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An improved direct RNA sequence method; its application to Vicia faba 5.8S ribosomal RNA

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ABSTRACT

We have developed a direct read-off sequencing procedure, based on the method of Stanley and Vassilenko using E. coli 5S ribosomal RNA as a model compound. Radioactive bands were transferred from an acrylamide gel fractionation in the first dimension onto a DEAE-cellulose thin layer plate. After in situ enzymatic digestion with RNase T_2, mononucleoside 3',5'-diphosphates were separated in the second dimension by electrophoresis at pH 2.3. Using this two-dimensional procedure the entire sequence of 163 residues of the previously unknown Vicia faba (broad bean) 5.8S ribosomal RNA was deduced.

INTRODUCTION

Several direct RNA sequencing methods have been developed recently based on either enzymatic or chemical degradation of end-labelled RNA, followed by analysis on high resolution polyacrylamide gels (1-6). Stanley and Vassilenko (7) have reported a quite different method relying on the chemical hydrolysis of RNA with hot formamide. Under defined conditions hydrolysis is limited to one "hit" per molecule resulting in the liberation of a single internal 5'-hydroxyl group which can then be labelled with γ-^{32}P-ATP and polynucleotide kinase. After fractionation on an acrylamide gel, bands were individually eluted and their 5' end groups were determined by alkaline hydrolysis followed by paper electrophoresis.

To overcome this somewhat laborious procedure, we have devised a "blotting" procedure to transfer the labelled fragments from the gel to a DEAE-cellulose thin layer (TL) plate. The transferred fragments were digested in situ with a RNase mixture (i.e. RNase T_2, T_1 and A) and the resultant nucleoside 3', 5'-diphosphate end groups were separated in a second dimension by electrophoresis at pH 2.3. This electrophoretic separation gave "sharp" spots and seemed an optimum system for "reading off" a sequence.

We have used the 5S ribosomal RNA (rRNA) of E. coli as a model compound of

known sequence to devise the method and to test a number of variables. We then sequenced broad bean (Vicia faba) 5.8S rRNA to test the efficiency of the method on an unknown compound. The method was simple, had a high transfer efficiency (between first and second dimension) and there was a clear separation of the four major nucleoside 3',5'-diphosphates. In the case of broad bean 5.8S rRNA four minor bases - Am, Gm and two residues of pseudouridine (ψ) - were detected in the sequence of 163 residues. Only at the ends of the molecule (residue 1 and residues 159-163) were other methods required to complete the sequence. Oligonucleotide maps determined previously (8) confirmed the sequence and provided evidence for residues 48 and 80 which were adjacent to the 2'-O-methylated residues, Am and Gm respectively, which were only tentatively assigned in the new procedure.

MATERIALS AND METHODS

Materials

5S rRNA (E. coli MRE 600) and yeast $tRNA^{Phe}$ were obtained from Boehringer Mannheim, Germany, formamide (AnalaR) from BDH. γ-^{32}P-ATP (3000 Ci/mmole) and (5')-^{32}P-pCp (2000 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, and New England Nuclear, respectively. RNase T_1 (Sankyo), RNase T_2 (Calbiochem), pancreatic RNase A (Worthington Biochemicals), RNase P_1 (Sigma), bacterial alkaline phosphatase (Boehringer), polynucleotide kinase and RNA ligase (P.-L. Biochemicals) were used without further purification. DEAE-cellulose TL plates (polygram CEL 300 DEAE, 40 x 20 cm, batch number 73, and polygram CEL 300 DEAE/HR-2/15, 40 x 20 cm) are products of Macherey-Nagel (Germany). Cellulose coated glass plates (20 x 20 cm, without fluorescence indicator) is a product of E. Merck. Glassware and reagents were sterilised if possible. All RNA samples used here were purified once using 8% acrylamide-7 M urea thin gels (9). The RNA was eluted from the excised gel in 0.5 M ammonium acetate, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.5% sodium dodecyl sulphate at 37°C for 2 hr without crushing.

Broad bean RNA was extracted from leaves by the naphthalene-1,5-disulphonic acid-phenol method and 5.8S rRNA purified as previously described (10).

