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Terminal-Sequence Studies of High-Molecular-Weight Ribonucleic Acid

THE 3′-TERMINI OF RABBIT RETICULOCYTE RIBOSOMAL RNA

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Sequences of the polynucleotide chains of RNA found in the large and small ribosomal subunits of rabbit reticulocytes have been determined from the 3′-end by use of periodate oxidation and condensation with [^{3}H]isoniazid and by stepwise degradation. By these methods the hexanucleotide sequences have been found as -pGpUpUpUpGpU for the 28 S RNA and -pGpUpCpGpCpU for the 6 S RNA of the large ribosomal subunit and the octanucleotide sequence -pGpApUpCpApUpUpA for the 18 S rRNA of the small ribosomal subunit. These sequences are present in at least 70% of all the RNA molecules and are discussed in relation to the specific cleavage of rRNA from its precursors and the role of multiple cistrons for rRNA in the DNA of higher organisms. The feasibility of using the method for longer sequence determinations is discussed.

The role of 28 S, 18 S and 6 S rRNA in the structure and function of the ribosome is still obscure. However, evidence from specific hybridization of rRNA species with DNA indicates that they are specific gene products of the DNA of both bacteria and the chromosomes of higher organisms (Ritossa, Atwood, Lindsley & Spiegelman, 1966). The quantitative studies of rRNA hybridization indicate that the cistrons that specify the sequences of rRNA are present as multiple copies varying from about 10 in *Escherichia coli* to as many as 1000 in *Xenopus laevis* (Perry, 1966). Other studies with the HeLa cell 45 S precursor of rRNA (Jeantur, Amaldi & Attardi, 1968) and fragmentation of the DNA containing ribosomal cistrons from *Xenopus laevis* (Brown & Weber, 1968) favour a model with alternating 28 S and 18 S RNA cistrons separated by non-homologous sequences making up a 45 S RNA cistron. The presence of the 45 S RNA precursor of the 18 S and 28 S RNA had been previously demonstrated by metabolic studies in HeLa and other cells (see Perry, 1966, and Penman, Vesco, Weinberg & Zylber, 1969, for review). However, Muramatsu & Busch (1967), using isolated nucleoli, consider that the 45 S RNA is a precursor of 28 S RNA alone.

Whatever the actual mechanism of synthesis of rRNA is, it is clear that a larger precursor is involved, and poses the following problems. (*a*) Do the rRNA species have a unique sequence? (*b*) Are the multiple DNA cistrons for rRNA duplicates of one master gene or are they autonomous? (*c*) Is the cleavage of the precursor rRNA highly precise?

These questions could be answered in part by determining a complete sequence of the RNA molecules, provided that they have a unique sequence, but this would seem to be an almost impossible task, although it has been attempted for the 16 S RNA of *E. coli* (Fellner, Ehresman & Ebel, 1970). A simpler approach has been used in the present work by determination of terminal sequences of the rRNA molecules (Hunt, 1965, 1970*a*).

This paper describes the determination of the 3′-terminal sequences obtained from the 28 S and 6 S RNA from the large ribosomal subunit and the 18 S RNA from the small ribosomal subunit of rabbit reticulocytes. Each of these RNA species has a unique terminal sequence.

MATERIALS AND METHODS

Materials. [G-^{3}H]- and [*carboxyl*-^{14}C]-Isonicotinic acid hydrazides were obtained from Amersham–Searle (Des Plaines, Ill., U.S.A.) at specific radioactivities of 200 mCi/mmol and 9.8 mCi/mmol respectively. Nucleosides, dinucleoside monophosphates and trinucleoside diphosphates were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) except for those oligonucleotides that could be readily isolated by DEAE-cellulose chromatography (Hunt, 1970*a*). Sodium metaperiodate (reagent grade) was obtained from Matheson, Coleman and Bell (East Rutherford, N.J., U.S.A.). Aniline was reagent grade from Allied Chemical Corp. (New York, N.Y., U.S.A.) and was redistilled and stored in a dark bottle at 4°C. Triethylamine from Eastman–Kodak Co. (Rochester, N.Y., U.S.A.) was redistilled before use. Sucrose was ribonuclease-free from Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). Other chemicals were reagent grade from

