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Isolation, identification, characterisation and stability of new cytoplasmic RNPs from *Vicia faba* seeds

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A new small cytoplasmic ribonucleoprotein complex (scRNPs) was isolated for the first time and its proteins and RNAs were identified. The results are summarised as follows:

(1) *Vicia faba* seeds soaked for 20 h has four complexes between the RNA and the proteins, three of them are major (complexes 1, 2 and 3 or C1, C2 and C3) and one minor (C4).
(2) Each complex contains two major proteins: complex 1 (C1) has 62 and 48 kDa patterns; C2 has 48 and 36 kDa; C3 has 36 and 25 kDa and C4 has 25 and 23 kDa.
(3) The differences between the highest molecular weight major proteins of each complex are approximately 12 ± 1 kDa (C1 has 62 kDa, C2 has 48 kDa, C3 has 36 kDa and the complex has 25 kDa).
(4) Each of the three major complexes contain the same species of uridine-rich RNA which was characterised as U1, U4, U5 and U6, in addition to the proteins mentioned above.
(5) The RNA in the complexes was found to be from the type of uridine-rich small ribonucleic acids that bind with proteins. They are called small ribonucleoproteins (snRNPs), and because they are found for the first time in cytoplasm and not extracted from the nucleus as in the literature, the name small cytoplasmic ribonucleoproteins (scRNPs) is suggested.
(6) The complexes were separated by a known method.
(7) The complexes were also separated using a new technique of separation.
(8) The RNA–protein complexes (scRNPs) were found to be strongly stable in guanidine–HCl (6 M), cyanobromide and sodium dodecyl sulphate (10%) at 100°C for 10 min. The complexes were slightly affected by proteinase K degrading enzyme and strongly stable with RNasel, which hydrolyses the ribonucleic acids.
(9) The results reveal that the proteins in the complexes have a specific function or enzymatic effect. Also, the uridine-rich RNAs (snRNA) U1, U4, U5 and U6 which are the same scRNA were found to have an important role in the splicing of the pre-mRNA to give mRNA.

INTRODUCTION

The seeds of *Vicia faba* always maintain their character (morphology and histology) when germinated, despite long storage. How, therefore, does the genetic material stay in the plant cell?

Eukaryotic cells contain a number of metabolically stable (Bochnig *et al.*, 1987), uridine-rich small nuclear RNAs (snRNAs). In addition to tRNA, 5S RNA and 5·8S RNA, plant nuclei contain six low-molecular-mass RNAs, from U1 to U6, respectively. Thus far, U-rich snRNAs have been isolated and characterised with respect to their size and in many cases to both primary and secondary structures from all eukaryotes examined (Michael *et al.*, 1980; Alain & Jean, 1983; Kiss *et al.*, 1985). These structures have shown that U-rich snRNAs are as follows:

(1) linear single-stranded molecules containing 106–215 nucleotide residues, depending on the molecular species;

(2) capped at their 5'-end with 2,2,7-trimethylguanosine (m_3G) for U1 to U5 snRNAs;
(3) contain modified nucleotides;
(4) occur in snRNP particles often associated with hnRNP particles;
(5) Highly conserved in nature with respect to their size as well as their primary and secondary structure; and
(6) metabolically stable.

snRNPs are the antigens recognised by antibodies of high specificity for m_3G caps which strongly react with snRNA caps and do not cross-react with m_7G containing hnRNA or mRNA caps. Therefore, they are used for immunoprecipitation of the isolated snRNPs (Michael *et al.*, 1980; Alain & Jean, 1983; Bringmann & Lührmann, 1986; Bochnig *et al.*, 1987).

The snRNAs associate with a set of proteins to form ribonucleoprotein particles (snRNPs). Twelve polypeptides have been identified as constituents of the major snRNPs U1 to U6. The snRNAs U1, U2 and U5 were organised within separate RNP particles while the U4 and U6 RNAs reside in one and the same ribonucleoprotein complex. RNA–protein complexes are resistant to dissociation in SDS-PAGE (Bringmann & Lührmann, 1986). The nuclear pre-mRNA splicing is mediated by a set of snRNAs (U1–U6) complexed with proteins, indicating that all major nucleoplasmic snRNPs, U1–U6, participate principally in the splicing of nuclear pre-mRNAs (Michael *et al.*, 1987; Paul *et al.*, 1987; Kiss *et al.*, 1988).

This work aims to investigate genetic materials in a complex form with protein and a recent report that the plant cell contains a RNA–protein complex in the form of snRNPs.

MATERIALS AND METHODS

Plant materials

Vicia faba seeds (Trio, from Nickerson-Zwaan BV Holland) were first washed with distilled water, then surface sterilised by washing for 60 s in 0·01% sodium azide and soaked for 20 h. The embryos were collected by removing the hull of the seeds.

Extraction of the crude RNA–protein complex

The crude of RNA–protein complex was extracted according to the method of Kiss *et al.* (1985).