Methods

(1) Hydrolysis in formamide and 5' end-labelling of RNA

0.1-0.5 µg of purified RNA dissolved in 1-2 µl of formamide was hydrolysed in a sealed, siliconised disposable micropipette (5 µl, Clay Adams) in a boiling water bath for 5 min. The heat-treated RNA was blown into 50 µl of 0.3 M sodium acetate (pH 5.5) and recovered by the conventional ethanol

precipitation method. The precipitated RNA was dissolved in 5 µl of 1 x polynucleotide kinase buffer (7) and 0.2-0.3 µl (2-3 units) of polynucleotide kinase. The mixture was transferred into an Eppendorf tube containing dried γ-^{32}P-ATP (3 pmoles) and incubated at 37°C for 60 min. An alternative reaction mixture (to obtain an even "phosphokination" profile for 5.8S rRNA) contained 2 µl of polynucleotide kinase buffer, 0.5 µl of dimethylsulphoxide and 0.2 µl of polynucleotide kinase. After incubation, one volume of formamide dye mixture (1) was added to the reaction mixture. Sometimes there were difficulties in obtaining a ladder. This may be caused either by degradation of RNA during its elution from acrylamide gels or by nonspecific RNase contamination of the T4 polynucleotide kinase.

(2) Gel electrophoresis and preparation of gel strips containing the radioactive ladders

The labelled sample was heated for 5 sec in a boiling water bath before loading 2-3 µl aliquots at time 0 and after 4 hr onto a long, 8% acrylamide-7 M urea thin gel (gel 80 x 30 x 0.05 cm: loading slots are 0.2 cm wide) (11). Electrophoresis was for 8 hr at constant voltage (2.9 kV) and about 35 mA. After the run, one glass plate was removed from the gel and two "used" X-ray films (43 x 35.5 cm) were placed end to end over the gel surface, completely covering it. The gel was cut into two halves along the contact line of the two films. The other glass plate was now removed, leaving the two halves of the gel stuck to each of the X-ray films. The strip of gel (3 mm wide, 38 cm long) containing the radioactive ladder was located by radioautography (3-6 hr, room temperature) and cut using the newly exposed film as a template. It was then transferred, after removing its Saranwrap covering, onto another "used" X-ray film (3 x 43 cm).

(3) Transfer to a DEAE-cellulose TL plate

Before use, DEAE-cellulose TL plates (polygram CEL 300 DEAE, 40 x 20 cm) must be washed with 25-50 mM EDTA (pH 4-5) by ascending chromatography until the front has almost reached the top (about 20 cm, for 2-2½ hr) and air dried. Plates were then washed with distilled water by ascending chromatography and air dried.

The dried plate was freshly wetted along the origin line (0.5-0.8 cm wide) using a 3MM paper soaked in 1.0 M ammonium acetate (pH 4.5). (The origin line is 2-3 cm from the edge that was dipped in the EDTA washing solution.) The cut gel strip on a "used" X-ray film was placed on the origin of the TL plate, and a plain glass plate and weight (usually two big bottles, ~1 kg each) were placed on it (see Fig. 1). After leaving overnight (10-15 hr) at

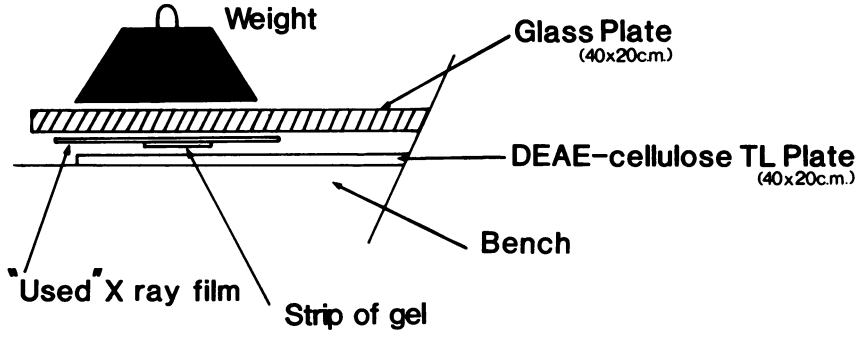

Figure 1. An illustration of the blotting procedure of 5' end-labelled fragments from the gel to the DEAE-cellulose TL plate. A space has been left between the glass plate and the "used" X-ray film as well as between the gel and the TL plate for clarity. In reality all these layers are in contact with one another. For details see Methods.

room temperature, the TL plate was separated from the gel strip, dried in air and finally soaked in distilled water (not methanol) for 2 min to remove urea and buffer and dried again.