Mallinckrodt (St Louis, Mo., U.S.A.) or Merck and Co. Inc. (Rahway, N.J., U.S.A.). Ribonuclease T_1 (EC 2.7.7.26) (Sankyo) was obtained through Calbiochem (Los Angeles, Calif., U.S.A.). Pancreatic ribonuclease (EC 2.7.7.16), recrystallized from ethanol, was from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and bacterial alkaline phosphatase (BAPF) (EC 3.1.3.1), ribonuclease-free, was from Worthington.

Ribosomal subunits were prepared from rabbit reticulocyte ribosomes by the method of Huez, Burny, Marbaix & Lebleu (1967) as modified by Labrie (1969). Narrow cuts were taken from the middle of the peaks, and the ribosomal subunits precipitated by adding $MgCl_2$ (final concn. 5mM) and lowering the pH to 5.4 with 10% (v/v) acetic acid. RNA was extracted by precipitation from 4M-guanidinium chloride (Cox, 1968).

Gel electrophoresis (*Loening*, 1967). Polyacrylamide gels were made by using 2.5% Cyanogum 41 (Fisher Scientific Co., Fairlawn, N.J., U.S.A.), 0.2% NN'-methylenebisacrylamide in 40mM-tris acetate–20mM-sodium acetate–2mM-EDTA (disodium salt), pH 7–8; 30μl of *NNN'N'*-tetramethylenediamine/g of Cyanogum 41 and 80μl of 10% (w/v) ammonium persulphate/100ml of gel were added to cross-link and polymerize the gels in Plexiglas (Perspex) tubes (10cm × 0.45cm diam.).

Electrophoresis was performed at 4°C at 100V for 2h. Gels were fixed in M-acetic acid for 10min and stained with 0.2% Methylene Blue in 0.2M-sodium acetate–0.2M-acetic acid (Peacock & Dingman, 1967).

Reaction of isoniazid with periodate-oxidized RNA. Periodate oxidation and condensation with [^{3}H]isoniazid was performed as described previously except that 0.01M-sodium acetate buffer, pH 5.0, was used for the condensation (Hunt, 1965, 1968). The residual [^{3}H]-isoniazid was removed, after two precipitations with ethanol from 0.1M-sodium acetate buffer, pH 5.0, by chromatography on Sephadex G-75 (Sigma Chemical Co.) in 0.1M-sodium acetate buffer, pH 5.0, precipitated with 2vol. of ethanol at 0°C and stored frozen in sterile water.

Stepwise degradation of RNA. Stepwise degradation was performed by the method of Steinschneider & Fraenkel-Conrat (1966). A typical protocol was as follows. RNA (1–1.5mg/ml) from either of the ribosomal subunits in 0.1M-sodium acetate buffer, pH 5.0, was oxidized with 100-fold molar excess of $NaIO_4$ in the dark. The RNA was precipitated twice from 0.1M-sodium acetate buffer, pH 5.0, with 2vol. of ethanol at 0°C and redissolved at the same concentration in 0.33M-aniline adjusted to pH 5.0 with conc. HCl, and incubated for 3h at 25°C to eliminate the terminal nucleoside. The solution was cooled to 0°C, 0.1vol. of 1M-sodium acetate buffer, pH 5.0, added and the RNA was precipitated with 2vol. of ethanol followed by reprecipitation with ethanol from 0.1M-sodium acetate buffer, pH 5.0. The RNA was then dissolved in 20mM-ammonium acetate buffer, pH 6.9, at double its former concentration, made 1mM in $MgCl_2$ and 20μg of ribonuclease-free alkaline phosphatase/ml was added. The mixture was incubated for 30min at 37°C to remove the 3'-terminal phosphate group, cooled, 0.1vol. of 1M-sodium acetate buffer, pH 5.0, was added, and the mixture was precipitated with 2vol. of ethanol. The whole procedure was repeated as many as five times.