Purification of the complex extract

The extract was purified through a Bio-Rex 70 cation-exchange column sodium from 200 to 400 mesh (h = 7·0 cm, r = 1·6 cm), and washed by 1 × HO buffer. The wash was collected as a maximal absorption at 254 nm, and concentrated by using ultrafiltration through a 20 kDa membrane.

Fractionation over glycerine gradient

The complex concentrate was fractionated over a glycerine density gradient (from 10 to 30% glycerine) according to the method II of Kiss *et al.* (1985). Fractions (10–30) were collected and subjected to dialysis and concentrated by ultrafiltration.

Complex separation on a Whatman DE-52 column

The concentrated fractions were applied to a Whatman DE-52 anion-exchange column (Pharmacia, r = 0·8 cm, h = 12 cm) and eluted with different concentrations of the KCl buffer according to Bringmann and Lührmann (1986). Peak fractions were collected and concentrated by ultrafiltration.

Complex and urea gel electrophoresis

The concentrated peak fractions from the DE-52 column were applied with a tRNA/5s RNA from yeast as a marker to a 5·7% native gel electrophoresis (complex gel) according to the method of Pieler (1984), and also to a 10% urea gel electrophoresis containing 7 M urea according to the method of Stegemann *et al.* (1987).

Gel staining and elution of complex bands

Both the urea and complex gels were first stained with ethidium bromide (Pieler, 1984), followed by 'Stains-all' staining according to Kay *et al.* (1964). Complex bands were cut and eluted according to Pieler (1984).

Precipitation, dialysis and concentration of the complex

Precipitation by ethanol was done according to the method of Pieler (1984), the precipitate was freeze-dried then dissolved in 50 μl water and subjected to microdialysis according to Marusyk and Sergeant (1980). The dialysed sample was collected, concentrated then kept at −20°C until use for 3'-end labelling.

3'-end labelling of the complex

The purified bands were 3'-end labelled by incubation with (5'-^{32}P) PCP (3000 Ci/mmol, Amersham) according to the procedure of England and Uhlenbeck (1978), then subjected to a 10% polyacrylamide gel (containing 8 M urea), at 1500 V for 3 h. The labelled fractionated bands were cut and eluted (Pieler, 1984) for the further steps.

Extraction of the complex by phenol method

The method of extracting RNA with phenol according to Pieler (1984) was used but by using (not discarding) the interphase which contains the RNA–protein complex (snRNPs). The interphase was carefully collected, washed with ether several times to remove the remaining phenol, centrifuged at 3000 rpm/30 s/4°C. The precipi-

tate was freeze-dried, then dissolved in a small volume of distilled water, and measured at 260 nm.

Fractionation and identification of the interphase complex bands

The interphase complex was fractionated on a complex gel (native gel) and stained with Stains-all. The three identified bands were cut, eluted from the gel (Pieler, 1984), then freeze-dried and dissolved in 50 μl distilled water. Microdialysis was done according to the method of Marusyk and Sergeant (1980).

Comparison between interphase and DE-52 column complex bands

A comparison between the complex bands separated from the DE-52 column and the interphase was done by 3'-end labelling and subjecting to a 8 M urea 10% gel electrophoresis, as mentioned by Pieler (1984).

Protein complex identification

The purified complex bands were applied to SDS-PAGE 12% according to the method of Laemmli (1970).

The stability tests of the RNA–protein complexes

Stability tests for cyano-bromide, proteinase K, guanidine–HCl (6 M), SDS 10% and RNase-A were done by special communication with Dr Corinna Lippmann (Inst. of Biochem., Thielallee 63, 1000 Berlin 33, Germany). The samples were subjected to 12% PAA/SDS, urea and native gel electrophoresis.

Separation of the complex by high-speed gel filtration

The RNA–protein complex samples were separated by using a column of silica-based aqueous gel, TSK-GEL G 3000 SW according to the method of Uchiyama *et al.* (1981), using a modified buffer system (50 mM Tris, 20 mM $MgCl_2$, pH 7·0). The run was done twice, at 260 and 280 nm, respectively, using the computer program 'Gold system' (LKB, Germany) loaded on an IBM computer, in connection with the HPLC from LKB.

RESULTS AND DISCUSSION

Extraction of the crude RNA–protein complex

The extraction of the cytoplasmic crude RNA–protein complex from the embryos of 20 h-soaked *V. faba* seeds was subjected to UV spectroscopy in the range 220–300 nm (Fig. 1). This showed a low absorption peak between the wavelengths 260 and 280 nm accompanied by three maximum absorptions at 272·7, 276 and 277·8 nm, respectively, indicating that there are probably three RNA–protein complexes.

Bio-Rex 70 cation-exchange column

The total crude extract was purified through a Bio-Rex 70 cation-exchange column at 254 nm in order to remove any cations which may affect the absorption, the elution was subjected to UV spectroscopy in the range 220–300 nm. The results shown in Fig. 1 reveal a higher absorption than that of the total crude extract. A maximum absorption at 277·1 nm was detected, representing the absorption of RNA and protein together as a complex according to the following observations:

(1) the purified crude material was collected at 254 nm which is similar to RNA absorption and means that the complex contained RNA; and
(2) the UV spectrum in Fig. 1 indicates that this purified crude complex contains protein because the maximum absorption is close to the protein area at 280 nm and not pure RNA; otherwise it would give a maximum absorption near 254 nm.