(4) In situ enzyme digestion on TL plate

A solution of RNase T_2 (0.2 u/µl), RNase T_1 (0.2 u/µl) and pancreatic RNase A (0.1 µg/µl) in 0.1 M ammonium acetate buffer (pH 4.5) was pipetted onto the origin line (0.5 cm wide) of the TL plate containing RNA fragments transferred from the gel, by streaking with a disposable micropipette (20 µl) using 130-150 µl/40 cm. The TL plate was wrapped with Saranwrap, sandwiched firmly between two glass plates (40 x 20 cm) with "bulldog" clips to prevent evaporation and incubated for 2-4 hr at 37°C. After the enzyme treatment, the plate was immersed in methanol for 5-10 min and dried.

(5) Electrophoresis

The TL electrophoresis apparatus has a metal plate (42 x 21 cm) for cooling with water (15°C). Electrophoresis buffer (pH 2.3) was a mixture of 500 ml of 8% acetic acid, 2% formic acid and 5 ml of 0.5 M disodium EDTA. For wetting the thin layer, the same solution (except made up in 20% acetone) was used and was applied gently with 3MM paper. Electrophoresis was at 400 V (20 V/cm) for 1 hr. After air drying, the plate was exposed to preflashed film against a phosphotungstate screen at -70°C (12). Some variation in the resolution of the G and U lines (see Figs. 3 and 5) has been observed and is probably the result of small variations in the pH of the electrophoresis.

(6) Detection of modified bases

Modified bases were detected as follows: A spot was cut out from the DEAE-cellulose TL and eluted with 0.1 ml of 30% triethylamine carbonate (TEC) in an Eppendorf tube for 3 hr. After centrifugation using an Eppendorf centrifuge (5412) for 5 min, an aliquot was pipetted out and freeze-dried in a tube. The freeze-drying was repeated twice by adding 50 µl of water for complete evaporation of TEC. The residue was dissolved in 5 µl of P_1 nuclease solution (0.1 µg/ml) in buffer (50 mM sodium acetate, pH 5.5) and left for 4 hr at 37°C. The resultant mononucleoside 5'-phosphates were fractionated on cellulose TL plates using two different solvent systems (propan-2-ol:HCl:H_2O, 70:15:15 v/v) for 16 hr and (isobutyric acid:H_2O:conc. NH_4OH, 100:58.5:1.5 v/v) for 12 hr (13). As markers, radioactively phosphorylated pU, pC, pA, pG, pGm, pCm and pΨ were all prepared from nuclease P_1 digest of yeast $tRNA^{Phe}$ using the sequencing method described in this paper. In addition, for 2'-O-methylated nucleotides, radioactive nucleoside 5'-monophosphates obtained by treatment with nuclease P_1 were separated by high voltage paper electrophoresis on Whatman No. 52 paper at pH 3.5 (3 kV constant, 25 min).

(7) Sequence analysis near the 5' and 3' ends of 5.8S rRNA

The 3' end of the RNA was labelled using (5')-^{32}P-pCp (14) and purified on 8% acrylamide-7 M urea thin gel. The 3' terminus was determined by digestion with RNase T_2 (0.5 units in 5 µl 0.1 M ammonium acetate, pH 4.5) followed by high voltage paper electrophoresis (pH 3.5, 3 kV constant, 25 min) to identify the mononucleoside-3'-phosphate. The sequence near the 3' end was analysed by the "wandering spot" method on heated RNA (see conditions below) on a DEAE-cellulose TL (polygram CEL 300 DEAE/HR-2/15, 40 x 20 cm) using 3% homomixture C (15). For the sequence near the 5' end, the 5' terminal phosphate was labelled with γ-^{32}P-ATP and T_4 polynucleotide kinase (16) and purified on an 8% acrylamide-7 M urea thin gel. After recovering RNA from the gel it was hydrolysed in boiling water for 2.5 min. The 5' terminus and the first several residues were determined from their M values after electrophoresis on EDTA-washed DEAE-cellulose TL at pH 1.9 (400 V, 3 hr) (15).

