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Low-Molecular-Weight (4.5S) Ribonucleic Acid in Higher-Plant Chloroplast Ribosomes

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A species of RNA that migrates on 10% (w/v) polyacrylamide gels between 5S and 4S RNA was detected in spinach chloroplasts. This RNA (referred to as 4.5S RNA) was present in amounts equimolar to the 5S RNA and its molecular weight was estimated to be approx. 33000. Fractionation of the chloroplast components showed that the 4.5S RNA was associated with the 50S ribosomal subunit and that it could be removed by washing the ribosomes with a buffer containing 0.01M-EDTA and 0.5M-KCl. It did not appear to be a cleavage product of the labile 23S RNA of spinach chloroplast ribosomes. When ^{125}I-labelled 4.5S RNA was hybridized to fragments of spinach chloroplast DNA produced by *Sma*I restriction endonuclease, a single fragment (mol.wt. 1.15×10^6) became labelled. The same DNA fragment also hybridized to chloroplast 5S RNA and part of the 23S RNA. It was concluded that the coding sequence for 4.5S RNA was part of, or immediately adjacent to, the rRNA-gene region in chloroplast DNA. A comparable RNA species was observed in chloroplasts of tobacco and pea leaves.

The low-molecular-weight fraction of total chloroplast RNA contains two major components, 5S RNA, originating from the 50S subunit of the chloroplast ribosomes, and tRNA species (4S RNA), which originate from the soluble phase of the chloroplast (Dyer & Leech, 1968; Payne & Dyer, 1971). In the course of purifying 5S RNA from spinach chloroplasts we observed a third low-molecular-weight RNA species that migrated on 10% (w/v) polyacrylamide-gel electrophoresis between the 5S and 4S RNA species. In the present paper we describe experiments designed to determine the origin of this RNA. Dyer and his colleagues (Dyer & Bowman, 1976; Dyer *et al.*, 1977) have made similar observations on the presence of a 4.5S RNA species in higher-plant chloroplasts and shown by oligonucleotide-'fingerprinting' analysis that the nucleotide sequence is quite different from those of chloroplast 5S RNA and 4S RNA species. A preliminary report on our findings has been published (Whitfeld *et al.*, 1977).

Experimental

Spinach (*Spinacea oleracea*, Yates hybrid no. 102) plants were grown in water culture in a glasshouse (Spencer & Whitfeld, 1967). Tobacco (*Nicotiana tabacum*, var. Virginia Gold) and broad-bean (*Vicia faba*, dwarf variety) plants were grown in soil in a glasshouse, and pea (*Pisum sativum*, var. Greenfeast) plants were raised in vermiculite under artificial illumination (16h day) at 15–20°C. Only young leaf material was used.

For the preparation of chloroplast RNA labelled *in vivo*, spinach plants at the six-leaf stage were placed in water containing [^{32}P]P_i (The Radiochemical Centre, Amersham, Bucks., U.K.) (50μCi/ml), for 8h before the leaves were harvested.

All the enzymes used, including restriction endonuclease *Sma*I (from the bacterium *Serratia marcescens*), were purchased from Boehringer Mannheim G.m.b.H, Mannheim 31, Germany.

Isolation of chloroplast RNA and DNA

Chloroplasts were isolated by the rapid procedure of Walker (1971), with the modifications detailed in Whitfeld *et al.* (1978). The preparation of chloroplast ribosomes, ribosomal subunits, and the fractions containing low-molecular-weight RNA has been described elsewhere (Whitfeld *et al.*, 1978). RNA was extracted from each of these preparations by a phenol/SDS or phenol/Sarkosyl procedure (Whitfeld & Higgins, 1976; Whitfeld *et al.*, 1978).

DNA was isolated from spinach chloroplasts that had been treated with deoxyribonuclease (Herrmann *et al.*, 1975), and was purified by centrifuging twice in CsCl gradients (Whitfeld *et al.*, 1978).

Abbreviation used: SDS, sodium dodecyl sulphate.

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The preparation of RNA from spinach leaf and root tissue involved an initial grinding of the tissue in liquid N_2 and then extraction with an SDS-containing buffer and phenol (Spencer & Whitfeld, 1966).

Gel electrophoresis of RNA

Electrophoretic analysis of RNA was carried out at 4°C on 2.2, 2.4 or 10% (w/v) polyacrylamide gels (90mm×6mm gels in Perspex tubes) in 0.036M-Tris/HCl/0.03M-sodium phosphate/1mM-EDTA, pH7 (Leaver, 1973), plus 0.05% SDS and 2mM-$MgCl_2$. Analysis of low-molecular-weight RNA under denaturing conditions was carried out on 9% polyacrylamide gels (acrylamide:bisacrylamide ratio of 10:1) in 99% (v/v) formamide (Boedtker *et al.*, 1973). Gels were washed for at least 30min and scanned at 260nm. All gels were subsequently stained in 0.005% Toluidine Blue in 20% (v/v) ethanol to verify that u.v.-absorbing bands coincided with stained bands.

Preparative gel electrophoresis for the isolation of pure 4.5S and 5S RNA species was carried out on 10% polyacrylamide slab gels (150mm×150mm×2mm) with a 20mm overlay of 3% polyacrylamide gel. Total chloroplast RNA (5–7mg) was loaded on a gel and run overnight at room temperature. The bands of RNA were located by examining the ethidium bromide-stained (0.5μg/ml) gel under u.v. light and cut out. RNA-containing gel strips were disintegrated by extrusion through a syringe, resuspended in 2vol. of buffer (0.5M-ammonium acetate/0.1mM-EDTA/0.1% SDS) for 30min at 20°C, and then centrifuged at 40*g* for 20min into a plug of silicone-treated glasswool, which effectively trapped the gel fragments, leaving the eluted RNA in the supernatant. The gel, trapped in the glasswool, was re-extracted twice more in the same buffer by resuspension and centrifugation, and the RNA precipitated from the combined supernatants by adding 2vol. of ethanol, and finally dialysed against water overnight.

