 µg tot ctRNA as described for S_1-mapping, followed by ethanol precipitation and resuspension in 50 µl reverse transcriptase buffer (100 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 145 mM KCl, 20 mM β-mercaptoethanol and 1 mM each of dATP, dGTP, dTTP and dCTP). Elongation of the primer was started by addition of 3 µl reverse transcriptase (16 units/µl). After 3 h at 42°C the reaction was terminated by addition of 2 µl of 0.5 M EDTA, followed by ethanol precipitation and alkaline hydrolysis of RNA as described above. Synthesized cDNAs were analysed on denaturing (8 M urea) PAA gels in parallel with products of sequencing reactions obtained from a DNA fragment of the coding strand, which was labelled at the same terminal position as the primer DNA but which extends on its opposite side beyond the 3' end of the primer.

Nucleotide Sequences

The sequences of 4.5S and 5S rRNA from maize were determined by J. Bedbrook and T. Dyer (1980, unpublished) together with the DNA sequences of the respective genes and the 23S/4.5S/5S intergenic regions. The authors kindly provided us with these sequences, which could be confirmed (except for position 97, in which we find T instead of C; see Figs. 3 and 5) by the various sequence ladders used in this work for alignment of S_1 and primer extension products.

RESULTS AND DISCUSSION

RNA Termini Within the 23S/4.5S Intergenic Region

In separate experiments the 3' terminally labelled coding strand of fragment $AvaII_{637}$ and the 5' terminally labelled coding strand of fragment $AvaII/DdeI_{287}$ (Fig. 1 and Fig. 2a) were hybridized with tot ctRNA, followed by degradation of single stranded regions with mung bean nuclease. In order to identify the 3' terminus of mature 23S rRNA, the 3' terminally labelled coding strand of fragment $AvaII_{637}$ was also annealed with isolated 23S rRNA and again degraded with mung bean nuclease. Frag-

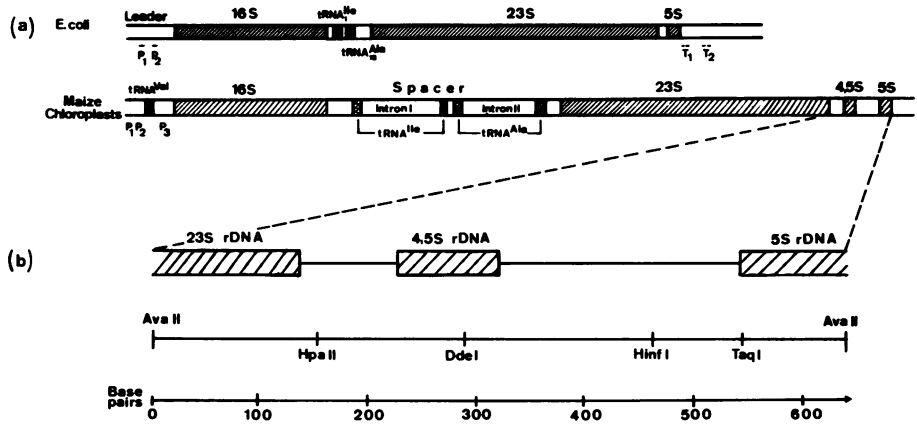

Fig. 1. a, Comparison of rRNA operons from E.coli (16) and maize chloroplasts (7). b, Enlargement of the region coding for the 3' terminal part of 23S rRNA, for 4.5S and 5S rRNA and for the intergenic regions. The restriction sites used for S_1 and primer extension mapping of processing sites in this region are also shown.