RESULTS

(1) Transfer of ^{32}P ladder from acrylamide gels to DEAE-cellulose TL

It was first necessary to achieve efficient transfer of the labelled ladder from the acrylamide gel to the DEAE TL. Using a simple blotting procedure (Fig. 1 and Methods) we compared (Fig. 2) the transfer efficiency

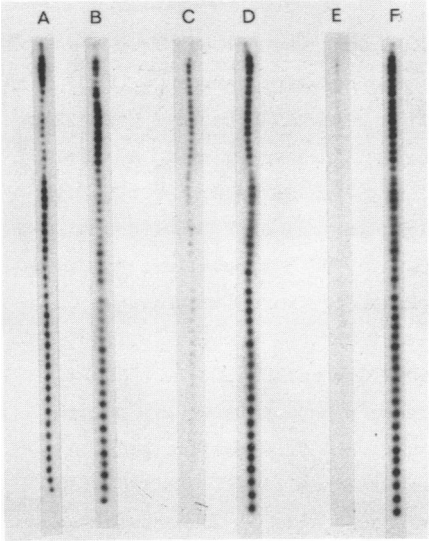

Figure 2. Effect of salt concentration on transfer efficiency. Autoradiograms of the material transferred to the DEAE-cellulose TL plates (B, D and F) and the residual material left on the gels (A, C and E). E. coli 5S rRNA fragments (lengths from 65 to 115 long) were transferred with 0.2, 0.5 and 1.0 M ammonium acetate buffer, pH 4.5, respectively.

of 5' end-labelled 5S rRNA fragments from acrylamide gels to DEAE-cellulose TL plates under three different conditions. The results show clearly that the highest concentration of the "blotting" buffer (1.0 M ammonium acetate, pH 4.5) gave the best (>90%) transfer. In addition, there was no detectable loss of resolution in the ladder after transfer.

Further experiments (data not shown) established that the conditions chosen for subsequent in situ RNase digestion (see Methods) did not cause diffusion of these bands on the thin layer.

(2) Application to E. coli 5S rRNA

Figure 3 shows an autoradiogram of the two-dimensional electrophoretic sequencing procedure applied to E. coli 5S rRNA. The spots fall into four vertical lines (labelled G, U, A and C) corresponding to the 5' end nucleoside 3',5'-diphosphates released. The sequence from residue 4 (a C residue) to residue 90 (a C residue) can be read off (numbering from the 5' end of the 5S rRNA). The only slight difficulty is that there is some diffusion of spots in the C line so that it is necessary to align with the ladder to establish the number of C residues in a "run" (e.g. C_{26-28}). The sequence deduced here agrees completely with the previously established sequence (17) except that G-G-G (see arrow) was observed instead of G-G-G-G at positions 83-86. We interpret this as a gel "compression" (1,18) caused by strong local base-pairing in this region. This procedure also shows the microheterogeneity of nucleotide sequence present in E. coli 5S rRNA. In addition to