Iodination of RNA

Samples (50μg) of 4.5S, 5S, 16S and 23S RNA were iodinated by the procedure of Getz *et al.* (1972) as modified by Whitfeld *et al.* (1978). Radioactivities of 5×10^6–7×10^6 c.p.m./μg of RNA were obtained.

DNA restriction, agarose-gel electrophoresis and hybridization

Digestion of spinach chloroplast DNA with endonuclease *Sma*I was carried out at 25°C in 15mM-

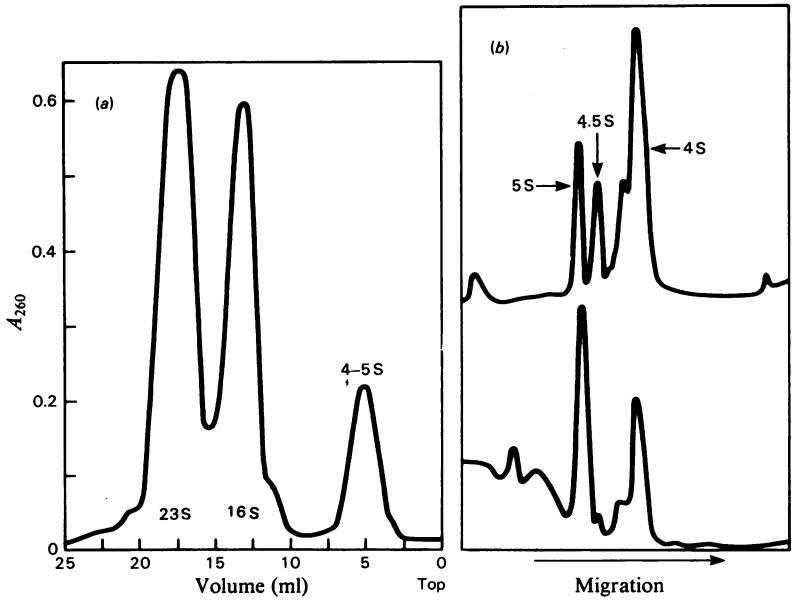

Fig. 1. (*a*) *Sucrose-density-gradient centrifugation of spinach chloroplast RNA and* (*b*) *polyacrylamide-gel electrophoresis of the low-molecular-weight RNA fraction*

A sample (1.0mg) of RNA was centrifuged at 24000rev./min in a Beckman SW 25.1 rotor at 3°C for 18h in a 5–20% (w/v) sucrose gradient in 0.15M-lithium acetate, pH6.0. The gradient was pumped through an Isco flow cell and fractions corresponding to A_{260} peaks were collected. The low-molecular-weight RNA fraction was recovered by precipitation with ethanol, dissolved in water and a 10μg sample was analysed on a 10% polyacrylamide gel [(*b*), upper trace]. A sample of *E. coli* RNA containing 5S and 4S RNA was electrophoresed on a parallel gel [(*b*), lower trace].

Tris/HCl (pH8.5)/6mM-$MgCl_2$/15mM-KCl/10mM-β-mercaptoethanol, and the fragments were separated on a 1% agarose slab gel (Whitfeld *et al.*, 1978). DNA fragments in the gel were denatured with alkali, neutralized, and transferred to cellulose nitrate membrane strips as described by Southern (1975). Hybridizations were carried out with 0.2–1.0μg of ^{125}I-labelled RNA and 20–30μg of unlabelled RNA in a volume of 1.7ml of 50% formamide/0.6M-NaCl/0.06M-trisodium citrate/25mM-Tris/HCl (pH7.0)/1mM-KI/0.1% SDS, at 47°C for 4–14h. Reactions containing ^{125}I-labelled 4.5S RNA contained unlabelled 4S, 5S, 16S and 23S RNA; those with ^{125}I-labelled 5S RNA contained unlabelled 4S, 4.5S, 16S and 23S RNA; those with ^{125}I-labelled 23S RNA contained unlabelled 4S, 4.5S, 5S and 16S RNA. Details of the washing procedures and radioautography have been described (Whitfeld *et al.*, 1978).

Results

When spinach chloroplast RNA was centrifuged on a sucrose gradient and the low-molecular-weight fraction recovered and electrophoresed on a 10% polyacrylamide gel, three major RNA species were observed (Fig. 1). By reference to a parallel gel run of *Escherichia coli* RNA it was established that the fastest-migrating component was 4S RNA and the slowest was 5S RNA. The middle component (designated 4.5S RNA) was unexpected and, to our knowledge, had not been described previously in the literature. As it was obviously present in significant amounts we considered it worthy of further investigation.

Electrophoresis of the chloroplast low-molecular-weight RNA fraction under denaturing conditions yielded the same pattern of three components, with the same relative mobilities, eliminating the possibility that the 4.5S RNA was a conformational isomer of 5S RNA. Supporting evidence for this conclusion was obtained by comparing the electrophoretic patterns of the RNA before and after heating at 75°C in the presence and absence of Mg^{2+} (Philipps & Timko, 1972). Equivalent patterns resulted in each case, no matter what treatment was applied (results not shown).