ments of the 3' labelled coding strand, nuclease resistant by hybridisation either with 23S rRNA or tot ctRNA are represented by bands a to e in lanes 5 and 6, respectively, of Fig. 2b. The group of bands, designated by a, corresponds to the 3' terminus of mature 23S rRNA, as it is the only signal detected after hybridisation with isolated 23S rRNA. The additional bands b, c, d and e obtained with tot ctRNA which contains also precursor RNAs (6,11) are therefore interpreted as indicating termini of processing intermediates in the 23S/4.5S rRNA intergenic region. Nuclease resistant fragments found after hybridisation of the 5' terminally labelled coding strand with tot ctRNA are designated as bands A to F in lane 5 of Fig. 2c. As evident from a comparison with known 4.5S rRNA sequences from tobacco (17), wheat (18) and maize (Bedbrook and Dyer, unpublished; see also Fig. 5), band A represents the 5' terminus of mature 4.5S rRNA and bands B to F are again interpreted as indicating the termini of RNA processing intermediates in the 23S/4.5S rRNA intergenic region. Comparable nuclease resistant products are not observed in a control experiment in which RNA was omitted (Fig. 2c, lane 6). The S_1 sites identified by using the 5' labelled coding strand were

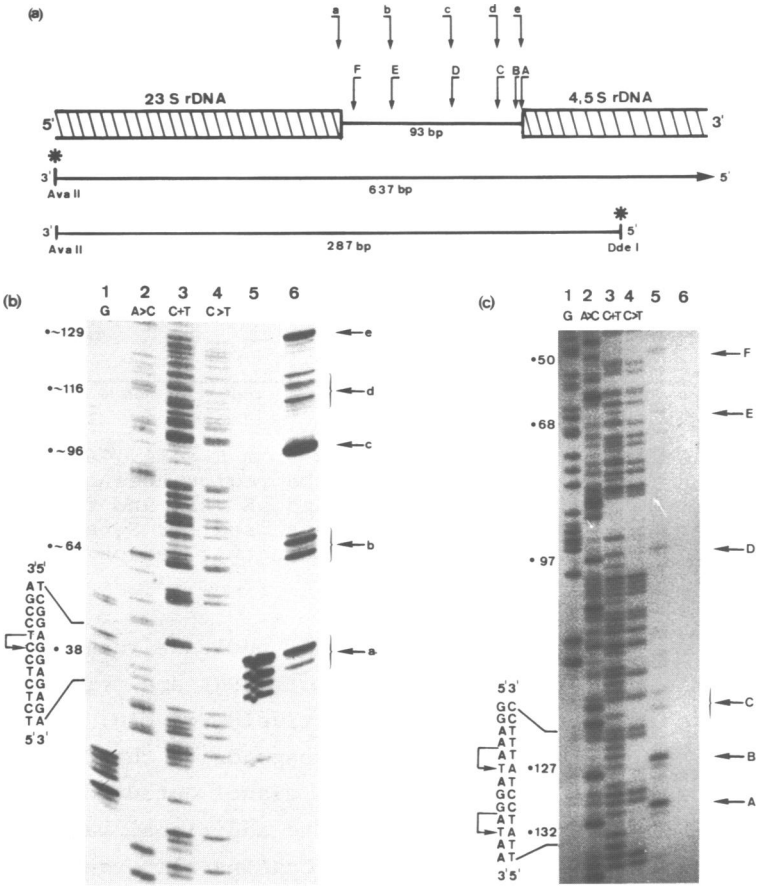

Fig. 2. S_1 mapping of RNA termini encoded in the 23S/4.5S intergenic region of the maize chloroplast rRNA operon. a, Schematic representation of the coding strand DNA fragments and labelled termini (✱) used. The vertical arrows on top indicate the positions of RNA termini identified with 5' terminally (A-F) or 3' terminally (a-e) labelled DNA probes. b, Gel electrophoretic analysis of mung bean nuclease resistant radioactive DNA fragments (a-e) obtained with the coding strand of fragment $AvaII_{637}$ annealed with mature 23S rRNA (lane 5) or tot ctRNA (lane 6). c, Gel electrophoretic analysis of mung bean nuclease resistant radioactive fragments (A-F) obtained with fragment $AvaII/DdeI_{287}$ 5' terminally labelled at its DdeI end and annealed with tot ctRNA (lane 5). Lane 6 shows a control in which tot ctRNA was omitted. In b and c sequence ladders (lanes 1-4) are given alongside. Numbering of nucleotide positions refers to the sequence depicted in Fig. 5. For the exact alignment of the S_1 bands with the positions of the respective sequence ladders a correction of 1 position and of 1.5 positions were made for 3' and 5' terminally labelled fragments, respectively, as exemplified by arrows in the small sequence sections depicted alongside lanes 1.