Glycerine 10–30% gradient

After ultracentrifugation, the gradients were fractionated using UV absorbance at 254 nm. The spectrum in Fig. 1 shows a sigmoid curve through 35 fractions; the area which gave the highest absorption (fractions 10–30) was collected for further characterisation.

DE-52 anion-exchange column

Figure 1 shows the results of a DE-52 anion-exchange column fractionation of the collected glycerine gradient fractions. The eluate was fractionated using a spectrophotometer at 254 nm; the fractions were subjected to the UV spectrum, ranging from 220 to 300 nm. Only fraction number 13 was noticed to show a profile of the complex as shown in Fig. 1 with higher absorption than those of the previous purification stages. Also fractions A, B and C showed a profile of pure RNA like Fig. 1.

The previous results are in accordance with those of Pieler (1984) and Kiss *et al.* (1987) who separated the complex from the nuclei but our complex is from the cytoplasm.

RNA–protein complex in the interphase

After the extraction of the RNA with phenol, it was clear that the interphase had an accompanying layer which may be a kind of RNA–protein complex. In a further study, through the UV spectrum, this layer was shown to be a RNA–protein complex as we expected because its absorption ranged between 260 nm (RNA area) and 280 nm (protein area) as elucidated in Fig. 2(e). This revealed that the interphase has three absorption maxima at 265, 269·6 and 275 nm, respectively, which are in the area between the RNA (254 nm) and protein (280 nm) absorptions.

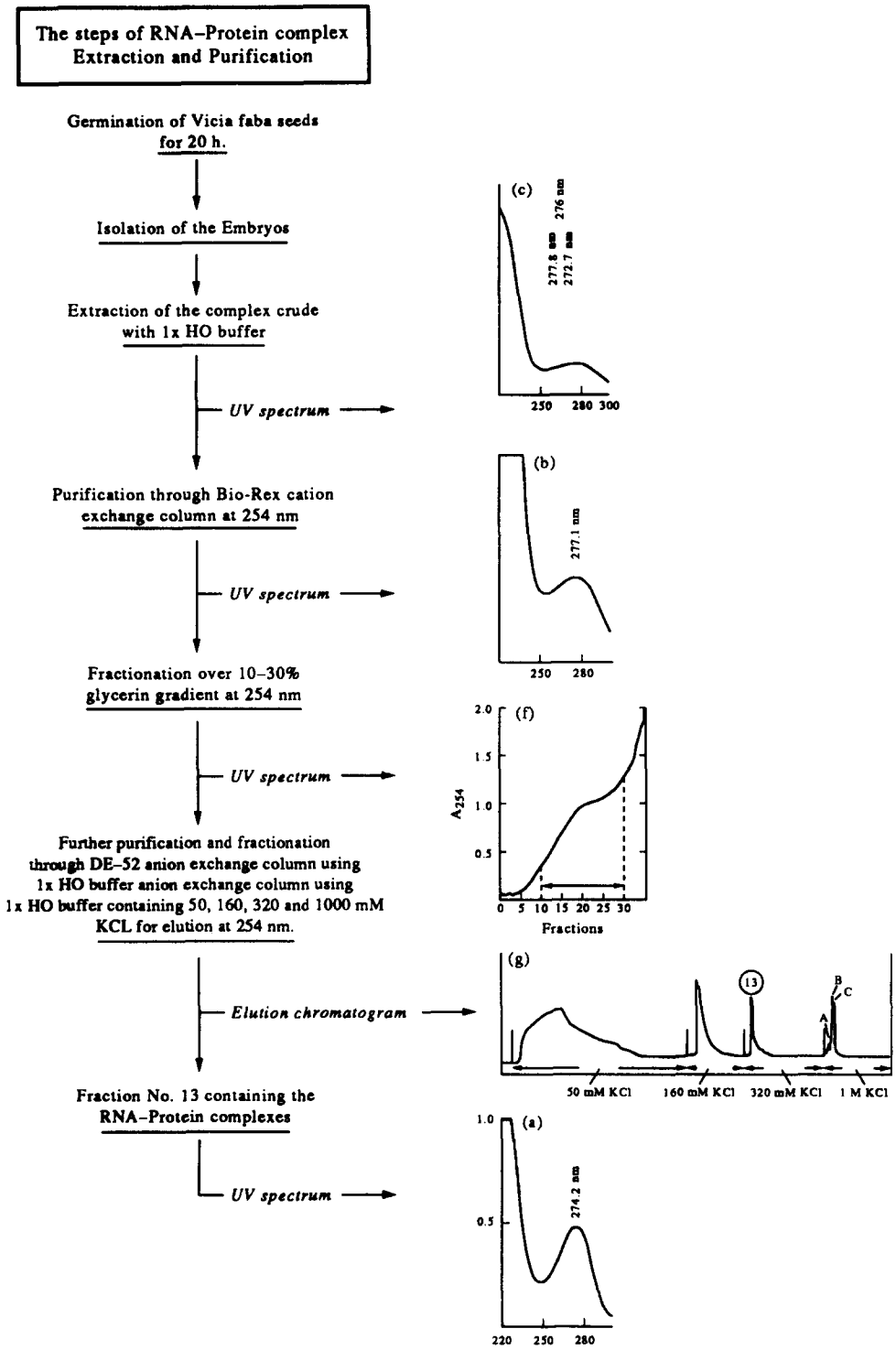

Fig. 1. The steps of RNA–protein complex—extraction and purification.