An estimate of the molecular size of the RNA was obtained by co-electrophoresis in formamide-containing gels of the chloroplast RNA and heated RNA from wheat-germ ribosomes (Fig. 2). The 5.8S RNA and 5S RNA arising from the wheat-germ cytoplasmic ribosomes migrated as additional reference markers in the gel. Assuming that the number of nucleotides in wheat germ 5.8S and 5S RNA is 157 and 118 (Payne & Dyer, 1971, 1972) and that the number in spinach chloroplast 5S RNA is 122 (Payne & Dyer, 1971) it was calculated that the spinach 4.5S RNA contained 103±1 nucleotides.

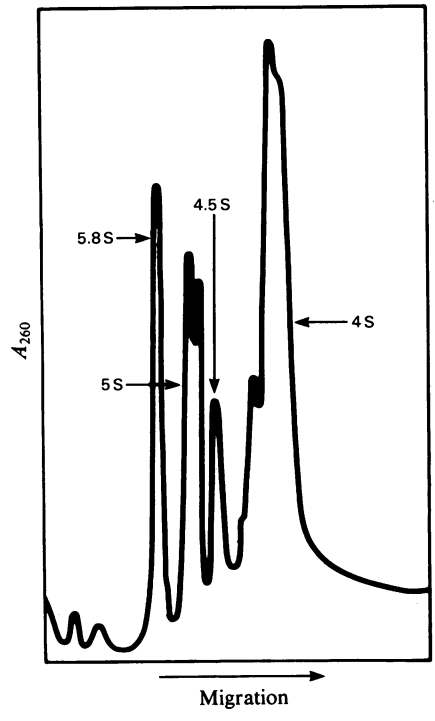

Fig. 2. *Polyacrylamide/formamide gel electrophoresis of low-molecular-weight RNA from spinach chloroplasts and heated wheat germ rRNA*

Wheat-germ rRNA, dissolved in 70% formamide, was heated to 60°C for 3min, quickly cooled, mixed with spinach chloroplast low-molecular-weight RNA in 70% formamide and layered on a 9% polyacrylamide gel (0.6cm×9cm) in 98% (v/v) formamide. After electrophoresis for 3h (3mA/gel) the gel was washed in 10% ethanol and scanned at 260nm.

Origin of 4.5S RNA

The possibility that the 4.5S RNA detected in chloroplast RNA preparations was due to contaminating nuclei or cytoplasm was discounted by examining RNA prepared from whole spinach leaf and root tissue. Fig. 3 shows that 4.5S RNA is present in leaf tissue, but not in root tissue. The proportion of 4.5S RNA relative to 5S RNA was much less in whole leaf tissue than in chloroplasts, as would be expected if the chloroplasts were the source of the 4.5S RNA.

When spinach chloroplasts were lysed with Triton X-100 and then centrifuged at 150000*g* for 4h to pellet the ribosomes, analysis of the RNA in the supernatant fraction showed it to be composed of 4S RNA only (Fig. 4*a*). All the 4.5S RNA as well as the

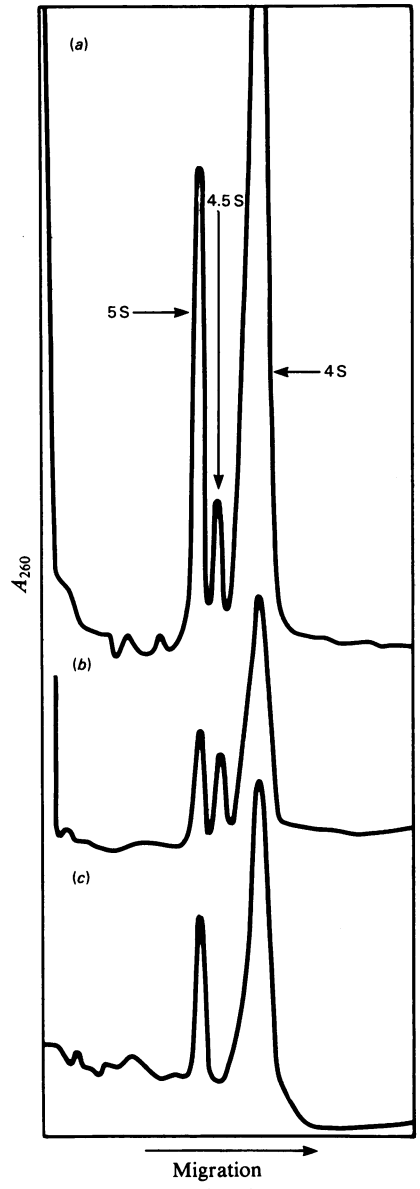

Fig. 3. *Polyacrylamide-gel* (10%) *electrophoresis of spinach RNA from* (*a*) *total leaf*, (*b*) *chloroplasts and* (*c*) *roots*