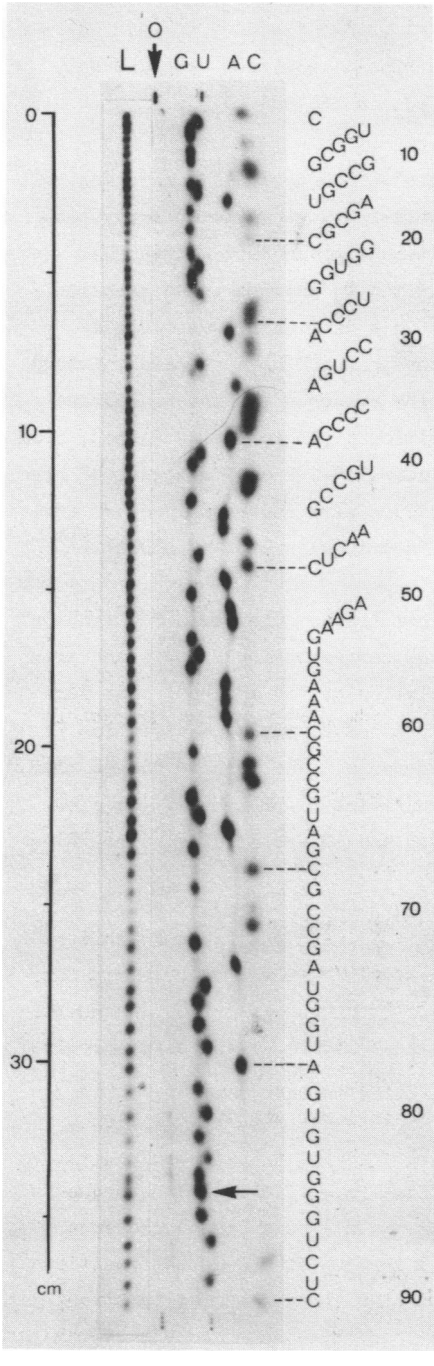

Figure 3. The two-dimensional sequencing procedure applied to E. coli 5S rRNA. The ladder (L) after transfer to the TL is aligned with the final two-dimensional pattern from which the sequence for residues 4-90 can be read off. The scale shows the original length of the autoradiogram. First dimension: 4 hr electrophoresis on 8% acrylamide gels, see Methods. Second dimension: DEAE-cellulose TL electrophoresis at pH 2.3, see Methods. O marks the origin of the second dimension.

the previously known G or U microheterogeneity at position 13 (17), position 12 is heterogeneous as there is a faint but distinct A spot besides the major spot C (see Fig. 4). Heterogeneity of 5S rRNA at position 12 had been previously observed in strain CA 265 but not in MRE 600 (17).

(3) Application to broad bean 5.8S rRNA

There was no difficulty in applying this method to the sequence analysis of broad bean 5.8S rRNA except that in preliminary experiments some spots were very faint on the two-dimensional autoradiogram (data not shown). We interpreted this as an uneven labelling in the T_4 phosphokinase reaction caused by localised secondary structure. This difficulty was overcome by the addition of 20% dimethylsulphoxide into the phosphokinase reaction mixture (see Fig. 5 and Methods). Figure 5 shows the evidence for the nucleotide sequence of broad bean 5.8S rRNA from position 1 (A) to 81 (A) (left), from position 86 (C) to 151 (C) (right). Residues 139-141 are "compressed" here (see arrow "e" in Fig. 5) and three G residues were observed in other experiments. Residues 82-85 which are not included in Figure 5 are shown in Figure 6.

The figures suggest the presence of minor bases. The spots ("a" and "c" arrowed) move a little more slowly than the corresponding U spots and are also labelled less strongly. After the elution of "a" and "c", they were analysed by chromatography on cellulose TL after P_1 nuclease digestion (see Methods) with an authentic marker ψ as reference. The results showed that both these unknown nucleotides were pseudouridine 5'-phosphate. As also shown with arrows "b" and "d", positions 48 and 80 are missing. They were

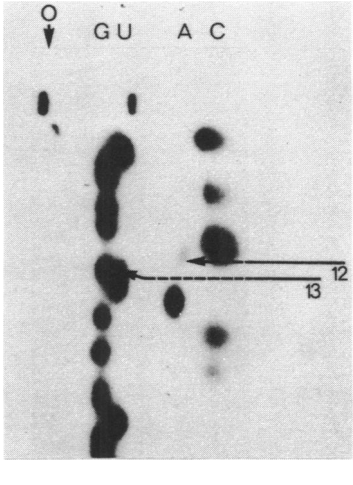

Figure 4. Magnified view of Fig. 3 from residues 4 to 24 to show microheterogeneity of sequence in E. coli 5S rRNA. The G,U heterogeneity at position 13 and the A,C at position 12 are shown by arrows pointing in each case to the spot in lower yield.