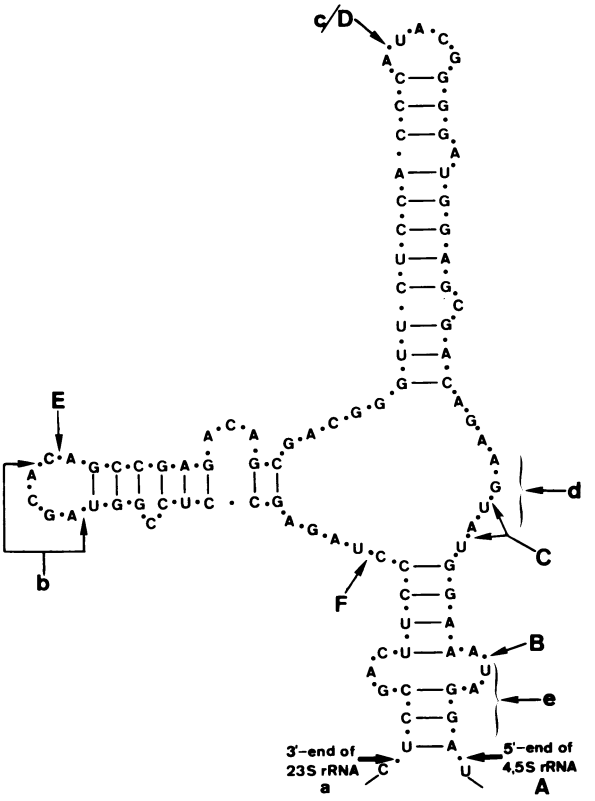

Fig. 3. Position of RNA termini (A-F and a-e) as deduced from Fig. 2 in the secondary structure proposed for the 23S/4.5S intergenic region from maize chloroplasts (19).

independently confirmed by primer extension experiments (data not shown).

The localization of the RNA termini, as identified above, are shown in a secondary structure proposed for the 23S/4.5S rRNA intergenic region (19) in Fig. 3. The processing sites are exclusively located in single stranded parts of this structure. Except for region B/e the break points do not resemble the symmetrical structure of prokaryotic RNaseIII cleavage sites (20,21). In most cases the cleavage sites deduced using the 5' terminally labelled DNA probe coincide exactly with (e.g. bands c and D) or are close to sites inferred from the 3' terminally labelled probe (e.g. bands b and E, d and C and e and B). This suggests that