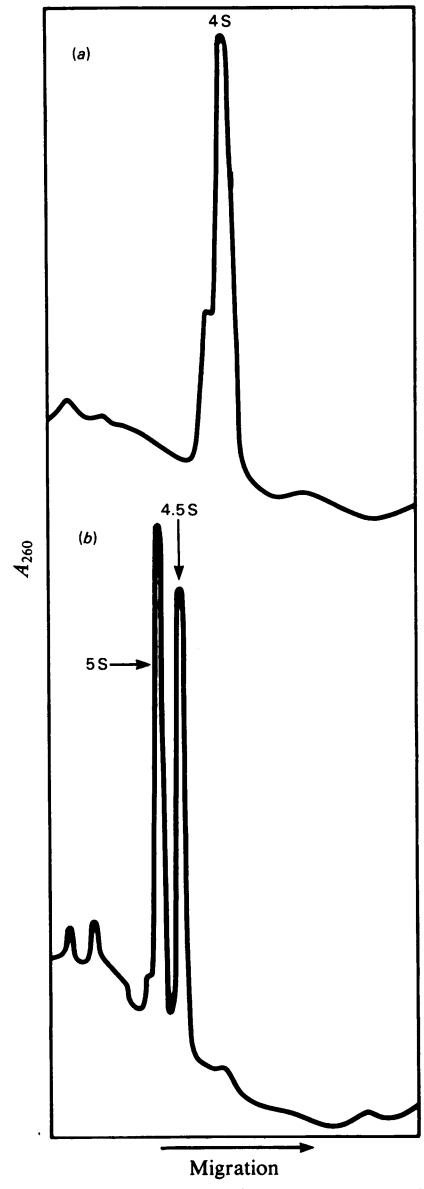

Fig. 4. *Polyacrylamide-gel* (10%) *electrophoresis of spinach chloroplast RNA extracted from* (*a*) *the post-ribosomal supernatant and* (*b*) *the ribosomal pellet*

Spinach chloroplasts in 10mM-Tris/HCl (pH 8.5)/50mM-KCl/10mM-$MgCl_2$, were lysed with Triton X-100 (final concn. 4%), clarified at 10000rev./min for 7min and the supernatant was layered over a 4ml cushion of 1 M-sucrose in the same buffer. After centrifugation for 3.5h at 60000rev./min at 4°C (Beckman L5-65 rotor) the green supernatant was removed and RNA extracted from it by shaking with phenol in the presence of 1% sodium dodecyl sulphate. The ribosomal pellet was rinsed once with buffer and resuspended for extraction of RNA. Electrophoresis was carried out for 3h at 5mA/gel as described in the Experimental section.

5S RNA was present in the RNA extracted from the ribosomal pellet (Fig. 4*b*). Chloroplast 70S ribosomes were treated with buffer containing 500mM-KCl, 2mM-$MgCl_2$ and the subunits separated by sucrose gradient centrifugation. RNA from the 50S and 30S subunits was analysed on both 2.2 and 10% polyacrylamide gels. Use of the 2.2% gels showed (Fig. 5*a*) that RNA from the 50S subunit has 23S RNA as a major component and three other high-molecular-

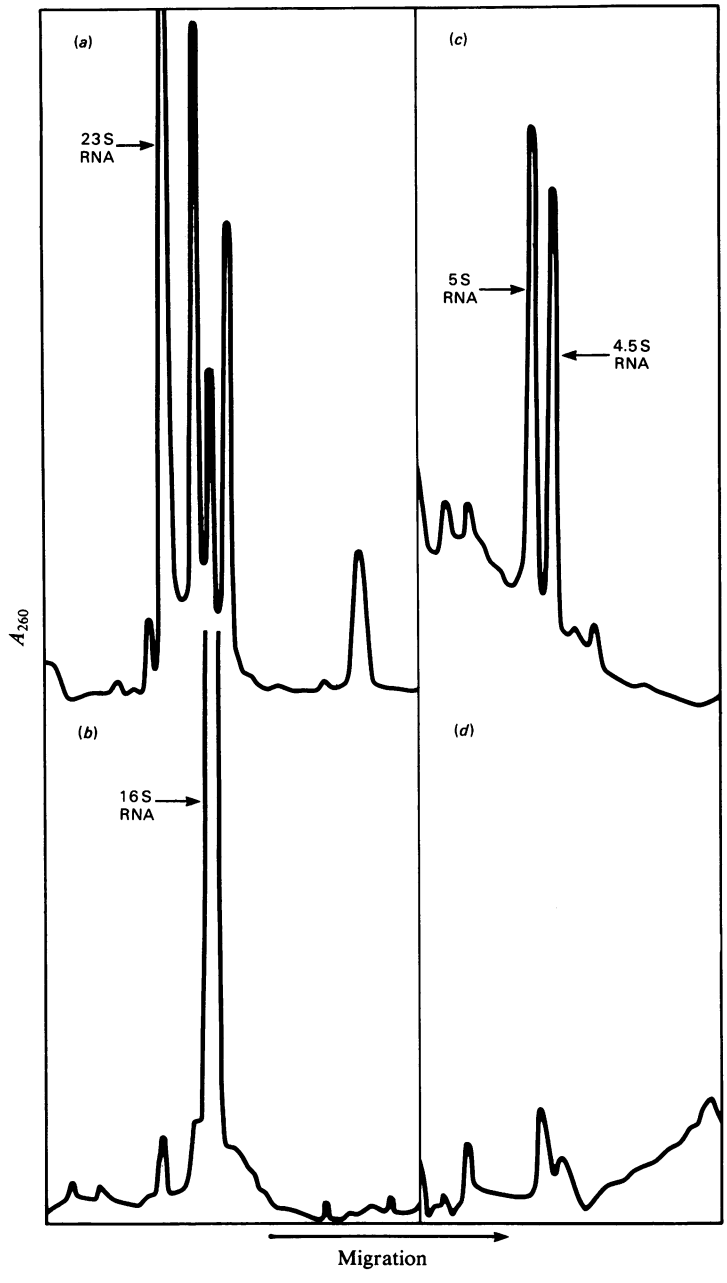

Fig. 5. *Polyacrylamide-gel electrophoresis of spinach chloroplast RNA extracted from 50S and 30S ribosomal subunits*
Chloroplast ribosomes, prepared as described in the legend to Fig. 4, were resuspended in 50mM-Tris/HCl (pH8.5)/500mM-KCl/2mM-$MgCl_2$, and fractionated on a 10-40% sucrose gradient in the same buffer in a Beckman SW 25.1 rotor at 24000rev./min at 4°C for 20h. Fractions containing the 50S and 30S subunits were separately pooled and the subunits recovered by precipitation with 1.5vol. of ethanol/10mM-$MgCl_2$. RNA was extracted and samples were analysed on 2.2% gels (*a*, *b*) and on 10% gels (*c*, *d*). (*a*) and (*c*) are the profiles of RNA from the 50S subunits and (*b*) and (*d*) those of RNA from the 30S subunits.