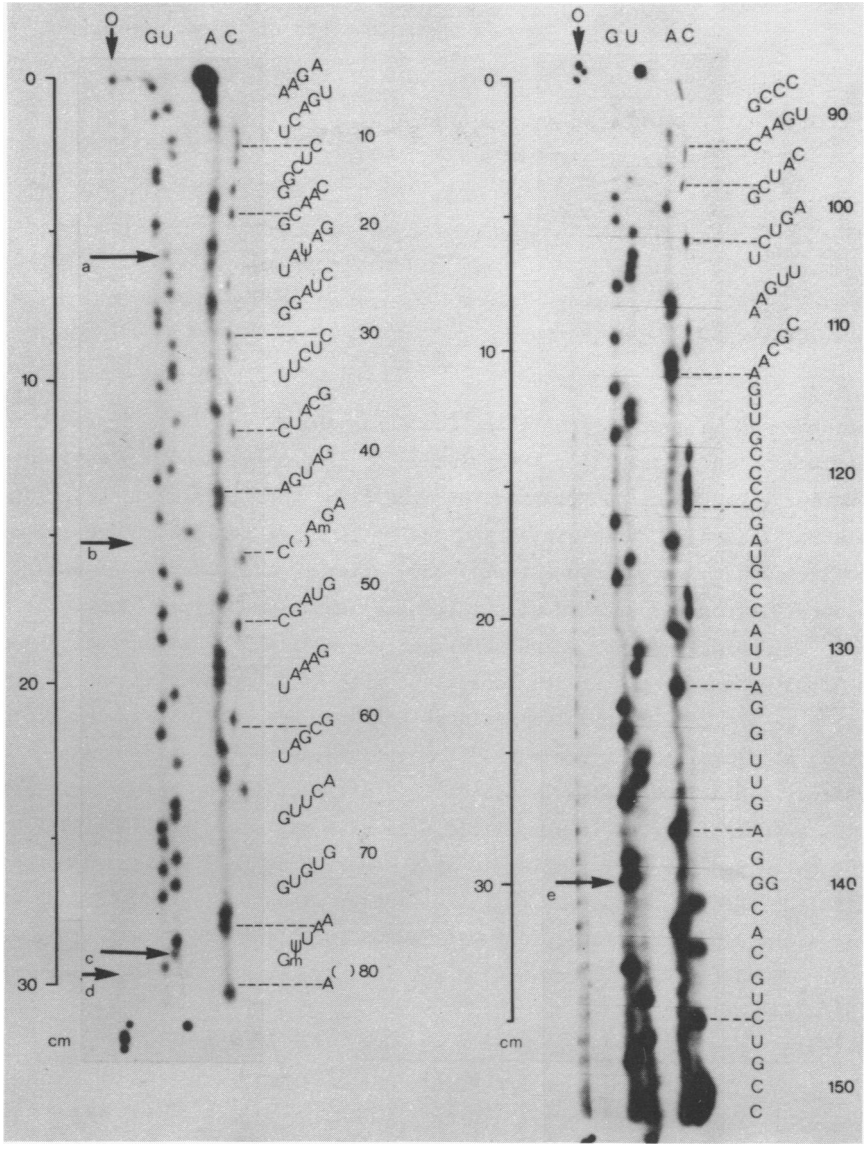

Figure 5. Two-dimensional sequencing of broad bean 5.8S rRNA. First dimension: 8% gel electrophoresis for either 4 hr (right) or 8 hr (left). Second dimension: DEAE-cellulose TL electrophoresis at pH 2.3 (see Methods). For identification of "a"-"e" see text.

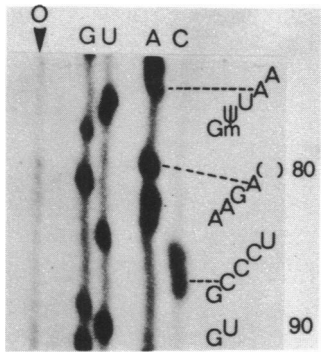

Figure 6. Two-dimensional sequencing of residues 75-91 of broad bean 5.8S rRNA.

also observed as gaps in the ladder (data not shown) and we interpreted these observations as evidence for the presence of 2'-O-methylated nucleosides known to be resistant to formamide heating (1). The immediately adjacent spots to "b" and "d" were eluted and the 5' terminal residue of these dinucleotides was characterised (see Methods) after P_1 nuclease digestion. Residue 79 cochromatographed with authentic marker Gm derived from yeast $tRNA^{Phe}$. Residue 47 (for which no marker was available) had chromatographic and electrophoretic properties consistent with Am. Residues 48 and 80 on the 3' side of the 2'-O-methylated residues have not been definitely assigned as they do not label in our protocol. However, these residues are almost certainly A and C respectively because (a) the mobility of these dinucleotides on the second dimension is compatible only with pApAp or pApCp and pGpAp or pGpCp (M values are very similar to that observed at pH 1.9 electrophoresis), (b) in the RNase T_1 catalogue (8), the further digestion of T43 (YYZGp) with RNase A gives products consistent with AmACG (position 47-50) and not with AmCCG. In addition, the presence of P30 (Xp) is compatible with the structure of GmC and not GmA.