Enzyme digestion. Ribonuclease T_1 and pancreatic ribonuclease digestion and dephosphorylation of carrier RNA were performed as described by Hunt (1970*a*). [^{3}H]Isoniazid-labelled RNA (1–2mg/ml) in water was digested with 40 units of ribonuclease T_1/mg or 40μg of pancreatic ribonuclease/mg, in the presence of alkaline phosphatase (10μg/mg of RNA) at 25°C for 2h.

Determination of nucleoside and oligonucleotide isonicotinoylhydrazones. Paper electrophoresis in 0.1M-triethylammonium formate buffer, pH 3.0, was performed as described by Hunt (1965, 1970*a*).

Column chromatography. DEAE-cellulose column chromatography to identify dinucleoside monophosphate isonicotinoylhydrazones was performed as described by Hunt (1970*a*). Separation of oligonucleotide isonicotinoylhydrazones according to size was accomplished as described by Tener (1967), on columns (50cm × 0.9cm diam.) of DEAE-Sephadex, with a linear gradient of sodium acetate buffer, pH 6.0, from 10mM to 1.0M in 7M-urea, at 4°C; the total volume of the gradient was 1320ml.

Radioactivity counting. The effluent from the column chromatograms was monitored for u.v. absorption, and for radioactivity by liquid-scintillation counting, with the apparatus described by Hunt (1968). Both the triethylammonium formate and sodium acetate–7M-urea solutions could be mixed with liquid scintillator up to a salt concentration of 0.6–0.8M without phase separation. For triethylammonium formate buffers a flow ratio of 4.3ml/h for column effluent to 28ml/h for liquid scintillator was used and 4.3ml/h for column effluent to 32ml/h for liquid scintillator for the 7M-urea–sodium acetate buffers. The scintillator solution contained Beckman BBS 3 solubilizer (Beckman Instruments Inc., Fullerton, Calif., U.S.A.), (1vol.), and toluene containing 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (5vol.).

RESULTS

Terminal-sequence determination of RNA from the large ribosomal subunit. Molecular-weight determination of the RNA isolated from sucrose-gradient fractionated ribosomal subunits by estimating the amount of isoniazid condensed on the RNA indicated a much lower value than the 0.7×10^6 daltons and 1.7×10^6 daltons found by sedimentation-velocity determinations (Petermann & Pavlovec, 1966) (Table 1). There was no evidence of gross contamination or breakdown of the RNA as determined by polyacrylamide-gel electrophoresis (Fig. 1). However, previous results had indicated that the 28S RNA from ribosomes had two terminal sequences (Hunt, 1965). By treatment of the RNA with 0.5% sodium dodecyl sulphate at 60°C it was possible to separate two RNA species by sucrose-gradient sedimentation from the RNA extracted from the large ribosomal subunit, each having only one terminal sequence (Hunt, 1970*b*). These two RNA species were a 28S RNA now with mol.wt. 1.5×10^6, with a terminal sequence-pGpU, and a '6S' RNA with the terminal sequence-PypU. In the sequence determinations described here unfractionated RNA was used since the sequences could be readily associated with each

Table 1. *Molecular weights of RNA preparations after various treatments*

Molecular weights of RNA were calculated by estimating the number of g of periodate oxidized RNA that condensed with 1 mol of isoniazid. RNA was determined by using an extinction coefficient at 260 nm of 23 in a 1 cm cell for a concentration of 1 mg/ml in 0.1 M-sodium phosphate buffer, pH 7.0. For the undegraded preparation, after sucrose gradient centrifugation the RNA from the small ribosomal subunit has mol. wt. $0.67 \pm 0.015 \times 10^6$ for the 18 S RNA, and for RNA from the large ribosomal subunit $1.15 \pm 0.02 \times 10^6$ for the 28 S RNA, except when treated with sodium dodecyl sulphate at 60°C when it is 1.53×10^6 (Hunt, 1970*b*).