weight species, which arise from 23S RNA as a result of the presence of nicks within the molecule (Leaver & Ingle, 1971). A low-molecular-weight component was also apparent in the RNA from 50S subunits (Fig. 5*a*). RNA from the 30S subunit migrated as a single major species (16S), there being no detectable low-molecular-weight species (Fig. 5*b*). Fractionation of the RNA from the 50S subunit on a 10% gel showed both the 4.5S and 5S RNA species to be present and in equimolar amounts (Fig. 5*c*). Only a trace of 4.5S and 5S RNA was detected in the preparation from the 30S subunit (Fig. 5*d*). Thus 4.5S RNA, like 5S RNA, was specifically associated with the chloroplast 50S ribosomal subunit.

A similar result was obtained if spinach chloroplast ribosomes were dissociated in buffer containing

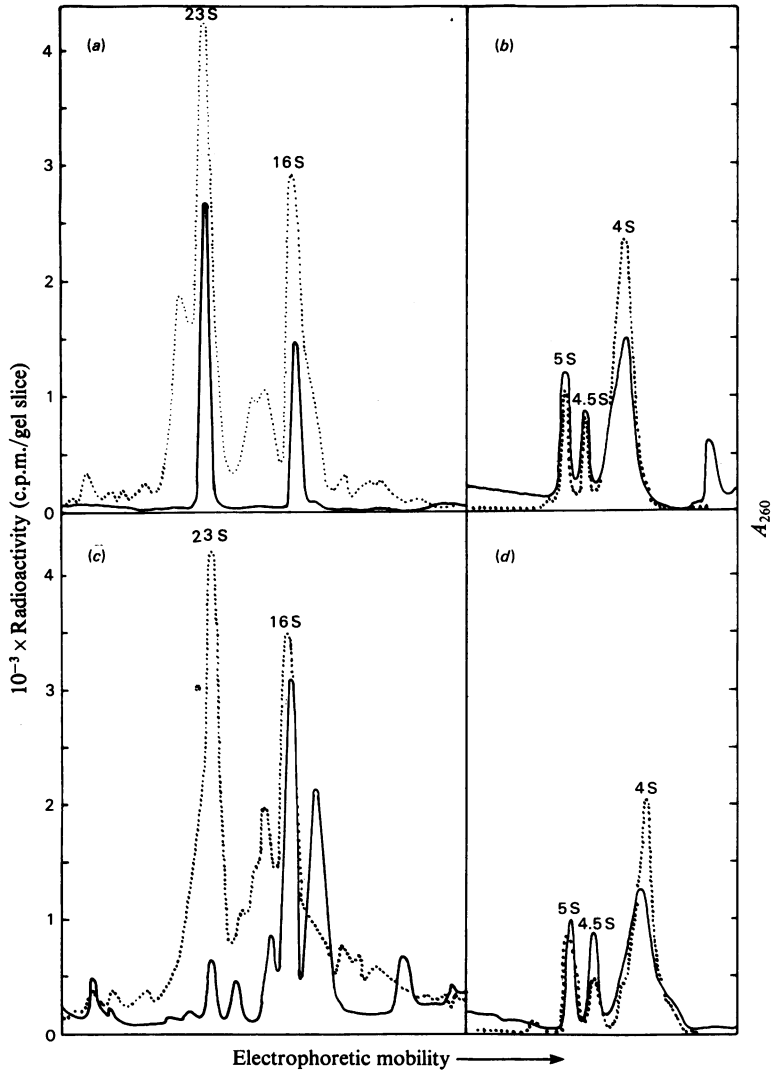

Fig. 6. *Polyacrylamide-gel electrophoresis of ^{32}P-labelled spinach chloroplast RNA*

Spinach plants were grown on [^{32}P]P_i (50μCi/ml) for 8h before harvesting. Chloroplasts were isolated and the RNA was extracted as described in the Experimental section. Samples were electrophoresed on 2.4 and 10% gels. The gels were washed briefly, scanned at 260nm, frozen and sliced into 1mm sections, which were dried and counted for radioactivity on an automated Geiger-counting system. (*a*) and (*b*) show the profiles of unheated RNA on 2.4 and 10% gels respectively; (*c*) and (*d*) show the corresponding profiles of heated RNA (60°C, 2min, followed by quick cooling). ——, A_{260}; · · · ·, radioactivity as c.p.m./1mm gel slice.

50mM-KCl and 0.1mM-EDTA. However, when high salt plus EDTA (500mM-KCl/10mM-EDTA) were used to dissociated the 70S ribosomes, both the 4.5S and 5S RNA were released from the 50S subunit and were recoverable from the top fractions of the sucrose gradient.