(4) The 5' and 3' end sequences of 5.8S rRNA

In theory the 5' end of an RNA should not be labelled by the sequencing procedure. But for 5.8S rRNA a strong A spot was present at the top of the gel. This was shown to correspond to residue 1 by preparing 5' end-labelled 5.8S rRNA (see Methods) and analysing for end group (an A), and for the adjacent four residues using more traditional sequencing methods (see Methods). The result implies that the 5' end of 5.8S rRNA is partially dephosphorylated. Correlated with this is the appearance of a high background in the A line (Fig. 5). The result also implies that partial dephosphorylation does not interfere with sequencing.

The 3' terminal 12 residues could not be sequenced under our usual labelling conditions. However, if more RNAs (1 μg or more) and less γ-^{32}P-ATP were used so that all the radioactive substrate was consumed, it was possible to sequence up to six nucleotides from the 3' end as shown in Figure 7 [residues 150 (C) to 158 (U)]. Therefore, to find the missing residues the 3' end of the RNA was labelled with (5')-^{32}P-pCp (see Methods) and the sequence was analysed using nearest neighbour analysis and wandering spot method after heating the RNA (see Methods, data not shown). Residues 142-163 were effectively established by these methods.

(5) Sequence of broad bean 5.8S rRNA

The primary nucleotide sequence is shown in Figure 8 with the alignment of oligonucleotide fragments obtained from complete RNase T_1 digestion for comparison. All residues were unambiguously deduced from the two-dimensional sequencing procedure developed here, except for residues 159-163 where more traditional sequencing methods were used (see above), and for residues 48 and 80 where data from previously published T_1 RNase catalogues was used (8). Four minor bases at positions 22 (ψ), 47 (Am), 78 (ψ) and 79 (Gm) were deduced from cellulose TL chromatography after elution of the spots from DEAE-cellulose TL (see Methods). To confirm that no other minor bases were present, all spots on the two-dimensional separation were eluted and chromatographed on cellulose TL (see Methods, data not shown). By contrast with 5S rRNA, no microheterogeneity was detected in 5.8S rRNA although this cannot be completely eliminated because of the high background in the A line.

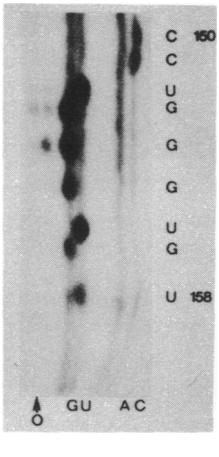

Figure 7. Two-dimensional sequencing of residues 150-158 of broad bean 5.8S rRNA.

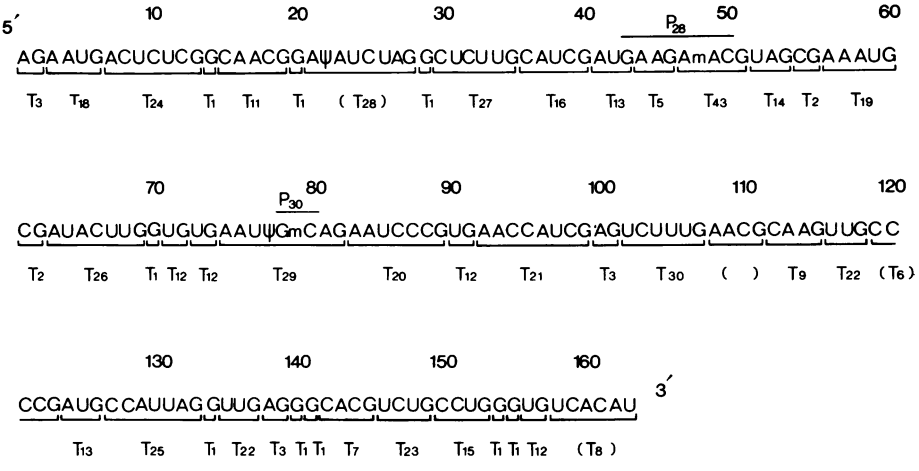

Figure 8. The primary nucleotide sequence of broad bean 5.8S rRNA. For comparison, the complete RNase T_1 products and two of the pancreatic RNase A (P_{28} and P_{30}) were aligned. The parentheses indicate sequence differences between the two reports.