Separation of the interphase content on HPLC

The separation of interphase extract by HPLC using a TSK-GEL G 3000 SW column and eluted by the modified Tris–HCl buffer pH 7·0 into RNA content at 254 nm (Fig. 3(A)) and protein content at 280 nm (Fig. 3(B)) reveals the following: (i) the interphase contains three protein peaks, C1, C2 and C3, at 82, 87 and 92 min, respectively, when separated at 280 nm; (ii) it contains three RNAs peaks, C1, C2 and C3, at 82, 86 and 96 min, respectively, when separated at 254 nm in addition to the other species of common RNAs; (iii) the three RNA peaks and the three protein peaks, C1, C2 and C3, are eluted after the same time, which means that the three proteins are bound to those RNAs making three complexes of RNA–protein complex. This hypothesis is supported by the interphase UV spectrum Fig. 2(e).

PAGE (native, urea and SDS)

The extracted fractions from different steps of purifi-

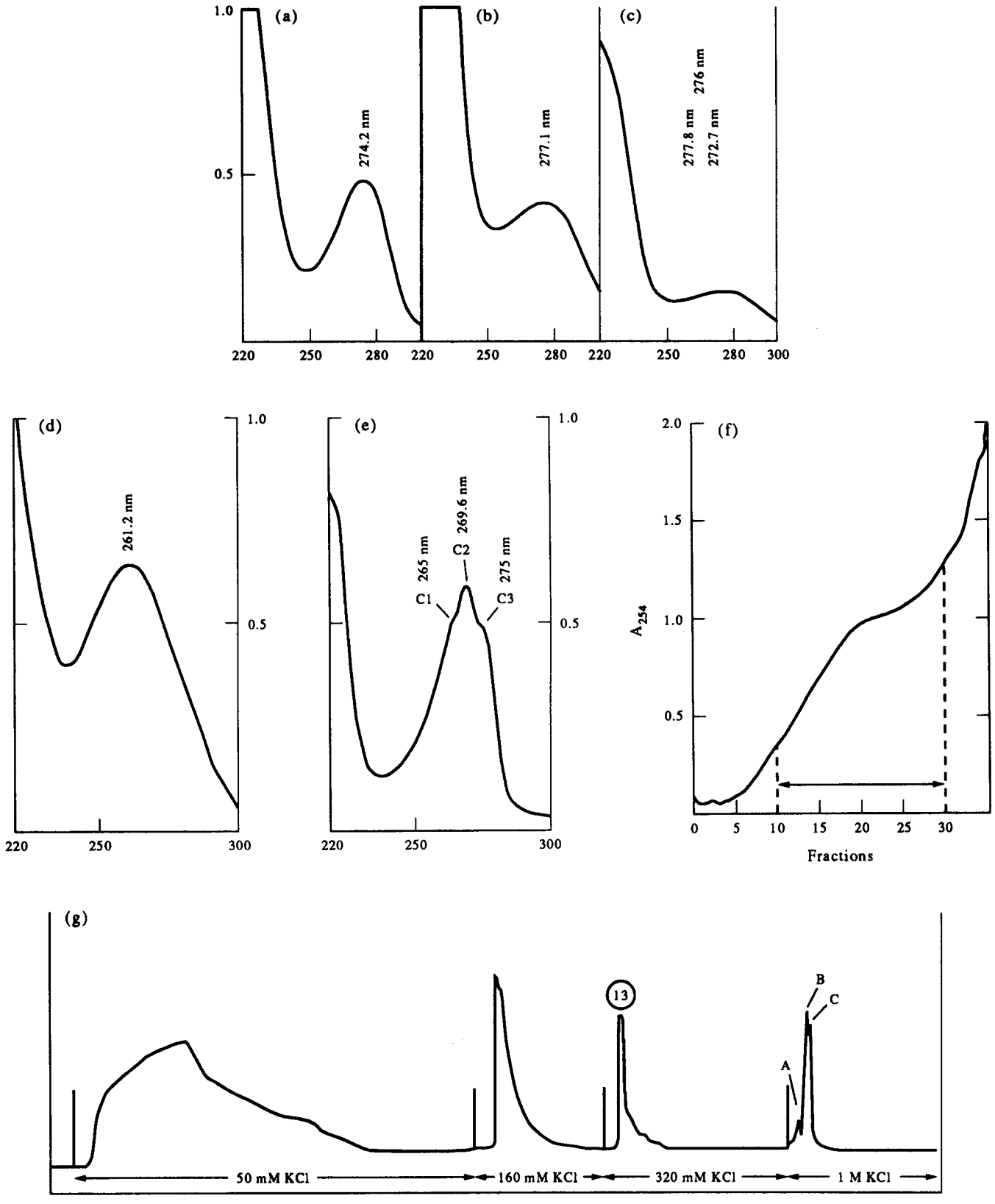

Fig. 2. UV spectrums for several fractions to follow the RNA–protein complexes. (a) Fraction 13 from the second column (DE-52). (b) Aliquot after the first column (Bio-Rex). (c) Total crude extract before any treatment. (d) Total RNA (phenol extract). (e) Interphase. (f) UV spectrum of the purified crude from the Bio-Rex column over 10–30% glycerine gradient at A_{254}. Collected fractions are shown by the arrows. (g) Ion-exchange chromatogram of the collected fractions from 10–30% glycerine gradient. 13, RNA–protein complex peak; A–C, RNA peaks.

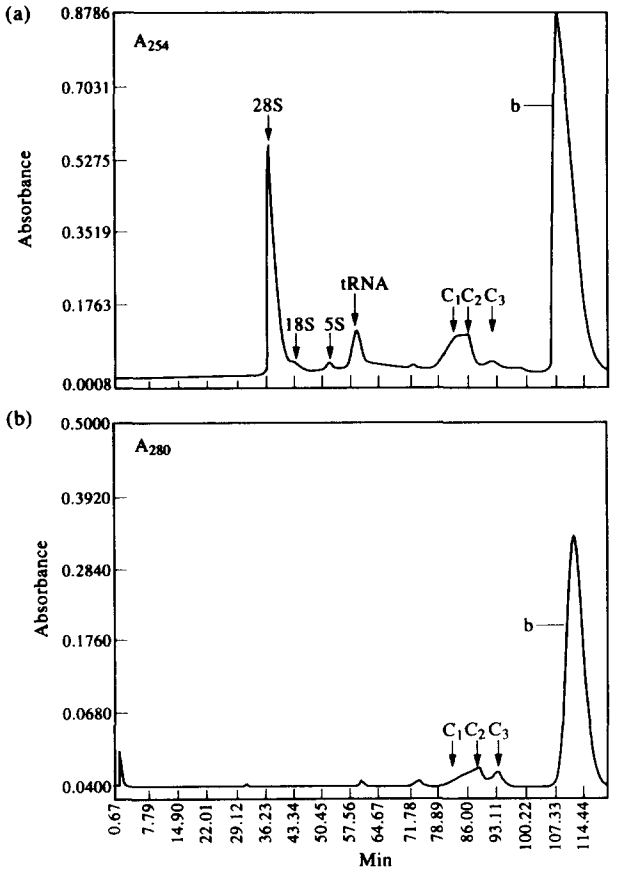

Fig. 3. HPLC charts of the interphase to look for the RNA–protein complexes (C_1, C_2 and C_3) at A_{254} and A_{280}; b is the buffer peak.