4.5S RNA is not a breakdown product of 23S RNA

The fact that the 4.5S RNA was a component of the 50S ribosomal subunit raised the possibility that it was a fragment of 23S RNA. Hidden nicks in spinach chloroplast 23S RNA are known to give rise to a number of cleavage products (Leaver & Ingle, 1971), and although RNA of size 4.5S has never been reported to be one of these products, it was conceivable that such a low-molecular-weight fragment could have been overlooked. Preliminary experiments showed that the proportion of 4.5S RNA, relative to 5S RNA, was not altered when spinach chloroplast RNA was heated at 65°C for 5min, although this treatment resulted in the conversion of more than 80% of the full-length 23S RNA molecules into specific cleavage products. This result, which indicated that the 4.5S RNA was not derived from 23S RNA, was supported by the results of the following experiment, in which advantage was taken of the fact that newly synthesized chloroplast 23S RNA does not contain hidden nicks (Ingle, 1968).

Chloroplast RNA was isolated from spinach plants that had been allowed to imbibe [^{32}P]P_i for 8h before harvesting and examined on 2.4% and 10% polyacrylamide gels. The pattern of unheated RNA on a 2.4% gel (Fig. 6*a*) showed the A_{260} peaks of 23S and 16S RNA coinciding with the major peaks of radioactivity. Additional peaks of radioactivity corresponding to precursors of 23S and 16S RNA were also present. Analysis of the unheated RNA on a 10% gel (Fig. 6*b*) clearly demonstrated the existence of radioactive newly synthesized 4.5S RNA. When heated RNA was examined on a 2.4% gel (Fig. 6*c*),

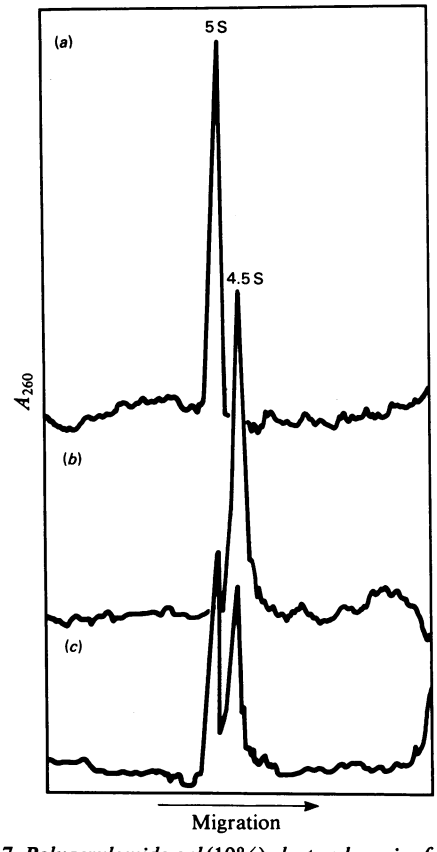

Fig. 7. *Polyacrylamide-gel (10%) electrophoresis of purified (a) chloroplast 5S RNA, (b) chloroplast 4.5S RNA and (c) a mixture of the two*

The 4.5S and 5S RNA were purified by preparative gel electrophoresis of spinach chloroplast RNA as described in the Experimental section. Samples were checked for possible cross-contamination by electrophoresis on analytical gels.

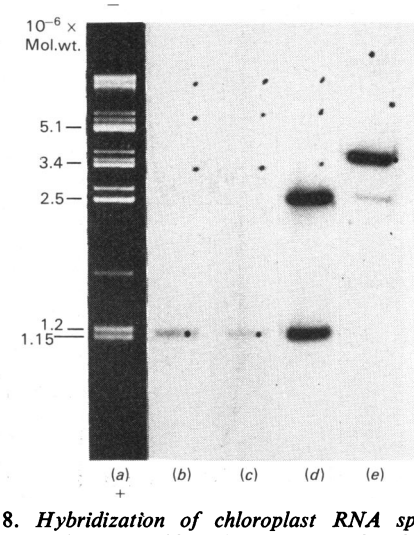

Fig. 8. *Hybridization of chloroplast RNA species to fragments of spinach chloroplast DNA produced by SmaI restriction endonuclease separated by electrophoresis on 1%-agarose slab gels*

Samples (4μg) of spinach chloroplast DNA were digested to completion with *Sma*I endonuclease and electrophoresed on an agarose slab gel. The ethidium bromide-stained gel was photographed under u.v. light (*a*) and the DNA fragments were then denatured and transferred to cellulose nitrate membranes for hybridization to ^{125}I-labelled RNA; (*b*)–(*e*) are radioautographs of membranes hybridized to 4.5S, 5S, 23S and 16S RNA respectively.

most of the 23S RNA, as reflected in the A_{260} scan, was seen to be dissociated into the usual cleavage products, whereas the newly synthesized 23S RNA (radioactivity) remained virtually intact. A parallel analysis on a 10% polyacrylamide gel (Fig. 6*d*) showed that heating the RNA did not significantly increase the amount of 4.5S RNA in terms of either A_{260} or radioactivity.

Location of the coding sequence for 4.5S RNA on spinach chloroplast DNA

The location and arrangement of the chloroplast 23S, 16S and 5S rRNA cistrons on a restriction-endonuclease 'map' of spinach chloroplast DNA are known (Whitfeld *et al.*, 1978). In view of the demonstrated close association of the 4.5S RNA with the chloroplast ribosomes it was decided to determine whether this RNA species was coded by the chloroplast DNA and, if so, whether it was transcribed from the region of DNA containing the rRNA genes. Answers to both these questions were obtained by hybridizing purified ^{125}I-labelled 4.5S RNA to the fragments of spinach chloroplast DNA produced by digestion with the restriction endonuclease *Sma*I.