Source of RNA	No. of stepwise degradations ...	$10^{-6} \times$ Mol.wt. None	One	Two	Three	Four	Five
Small ribosomal subunit		0.46 ± 0.03	0.47 ± 0.06	0.62	—	0.42	0.42
Large ribosomal subunit		0.77 ± 0.02	0.83 ± 0.02	1.04	0.90	—	—

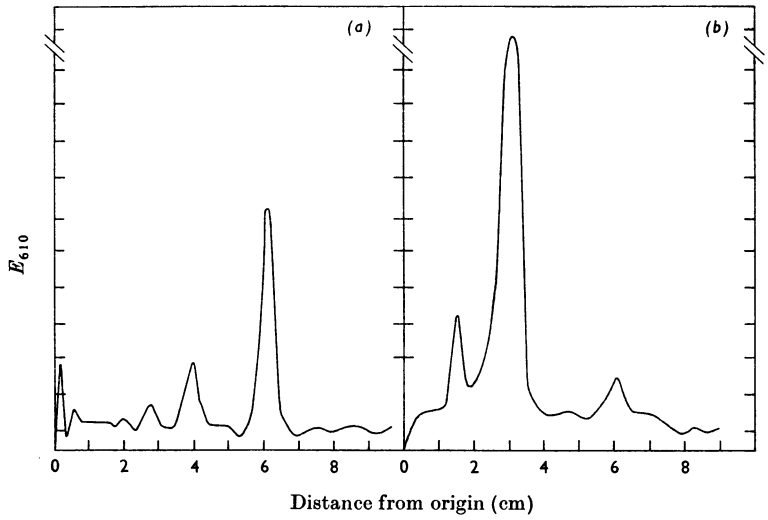

Fig. 1. Densitometer scans of polyacrylamide-gel electrophoretograms of RNA extracted from (*a*) the small and (*b*) the large ribosomal subunit by guanidinium chloride. Direction of migration was from left to right and gel concentration was 2.4%.

original sequence. The general strategy used was to digest the isoniazid-coupled RNA with pancreatic ribonuclease and ribonuclease T_1 and to characterize the products according to size by chromatography on DEAE-Sephadex in 7 M-urea, and by sequence of tri- or di-nucleotide-isonicotinoylhydrazones by DEAE-cellulose chromatography. Identity of nucleoside isonicotinoylhydrazones was determined by paper electrophoresis. The RNA was subjected to one or more cycles of stepwise degradation followed by periodate oxidation and condensation with isoniazid. End-group analyses were then performed as described above. Important aspects of the stepwise degradation procedure are to ensure that (*a*) the RNA is undegraded so that no new periodate-oxidizable ends are formed after treatment with alkaline phosphatase, and (*b*) the reaction is as close to quantitative as possible, so that yields of new sequences are as high as possible after several steps. Criterion (*a*) is fulfilled in these experiments, as shown in Table 1, since the number of oxidizable ends of RNA molecules after as many as five cycles of stepwise degradation tended to decrease rather than increase.

Table 2 is a summary of the results obtained from end-group analysis of the RNA from the large ribosomal subunit after each step. Preliminary studies by paper electrophoresis had indicated the presence of a -GpU sequence and a -PypU sequence due to the presence of uridine isonicotinoylhydrazone after digestion with pancreatic ribonuclease or ribonuclease T_1, but could not identify the sequence

Table 2. *Digestion products of izoniazid-labelled RNA from the* 60S *ribosomal subunit*