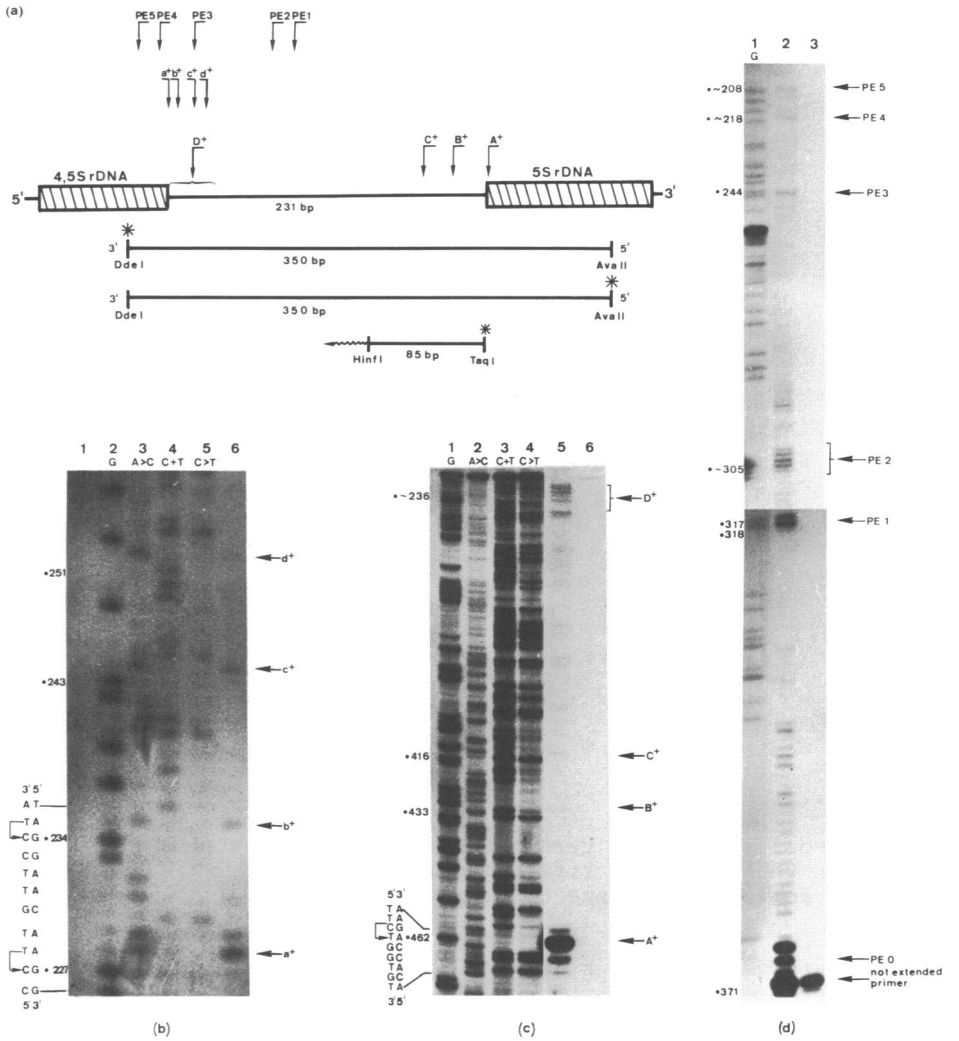

Fig. 4. S_1 and primer extension mapping of RNA termini encoded in the 4.5S/5S intergenic region. a, Schematic representation of the coding strand DNA fragments and labelled termini (✱) used. The vertical arrows on top indicate the positions of RNA termini identified with 5' terminally (A^+-D^+) or 3' terminally (a^+-d^+) labelled DNA probes in S_1 experiments. The vertical arrows marked by PE1-PE5 indicate positions corresponding to bands obtained by primer extension experiments. In lane 6 of panel b and in lane 5 of panel c the gel electrophoretic analysis of mung bean nuclease resistant radioactive fragments obtained with the 3' terminally (a^+-d^+) or 5' terminally (A^+-D^+) labelled coding strand of fragment $DdeI/AvaII_{350}$ annealed with tot ctRNA are shown alongside with sequence ladders and with controls in which tot ctRNA was

omitted (lane 1 of panel b and lane 6 of panel c). In panel d gel electrophoretic analysis of products (PE1-PE5) obtained by primer extension of the 5' terminally labelled coding strand of fragment HinfI/TaqI$_{85}$ with tot ctRNA as template is shown (lane 2) alongside with a G-ladder derived from fragment HpaII/TaqI$_{394}$ labelled 5' terminally at its TaqI end (lane 1) and a control in which reverse transcriptase was omitted in the primer extension reaction (lane 3). The 3 bands marked PEO are believed to originate from filling the 3' terminal HinfI site with the protruding 5' end of the complementary DNA as template. Corrections for exact alignment with sequence positions of the "ladders" are exemplified in the small sequence sections depicted alongside lanes 1 (panels b and c). Numbering of nucleotide positions refers to the sequence depicted in Fig. 5.