The 4.5S and 5S RNA were purified by preparative gel electrophoresis (see the Experimental section) and were found to be free of detectable cross-contamination when analysed on 10% polyacrylamide gels (Fig. 7). As an additional precaution against the possibility of trace amounts of cross-contamination giving rise to spurious results, excess unlabelled 5S RNA was added to hybridizations

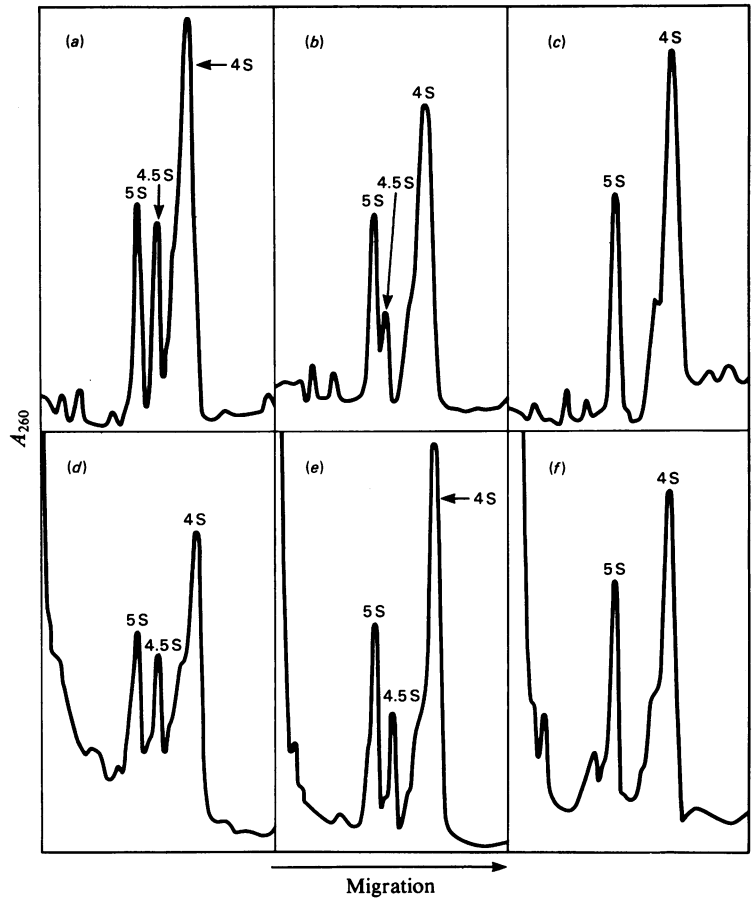

Fig. 9. *Polyacrylamide-gel electrophoresis of RNA extracted from chloroplasts of (a, d), tobacco (b, e) pea and (c, f) broad-bean plants*

Approx. 40μg of total chloroplast RNA in each case was layered on either non-denaturing 10% polyacrylamide gels (*a*, *b*, *c*) or denaturing 9% polyacrylamide gels in 98% formamide (*d*, *e*, *f*). In the latter case the RNA samples, dissolved in 70% formamide, were heated to 70°C for 3min before electrophoresis. Gels were washed in 10% ethanol and scanned at 260nm.

involving ^{125}I-labelled 4.5S RNA, and vice versa. Fig. 8 shows that 4.5S RNA hybridized to a single fragment produced by *Sma*I endonuclease. The same fragment (mol.wt. 1.15×10^6) also became labelled when ^{125}I-labelled 5S RNA was used as the hybridization probe (Fig. 8). ^{125}I-labelled 23S RNA hybridized in part to this fragment also, although the major band of radioactivity coincided with the 2.5×10^6-mol.wt. DNA fragment (Fig. 8). The 16S RNA did not hybridize to the 1.15×10^6-mol.wt. fragment, but mainly to a fragment of mol.wt. 3.4×10^6. From these data it was concluded that the 4.5S RNA must be transcribed from that region of the chloroplast DNA which is adjacent to the 23S and 5S RNA cistrons.

4.5S RNA in chloroplasts from other plants

Chloroplasts were isolated from the leaves of tobacco, pea and broad-bean plants and the RNA was extracted and electrophoresed on 10% polyacrylamide gels. The pattern for tobacco RNA (Figs. 9*a* and 9*d*) closely resembled that for spinach RNA, with a prominent peak of 4.5S RNA lying midway between the 5S and 4S RNA peaks. Also, as in spinach, the amount of tobacco 4.5S RNA was approximately equimolar to that of the chloroplast 5S RNA. In pea chloroplast RNA, there was a species which, in a non-denaturing 10% gel, migrated marginally ahead of 5S RNA (Fig. 9*b*). When examined in a formamide-containing gel, however, this RNA species migrated in the same relative position as the 4.5S RNA of spinach and tobacco chloroplasts (Fig. 9*e*). The amount of this presumptive 4.5S RNA in pea chloroplasts was clearly less than that in spinach, being equivalent to only half the amount of the 5S RNA.

Analysis of the RNA from *V. faba* chloroplasts showed the presence of only two low-molecular-weight RNA species, 5S and 4S, regardless of whether electrophoresis was carried out under denaturing or non-denaturing conditions (Figs. 9*c* and 9*f*). This apparent lack of a 4.5S RNA species in *V. faba* chloroplasts will be referred to again in the Discussion section.