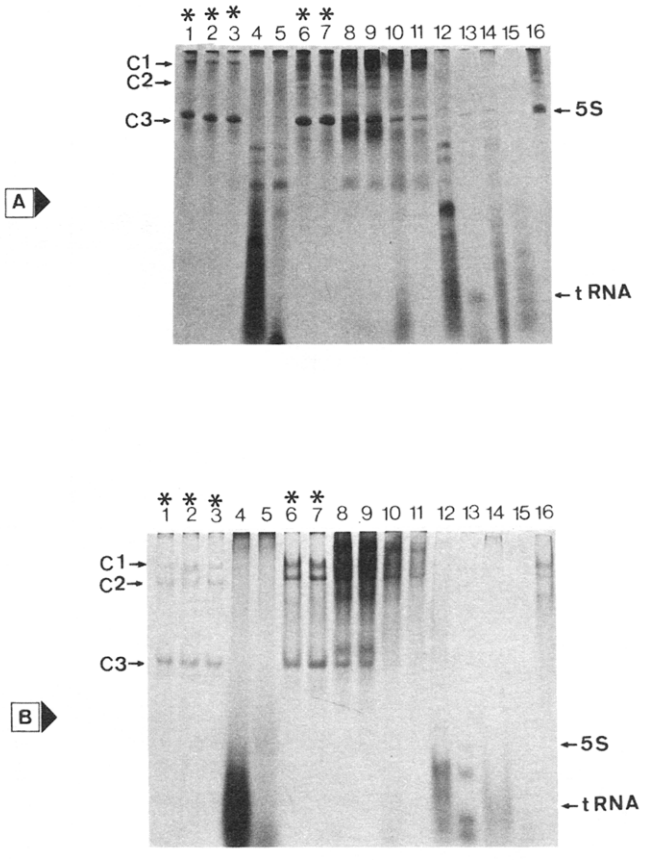

Fig. 4. Stability of the complexes (snRNPs) for the SDS and RNase 1, and comparison between the complexes from several stages of separation on (A) urea and (B) native gel. *, where the clear C1, C2 and C3 are complexes; 1, interphase complexes; 2, 1 and treated with 10% SDS/100°C/10 min; 3, 1 and treated with RNase 1; 4, phenol extracted total RNA from 24 h-germinated seed; 5, 4 and treated with RNase 1; 6, complexes of fraction 13 from second column; 7, 6 and treated with RNase 1; 8, compl. gradient No. 10 (fractions 10–25 concentrated); 9, 8 and treated with RNase 1; 10, concentrated aliquot after the first column; 11, 10 and treated with RNase 1; 12, fraction (B) from second column; 13, 5SrRNA treated with RNase 1; 14, 5SrRNA marker from spinach chloroplast; 15, tRNA marker from spinach chloroplast; 16, complexes of fraction 14 from second column.

cation and also the interphase were subjected to PAGE analysis (native, 7 M urea and SDS 12% gels). The gels were stained with ethidium bromide (for RNA), Stains-all (for RNA and protein together either free or bound with each other) and Coommassie blue for SDS-PAGE. Staining with Stains-all depends on colours, where conjugated proteins stained red–violet, unconjugated proteins stained red and DNA/RNA stained blue to blue–violet (Kay *et al.*, 1964).

Figure 4 shows the collected fractions from glycerine

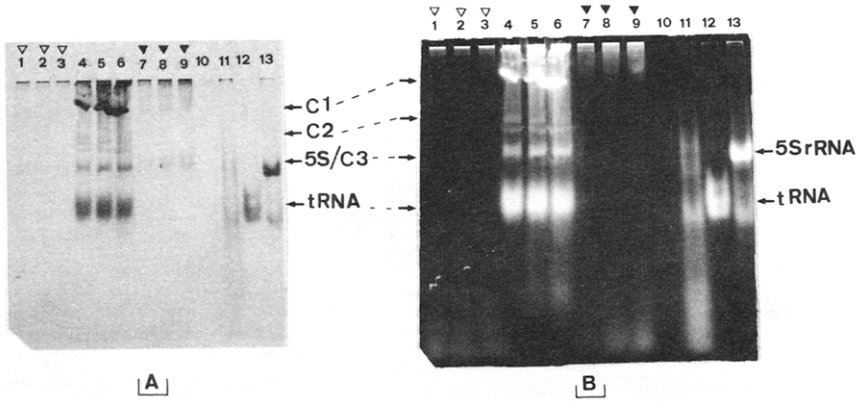

Fig. 5. Fractionation of the three snRNPs complexes from fraction 13 of *Vicia faba* 20 h-germinated seeds; total extract of 1, 2 and 3 day-germinated seeds and phenol extract of both total extracts and the fraction 13 on 10% PAA 6 M urea gel. (A) Stains-all staining; (B) ethidium bromide staining. ∇, Where the complexes in total extract 1, 2 and 3 day-germinated seeds are present; ▼, where the complexes snRNPs C1, C2 and C3 are present in fraction 13; 1–3, total extract of 1, 2 and 3 day-germinated *V. faba* seeds; 4–6, phenol extract of 1, 2 and 3 day-germinated *V. faba* seeds; 7, 0·1 OD A_{260} of the fraction 13 complexes; 8, 0·15 OD A_{260} of the fraction 13 complexes; 9, 0·2 OD A_{260} of the fraction 13 complexes; 10,11, phenol extract of fraction 13 complexes; 12, tRNA marker from yeast; 13, 5S rRNA and tRNA marker from yeast.

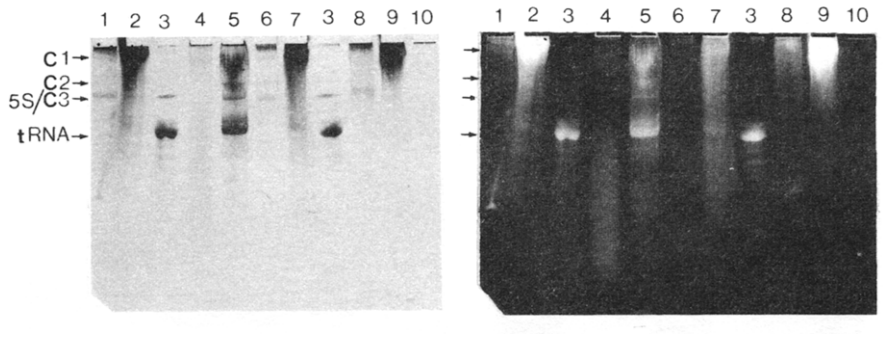

Fig. 6. Stability of the interphase complexes (snRNPs) and RNAs for guanidine–HCl 6 M and RNase 1 on 6 M urea gel. (A) Stains-all staining; (B) ethidium-bromide staining. 1, Interphase complexes C1, C2 and C3 without treatment; 2, phenol-extracted total RNA from 20 h-germinated seeds; 3, tRNA marker including 5srRNA from yeast; 4, protein extract from 24 h-germinated seeds; 5, phenol extracted total RNA from 24 h-germinated seeds; 6, 1 and treated with guanidine–HCl 6 M; 7, 2 and treated with guanidine–HCl 6 M; 8, 1 and treated with RNase 1; 9, 2 and treated with RNase 1; 10, 5 and treated with RNase 1.