processing in this region does not follow a pathway starting at only one of these sites, as this would lead to only one common site, the initiating site of processing, observed in the S_1 experiments with the DNA probe extending from a 5' label within the 4.5S rDNA and with the DNA probe extending from a 3' label within the 23S rDNA. In view of several common S_1 sites processing of the 23S/4.5S intergenic RNA appears to consist of a random succession of specific cleavage reactions which is in general agreement with rRNA processing in prokaryotic systems (22).

RNA Termini Within the 4.5S/5S Intergenic Region

Screening of RNA termini in this region was performed by using in separate experiments the 3' or 5' terminally labelled coding strand of fragment DdeI/AvaII$_{350}$ as depicted in Figs. 1 and 4a. The mung bean nuclease resistant fragments designated as a^+ to d^+ (3' terminally labelled probe) and A^+ to D^+ (5' terminally labelled probe) and their positions in respect to a sequence ladder are depicted in Fig. 4b (lane 6) and Fig. 4c (lane 5). The corresponding products are RNA specific as they are absent in control experiments in which RNA has been omitted. The strong band a^+ corresponds to the 3' end of mature 4.5S rRNA, although it differs by one position from the 3' end of mature 4.5S rRNAs as identified by RNA sequencing (quoted above; see also Fig. 5). This difference is probably due to a general limitation of specificity of S_1 or mung bean nuclease in respect to the single/double strand borders of the substrates (23); this can lead either to a few unpaired nucleotides undigested or, especially at AT rich regions, to a nibbling of a few positions at double stranded termini.