DEAE-Sephadex column chromatography was used to separate the digestion products according to size; chromatography on DEAE-cellulose was used to identify some of the oligonucleotides, and the nucleoside hydrazones were identified by paper electrophoresis. The percentages noted for each oligonucleotide recovered from column chromatography were calculated by measuring the area of the peaks and converting this into total c.p.m. by using the formula c.p.m. $= (A \times V_t \times F)/V_c$, where A is the area of the peak in in^2, V_t is the total flow rate from the column in ml/h, F is a factor from the scale of graph and is found by dividing the c.p.m./in scale by the chart rate in in/h, and V_c is the effective volume of the flow cell in ml obtained by multiplying the cell volume (3.74 ml) by $V_A/(V_S+V_A)$, where V_A is the flow rate in ml/h of the proportion of effluent mixed with the scintillator flowing at the rate V_S ml/h. Normal values for V_t varied from 35 to 42 ml/h. V_A was 4.3 ml/h and V_S for 7 M-urea buffers 32 ml/h and 27 ml/h for triethylamine buffers. Efficiency of ^{3}H-counting was 12% for both conditions. Total recovery from the columns is given in parentheses. The percentages for each identifiable peak obtained by paper electrophoresis was obtained after correction for background caused by breakdown (obtained by running an undigested control). Where a peak cannot be identified as a nucleoside isonicotinoylhydrazone, it is designated oligo-1,-2 etc. Material remaining at the origin is also included. Figures in parentheses indicate a duplicate on a new sample.

Treatment of RNA before oxidation and condensation with isoniazid	Enzyme used	Fractionation method (%): 7 M-Urea–DEAE-Sephadex column	DEAE-cellulose column	Paper electrophoresis
None	Pancreatic ribonuclease	Not run	Mono 75 GpU 25	U 45* GpU 49
	Ribonuclease T_1	Not run	Mono 59 CpU 41	U 43 CpU 50
One stepwise degradation	Pancreatic ribonuclease	Not run	Mono 67 GpC 21 GpU 10 Unknown 2 (86% recovery)	GpC 25 (20) G 21 (29) A 6 (0) C 12 (10) GpU and origin 26 (10) (18)
	Ribonuclease T_1	Mono 46 Di 8 Tri 8 Tetra 30 Penta 1 Hexa 4 Residue 3 (100% recovery)	Not run	U 4 (11) C 32 (30) CpU 5 (9) Oligo 29 (27) Origin 26 (22)
Two stepwise degradations	Pancreatic ribonuclease	Mono 77 Di 19 Tri 4 (68% recovery)	Not run	U+G 35 A 8 C 8 Origin 30
	Ribonuclease T_1	Mono 23 Di 7 Tri 52 Tetra 6 Penta 9 Residue 3 (91% recovery)	Mono 23 UpCpG 21 UpUpU 36 Residue 19 (78% recovery)	C 8 Oligo-1 25 Oligo-2 31 Origin 12
Three stepwise degradations	Pancreatic ribonuclease	Not run	Not run	U 12 C 13 Origin 44
	Ribonuclease T_1	Mono 19 Di 56 Tri 15 Tetra 10 (67% recovery)	Mono 32 UpC 21 UpU 31 Other (tri) 15 (59% recovery)	UpC 14 Origin 59

* Isoniazid-labelled RNA was purified by sucrose-density-gradient centrifugation before enzymic digestion.

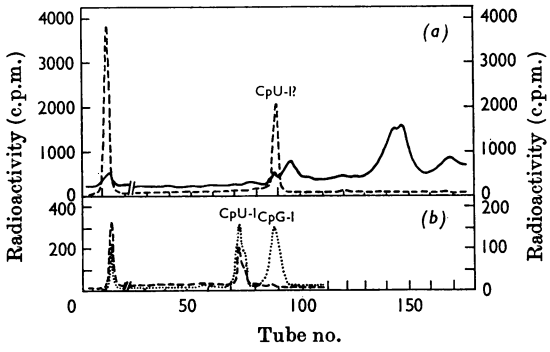

Fig. 2. Column chromatography on DEAE-cellulose of the products of ribonuclease T_1 digestion of [^{3}H]isoniazid-labelled RNA from the large ribosomal subunit. Elution was by a concave gradient of 5mM-triethylammonium formate buffer, pH4.0, to 0.5M-triethylammonium formate buffer, pH5.0, at 4°C. (*a*) Original digest showing an oligonucleotide isonicotinoylhydrazone close to CpG- or ApC-isonicotinoylhydrazone. ——, E_{260} of carrier ribonuclease T_1-digested RNA; ----, ^{3}H radioactivity. (*b*) Rechromatography of the oligonucleotide hydrazone peak in (*a*). ——, E_{260} of carrier ribonuclease T_1-digested RNA; ----, ^{3}H radioactivity from the isolated peak; ...., ^{14}C radioactivity from ^{14}C-labelled CpU- and CpG-isonicotinoylhydrazones added as carriers for identification. Both the extinction and radioactivity were monitored automatically. Flow rate: (*a*) 39ml/h; (*b*) 36ml/h (3.5ml/tube). Only c.p.m. are shown on the ordinate. Where a break in the graph occurs the c.p.m. are those of the appropriate ordinate.