gradients (lanes 8 and 9), Bio-Rex column (lanes 10 and 11), DE-25 column as fraction 13 (lanes 6 and 7) and the interphase (lanes 1–3) where they were subjected to native gel 5·7% and stained with Stains-all. The results showed that they all have the three complex bands C1, C2 and C3, and the interphase and fraction no. 13 from the DE-25 column are the clearer bands coloured blue–reddish which is a mixture between blue–violet (RNA) and red (protein). The difference in colour between the complex bands is due to the RNA (blue–violet)/protein (red) ratio in the complex.

It is remarkable that the migration of the complex band (C3) is similar to 5S rRNA on urea gel (Fig. 5(A), lanes 7–9), but not on native gel (Fig. 4). This indicates that the protein affected its migration on native gel, otherwise it would be like urea gel in migration. On the other hand, in fraction 13 (Fig. 5(A), lanes 7–9) C1, C2 and C3 complex bands appeared when stained with Stains-all and did not appear when stained with ethidium bromide (Fig. 5(B), lanes 7–9) indicating that RNAs in the complexes are bound or covered strongly with protein.

Stability of the RNA–protein complexes

The three complexes C1, C2 and C3 either of the interphase or fraction no. 13 were found to be strongly stable (i.e. no differences or changes between treated and untreated samples) for

(1) SDS 10%/100°C/10 min which acts as protein denaturating agent; see the results present in Fig. 4 (lane 1 untreated, lane 2 treated) stained with Stains-all on urea gel;
(2) guanidine–HCl 6 M which acts as denaturating salt; see the results in Fig. 6 (lane 1 untreated, lane 6 treated) stained with Stains-all and ethidium-bromide on urea gel;
(3) cyanobromide which degrades the proteins containing methionine; see the results in Fig. 7 (lane 1 untreated, lane 3 treated) on 12% SDS-PAGE; and
(4) RNase 1 which hydrolyses the RNA; see the results in Fig. 6 (lane 1 untreated, lane 8 treated) stained with Stains-all and ethidium bromide on urea gel.

On the other hand, the complex proteins were slightly affected by proteinase K, the protein degrading enzyme (hydrolyses the peptide bonds after the amino acid arginine). The results show two dark bands down the gel in addition to the dark tail and also disappearance of the first main protein as mentioned in Fig. 7 (lane 1 untreated, lane 3 treated) on 12% SDS-PAGE.

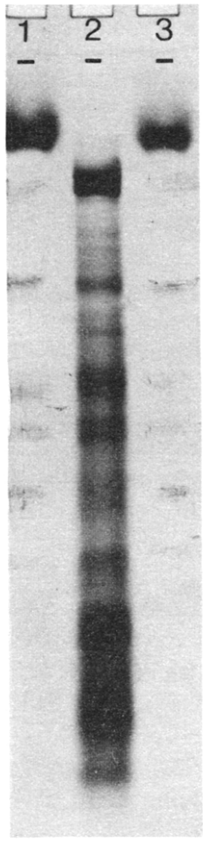

Fig. 7. Stability of the interphase proteins for proteinase K and cyano-bromide on SDS-PAGE 12% stained with Coommassie blue. 1, Interphase protein complex; 2, 1 treated with proteinase K; 3, 1 treated with cyano-bromide.