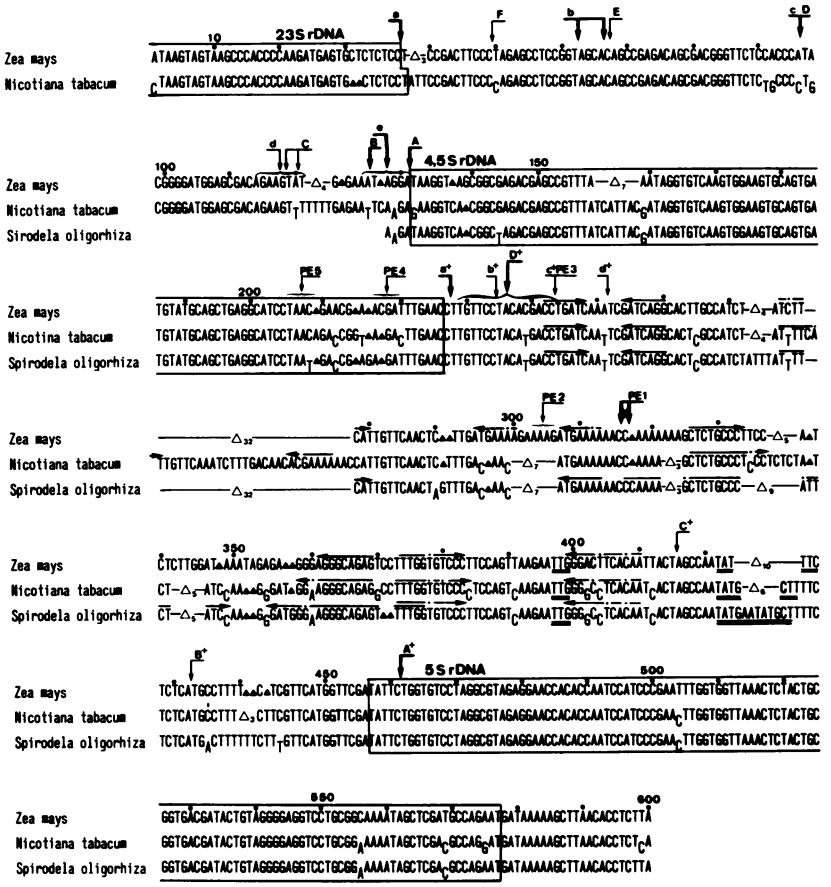

Fig. 5. Comparison of nucleotide sequences of the RNA-like DNA strands from the 23S/4.5S and 4.5S/5S intergenic regions of Zea mays, Nicotiana tabacum and Spirodela oligorhiza chloroplasts. Sequences of structural genes are framed. 5' and 3' termini of these genes are deduced from RNA sequencing data in the case of maize and tobacco 4.5S and 5S rRNA (Bedbrook and Dyer, unpublished; 17,24), S_1 mapping in the case of maize and tobacco 23S rRNA (27) or DNA sequence comparison in the case of Spirodela 4.5S and 5S rRNA (8). Vertical arrows indicate S_1 sites (a-e, A-F, a^+-d^+, and A^+-D^+) or primer extension sites (PE1-PE5) as described in Figs. 2 and 4. The thickness of arrows approximately corresponds to the strength of the bands on the autoradiographs. Pairs of horizontal arrows above the respective sequence indicate inverted repeats of at least five nucleotides. Double underlined sequences proximal to the 5S rRNA genes mark the prokaryotic-like promoter sequences proposed earlier (8,25,28). Positions deleted as compared to one or both of the other given sequences are symbolized by ▲ or Δ (in the case of stretches longer than 2 positions).

Similarly, the strong band A^+ corresponds to the 5' terminal region of mature 5S rRNA but shows a difference of even 5 nucleotide positions as compared to RNA sequencing data (Bedbrook and Dyer, unpublished; see also Fig. 5); the extensive nibbling in this case apparently is due to a high AT content of this region. The faint bands b^+ to d^+ are interpreted as indicating processing sites of precursor rRNA located close to the 3' end of mature 4.5S rRNA. No mung bean nuclease protected products of higher chain lengths could be detected in this experiment when the gel electrophoresis was run for a longer time (data not shown). Bands B^+ to D^+ together with several fainter undesignated bands between C^+ and D^+ of lane 5 in Fig. 4c are interpreted as representing processing sites upstream of the 5S rRNA sequence. The group of bands marked with D^+ coincides with the S_1 sites b^+, c^+ and d^+ identified with the 3' labelled DNA probe. This coincidence of S_1 sites observed with DNA probes comprising the entire 4.5S/5S intergenic region from both sides and major parts of the two flanking structural genes led us to the conclusion that 4.5S rRNA and 5S rRNA are part of a common precursor RNA. For maturation of the 3' end of 4.5S rRNA and of the 5' end of 5S rRNA the precursor RNA is first cleaved at a site positioned about 20 nucleotides downstream of the 4.5S rRNA sequence. A very fast rate of this cleavage reaction apparently is the reason why 5S rRNA sequences cannot be detected in large rRNA precursors (6, 7) and why the kinetics of *in vivo* labelling is similar for 5S rRNA and the immediate precursors (and not the mature species) of 16S and 23S rRNA (6). It is noteworthy in this connection that in the case of *E.coli* rRNA operons 5S rRNA originally also was thought not to be contained in the large precursor rRNA; only by using a RNaseIII mutant strain in which processing is deficient, could the existence of a large precursor rRNA which contains also 5S rRNA be demonstrated (22).

The connection of 4.5S rRNA and 5S rRNA on a common precursor could also be demonstrated by primer extension mapping using the 5' labelled coding strand of fragment HinfI/$TaqI_{85}$ (see Figs. 1 and 4a) as primer for reverse transcriptase after hybridization with tot ctRNA. The band designated PE3 in lane 2 of Fig. 4d represents a cDNA which ends exactly at a position which had alrea-

dy been identified as a processing site in the S_1 experiment using the 3' labelled DNA probe (Fig. 4b, band c^+; see also Fig. 5).