DISCUSSION

The improved Stanley and Vassilenko RNA sequencing method reported in this paper is suitable for the rapid sequence analysis of 5S rRNA from *E. coli* (MRE 600) and 5.8S rRNA from broad bean. Quite recently, Gupta and Randerath (19) have developed independently a somewhat related blotting strategy followed by polyethyleneimine TL chromatography in the second dimension. Compared to their method, our method provides improved transfer efficiency of the RNA fragments from gel to DEAE-cellulose TL plate and a simpler one-step transfer. We have also shown that our method has the resolution necessary to sequence an unknown RNA molecule 163 nucleotides long, whereas others (19) have so far applied the method to shorter tRNA molecules of known sequence. Diffusion is minimal in our second-dimensional electrophoretic system, thus to a large extent maintaining the high resolution present in the first dimension. But to maintain this resolution it is essential to prewash the DEAE-cellulose TL plates with EDTA solution as detailed in Methods. This prevents "streaking" and facilitates the *in situ* RNase digestion, probably by removing impurities.

For the sequence analysis of RNAs using this method, long thin acrylamide gel (80 cm long) has been proved to be very effective not only because less

material can be used but also because resolution is increased. Even at the 5' end of 5.8S rRNA the distance between adjacent bands of the ladder was 2.5 mm.

The method developed here clearly supercedes the enzymatic degradative methods (1-5) applied to end-labelled RNA in both sequence accuracy and rapidity. Our method also has advantages over the chemical degradative procedure of Peattie (6). Thus the sequence accuracy is probably higher and it is impressive that sequence microheterogeneity was easily detected. The ability to detect and further analyse for minor bases is also an important advantage.

In Figure 9 we compare the primary sequence of broad bean 5.8S rRNA with that of yeast (20) and human (21). Using the alignment shown, which includes a few deletions and additions, broad bean is 67% homologous to human and 69% homologous to yeast 5.8S rRNA. Yeast is 73% homologous to human. This is a slightly greater extent of homology than for the same 5S rRNA species, suggesting that 5.8S rRNA is, like 5S rRNA, highly conserved in evolution. Some regions at least must have a function critically dependent on sequence. There has been no previous report of 2'-O-methylated adenylic acid in 5.8S rRNA and it will be interesting to know whether other plants, especially

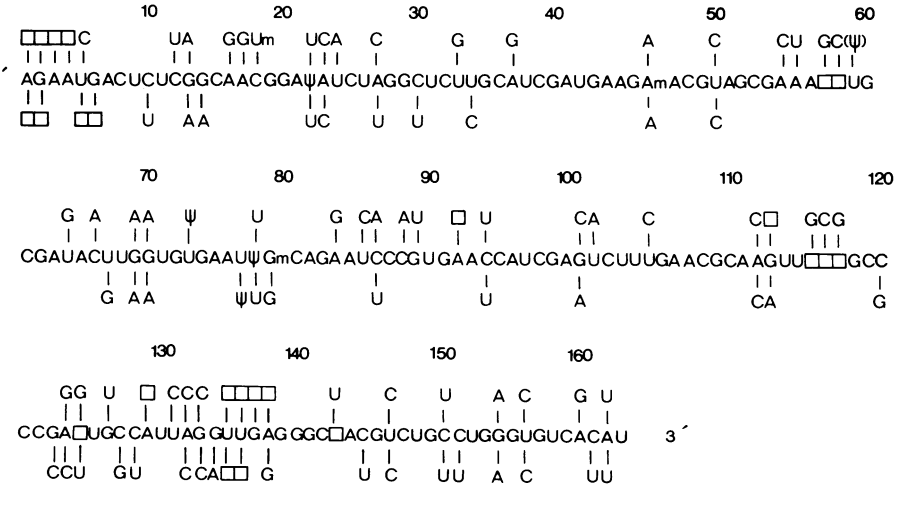

Figure 9. Comparison of the nucleotide sequence of human (upper line), broad bean (middle line) and yeast (lower line) 5.8S rRNA. The base positions are numbered as in Fig. 8. Only differences are shown and boxes show gaps placed in the sequence to obtain maximal homology.

lower plants, have this same modification or not.

In conclusion, the method reported here is simple and accurate and should be well suited to an analysis of any low molecular weight RNA. In particular it should allow one to sequence 5S or 5.8S rRNA from a large number of species quickly to obtain a more accurate molecular phylogenetic tree.

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