Discussion

The experiments described in this paper provide convincing evidence of the existence of a 4.5S RNA species in the 50S ribosomal subunit of spinach chloroplasts. RNA of this size has been reported to occur in *E. coli* and in many animal cells. The 4.5S RNA of *E. coli* is present in the supernatant fraction (Griffin, 1975), not in the ribosomes, and in an amount such that it is only readily detectable as a radioactively labelled species. Although its nucleotide sequence is known (Griffin, 1975), as is that of the precursor RNA from which it is processed (Bothwell *et al.*, 1976), its role within the bacterial cell is still conjectural. In animal cells, such as HeLa cells, there are a number of low-molecular-weight RNA species localized in the nucleus and one of these has an electrophoretic mobility intermediate between that of 4S and 5S RNA (Ro-Choi & Busch, 1974). This RNA species, which is present in amounts such that it can be detected by u.v. absorbance, is also of unknown function. Because the 4.5S RNA of spinach was not a nuclear component (it was not present in the total RNA from spinach root tissue), and because it was strongly associated with the chloroplast ribosomes, it could in no way be equated to the animal or bacterial species of 4.5S RNA.

It is known that 5S RNA from *E. coli* can exist in two conformational states which migrate as distinct species on polyacrylamide gels (Philipps & Timko, 1972). Such a phenomenon was not responsible for the appearance of a 4.5S RNA species in spinach chloroplasts. Likewise, because of its strict localization on the ribosomes, the chloroplast 4.5S RNA could not be considered an aberrant tRNA. Both these observations support the conclusions of Dyer & Bowman (1976), based on oligonucleotide 'fingerprinting' data, that chloroplast 4.5S RNA is different from 5S and 4S RNA.

Like 5S RNA, 4.5S RNA could be dissociated from the ribosomes by washing them in a KCl/EDTA buffer. It has not been determined whether any protein remains associated with the 4.5S RNA under these conditions. Because of this property, 4.5S RNA does not appear to be strictly analogous to the 5.8S RNA of eukaryote cytoplasmic ribosomes, which is not dissociated under comparable conditions. Nor does it appear equivalent to 5.8S RNA in that 4.5S RNA migrates on gels as a discrete species without prior heating of the chloroplast RNA. If 4.5S RNA is base-paired to the 23S RNA then very few hydrogen bonds can be involved, because it must dissociate at temperatures no greater than 5°C.

Considerable attention was paid to the possibility that 4.5S RNA originated as a cleavage product of nicked 23S RNA, particularly in view of the observation by Dyer & Bowman (1976) that 4.5S RNA, in contrast with most mature RNA species, lacked a terminal 5′-phosphorylated nucleotide. The two strongest arguments against such an explanation are firstly that the 4.5S RNA appeared as a discrete species even when the nicked 23S RNA was held together by keeping it at 4°C in the presence of 2mM-Mg^{2+}. Secondly, in a situation where newly synthesized chloroplast 23S RNA which lacks hidden nicks could be monitored by virtue of its radioactivity and shown to be stable to heat, radioactive 4.5S RNA was also present.

It is apparent that the close association between the 4.5S, 5S and 23S RNA of spinach chloroplasts goes beyond their assembly into a 50S ribosomal subunit. The implication that the coding region for 4.5S RNA lay adjacent to that for 5S RNA in the chloroplast

DNA molecule came from earlier experiments (Whitfeld *et al.* 1978) when it was observed that a spinach chloroplast 5S RNA preparation, which contained 20–40% 4.5S RNA, hybridized to a single fragment of chloroplast DNA produced by *Sma*I restriction endonuclease. That this was indeed the case has now been confirmed in that individually purified 4.5S and 5S RNA molecules have both been found to hybridize to the same DNA fragment in a *Sma*I-endonuclease digest of spinach chloroplast DNA. As part of the 23S RNA also hybridized to the same 1.15×10^6-mol.wt. DNA band, the coding region for 4.5S RNA must lie either between the 23S RNA and 5S RNA cistrons or just distal to the 5S RNA cistron. It should be possible to define its exact location if a restriction endonuclease that cleaves spinach chloroplast DNA between the 23S and 5S RNA cistrons can be found.

It is likely, then, that the coding region for 4.5S RNA is part of the rRNA gene region in chloroplast DNA. This would be in agreement with the observation made by Hartley (1977) that the kinetics of labelling of 4.5S RNA in intact spinach plants are similar to those of the 16S and 23S RNA, leading to the suggestion that the 4.5S RNA might be derived from precursor to 23S RNA or 16S RNA. It also is in line with the view put forward by Dyer & Bowman (1976) that the 4.5S RNA is a piece of spacer RNA transcribed from the rRNA genes. If this is the case then it must follow that the primary transcript containing rRNA and spacer RNA is assembled into the ribosomal subunit intact and only later is processing into mature rRNA and spacer 4.5S RNA completed. Studies on the processing of a 30S rRNA transcript in *E. coli* (Lund & Dahlberg, 1977) have shown that tRNA molecules may occur in the spacer regions. Nucleotide-sequence data on chloroplast 4.5S RNA (Dyer *et al.*, 1977) make it unlikely that such RNA species are either tRNA molecules or precursors thereof.

In extending our observations to the chloroplasts of other plants we had hoped to show that 4.5S RNA was common to all species. Althouth tobacco chloroplasts contained a 4.5S RNA similar to that of spinach chloroplasts, and pea chloroplasts also contained a similar RNA species, albeit it was present in lesser amounts, the chloroplasts of *V. faba* did not appear to contain a comparable RNA species. In view of the data of Dyer & Bowman (1976), our negative result for *V. faba* chloroplasts may be misleading. Dyer & Bowman (1976) have found that in certain plants an RNA species directly analogous to the 4.5S RNA described in the present paper migrates in the region of 4S RNA and is only 80 nucleotides long. In our experiments such an RNA species would have been overlooked. Furthermore, to establish that such an RNA was comparable with the 4.5 S RNA of spinach it would be necessary to compare oligonucleotide 'fingerprints' of the two types of RNA. Thus it may still be that a 4.5S-like RNA species is a component of all chloroplast ribosomes.

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