The primer extension products designated by PE1, PE2, PE4 and PE5 do not coincide with S_1 bands. It is also noteworthy that they occur at positions close to the 3' sides of palindromic sequences (PE1 and PE2; see also Fig. 5) and in interior regions of the 4.5S rRNA (PE4 and PE5), whereas PE3 is positioned at the 5' side of a palindrom (see Fig. 5). This supports the interpretation that only PE3 represents a genuine RNA terminus whereas the other primer extension products rather reflect pausing sites of reverse transcriptase at hair pin structures of the RNA template. From the mere existence and position of all the primer extension products (PE1-PE5) it is, however, clear that 5S rRNA precursor sequences reach far beyond the putative promoter sites postulated earlier (25) for the region between position 400 and 430 (see Fig. 5).

In this connection it should also be mentioned that we were not able to observe products corresponding to PE3-PE5 when the 5' labelled fragment AvaII/Taq_{93} (the rightmost fragment of Fig. 1) was used as a primer for reverse transcriptase catalized extension. In this case the only cDNA product obtained (data not shown) corresponds to the site PE1. Apparently the fragment AvaII/Taq_{93} during hybridization is competed out largely by mature 5S rRNA and therefore is less suitable for detection of longer precursors. In contrast to this, fragment TaqI/$HinfI_{85}$ contains no sequences complementary to mature 5S rRNA and can hybridize with precursor RNA only, thus leading to precursor signals in higher yield and/or from longer precursors.

In no case the primer extension experiments yielded a signal corresponding to the postulated promoter like sequences 20-50 bp upstream of the 5S rRNA gene. The only S_1 products terminated within this region are bands B^+ and C^+ in Fig. 4c; however, in view of the many longer precursors these products must be interpreted as indicating further processing sites of the 5S rRNA precursor rather than initiation sites of an independent 5S rRNA transcription.

From Southern hybridisation data obtained with tobacco 5S rRNA

(26) and maize 5S rRNA specific DNA probes (our own data, not shown) the existence of additional 5S rRNA genes at other positions of the chloroplast genome can be excluded. Therefore a separate transcription of 5S rRNA caused by such genes as discussed by Hartley (6) can also be ruled out.

Sequence Comparison of the 23S/4.5S and 4.5S/5S Intergenic Regions

A comparison of the 3' end of the 23S, the 4.5S and the 5S rRNA structural genes and intergenic regions from Zea mays (Bedbrook and Dyer, unpublished), Nicotiana tabacum (27,28) and partly of Spirodela oligorhiza (8) is presented in Fig. 5. It shows that the postulated promoter sequences proximal to the 5S rRNA gene contain deletions and base changes and are thus not conserved, as would be expected for a functional promoter. In addition, the -10 consensus sequence of maize chloroplasts (TATTTC) would not fit with the bacterial -10 consensus sequence (TATAAT) or with chloroplast -10 consensus sequences, in which the 3' terminal T position is highly conserved in all functional promoters (29,30). The sequence comparison on the other hand shows that the region immediately downstream of 4.5S rDNA which includes a palindromic haptanucleotide sequence (position 242-260) is highly conserved. A stem structure can be formed between the 3' terminal region of 4.5S rRNA and the 5' terminal region of 23S rRNA of maize chloroplasts (2). This structure, which includes the six positions of the 3' flanking sequences of the 4.5S rRNA sequence (CUUGUU; see Fig. 5), was proposed to function as a substrate recognized by an RNaseIII like enzyme (2). As strong sequence conservation extends much further to about 40 positions beyond the 3' end of 4.5S rRNA and as this region does contain several distinct cleavage sites it is likely that the entire region, in particular the stem loop structure which can be formed by the palindromic sequence, is functioning as a signal for 23S/4.5S/5S rRNA processing. In contrast, the regions around PE1 and PE2 are highly divergent, which lends further support that these products do not indicate functional sites.

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