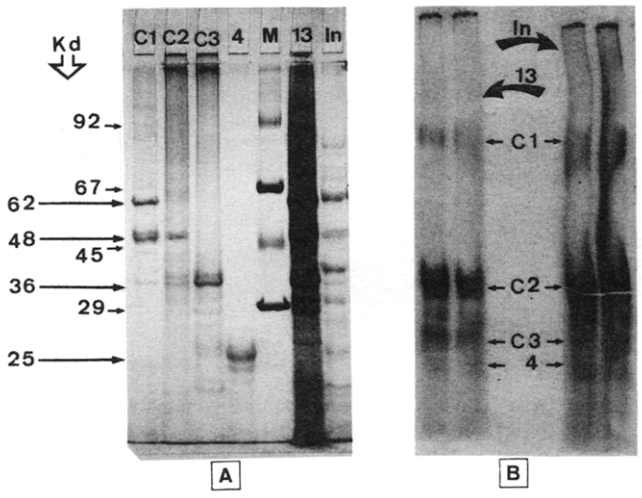

Fig. 8. (A) Identification of the complex proteins and estimation of their molecular weight on SDS-PAGE 12% stained with Coommassie blue. C1, First RNR–protein complex and has two proteins with a molecular weight of 62 and 48 kDa, respectively; C2, second RNA–protein complex and has only one protein with a molecular weight of 48 kDa; C3, third RNA–protein complex and has one protein with a molecular weight of 36 kDa; 4, fourth RNA–protein complex and has one protein with a molecular weight of 25 kDa (it was difficult to obtain high enough concentration of this complex so it was not in the main study); M, P_4 protein marker contains phosphorylase B 92·0, albumin 67·0, ovalbumin 45·0, carbonic anhydrase 29·0; 13, fraction no. 13 eluted from the second column (DE-52) by NaCl 1 M, and has all four RNA–protein complexes (C1, C2, C3 and C4); In, interphase RNA–protein complexes and has the same four complexes like fraction 13. (B) Identification of the complex bands in both fraction 13 and the interphase on native gel 5·7% stained with Stain-all. 13 and IN, fraction no. 13 and total interphase; C1, C2, C3, 4, the four complexes of both fraction 13 and interphase.

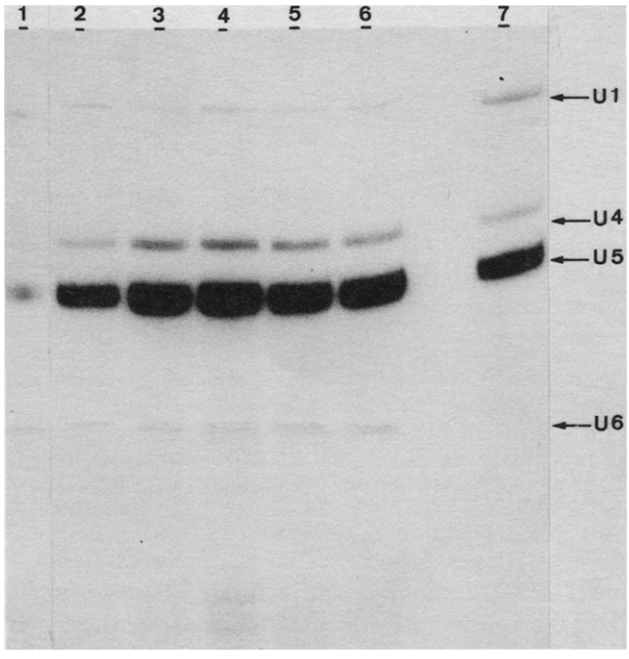

Fig. 9. This film after 30 s exposure to the radioactive 3' labelling gel. Identification of the three snRNPs complexes isolated from fraction 13 of *Vicia faba* 20 h-germinated seeds by comparison with total snRNPs (U1, U4, U5 and U6) of *V. faba* leaves of the same plant and also with the same snRNPs from Hela cells on urea 8 M gel. 1 and 2, *Vicia faba* leaves total snRNPs (U1, U4, U5 and U6) as marker from Dr Corinna Lippmann, Institut für Biochemie, FU, 1000 Berlin 33; 3, Hela cells total snRNPs (U1, U4, U5 and U6) as marker obtained from the same person; 4–6, the three RNA–protein complexes C1, C2 and C3 isolated from fraction 13, respectively; 7, total interphase snRNPs complexes of 20 h.

Identification of the complex proteins

The three complex bands C1, C2 and C3 were cut from the native gel, eluted, then concentrated and subjected to 12% SDS-PAGE. The results presented in Fig. 8(A) show that each complex contains two major proteins where C1 has a molecular weight of 62 and 48 kDa, C2 48 and 36 kDa and C3 36 and 25 kDa, respectively, and two minor proteins where C1 has a molecular weight of 36 and 25 kDa, C2 62 and 19 kDa and C3 29 and 20 kDa, respectively. The interphase and fraction 13 has a fourth complex band (Fig. 8(B)) when plotted with a high concentration on the native gel stained with Stains-all. It contains only one major and one minor protein with 25 and 23 kDa, respectively. The difference between the highest molecular weight major proteins of each complex indicated a relationship which was 12 ± 1 kDa (C1 has 62 kDa, C2 has 48 kDa, C3 has 36 kDa and complex four has 25 kDa. These results reveal that these proteins have a specific function, maybe as a subunit of an enzyme.

Identification of the complex RNAs

Results in Fig. 9 show that each of the complexes C1, C2 and C3 contain the same types of snRNAs U1, U4, U5 and U6, where U4 and U5 are major and U1 and U6 are minor, in addition to tRNAs and 4·5S RNA. The total interphase (lane 7) contains the same type of RNAs in addition to another type of RNA close to U1 which might be 5·8S RNA (as mentioned by Bochnig *et al.*, 1987).

These complexes were found in the cytoplasmic extract and not in the nucleus. The nucleus should be first isolated from the cell then hydrolysed to get the snRNAs previously reported (Michael *et al.*, 1980; Alain & Jean, 1983; Kiss *et al.*, 1985; Bringmann & Lührmann 1986; Bochnig *et al.*, 1987); thus the authors suggest that these complexes are called small cytoplasmic RNA (scRNA) instead of small-nuclear RNA. According to their knowledge these findings are original.

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