that the -PypU end released after ribonuclease T_1 digestion. DEAE-cellulose chromatography with triethylammonium formate readily identified a dinucleoside monophosphate isonicotinoylhydrazone as GpU-isonicotinoylhydrazone in the pancreatic ribonuclease digest. The dinucleoside monophosphate isonicotinoylhydrazone released by ribonuclease T_1 digestion has a chromatographic mobility closest to ApC- or GpC-isonicotinoyl hydrazone (Fig. 2*a*). Rechromatography of this compound was accomplished by adjusting the pH of the eluate containing the peak of radioactivity to 5.0 and adsorbing it to the standard DEAE-cellulose column, after which chromatography under standard conditions was possible. When this was done the position of the compound moved closer to that of CpU-, CpA- or UpC-isonicotinoylhydrazone and its identity was established by cochromatography with ^{14}C-labelled CpU-isonicotinoylhydrazone (Fig. 2*b*), confirming the sequence -pGpCpU (Table 3).

Sequences after one stepwise degradation. The first stepwise degradation was not complete, as was demonstrated in the pancreatic ribonuclease digest when chromatographed on DEAE-cellulose (Fig. 3). However, the extent of reaction was sufficient to characterize GpC-isonicotinoylhydrazone, confirming a tetranucleotide sequence -PypGpCpU. Guanosine isonicotinoylhydrazone was identified by paper electrophoresis as predicted from the sequence -PypGpU. The ribonuclease T_1 digest chromatographed on DEAE-Sephadex in 7M-urea (Fig. 4*a*) contained a tetranucleoside triphosphate- and a nucleoside-isonicotinoylhydrazone. The latter was identified as cytosine isonicotinoylhydrazone by paper electrophoresis.

The cytosine isonicotinoylhydrazone confirms the sequence -pPypGpCpU and the tetranucleoside triphosphate isonicotinoylhydrazone implies a sequence pGp(NpNpPypG)pU, the tetranucleotide being NpNpPypG, when N can be any nucleoside except guanosine.

Sequences after two stepwise degradations. Pancreatic ribonuclease released guanosine and uridine isonicotinoylhydrazones. The guanosine isonicotinoylhydrazone was expected from the sequence -pPypGpCpU (Table 3), hence the uridine isonicotinoylhydrazone must be from the -pGpNpNpNpGpU sequence, making it -pGpNpPypUpGpU. Ribonuclease T_1 released two trinucleoside diphosphate isonicotinoylhydrazones (Figs. 4*b* and 5*a*); their positions on the DEAE-cellulose column chromatogram were close to those of UpCpG- or CpUpG-isonicotinoylhydrazone and ApApG-isonicotinoylhydrazone respectively. One trinucleoside diphosphate isonicotinoylhydrazone is expected from the sequence -pGpNpPypUpGpU, and would have the sequence NpPypU-isonicotinoylhydrazone; the other predicts a sequence -pGpNpPypGpCpU. Since the trinucleoside diphosphate isonicotinoylhydrazone close to the UpCpG-, CpUpG-isonicotinoylhydrazone region cochromatographs with ^{14}C-labelled UpCpG-isonicotinoylhydrazone, it must be the product of an original sequence pGpUpCpGpCpU.

Sequences after three stepwise degradations. Pancreatic ribonuclease released cytosine and uridine isonicotinoylhydrazone, predicting sequences -PypC and -PypU. Ribonuclease T_1 released UpC-isonicotinoylhydrazone and UpU-isonicotinoylhydrazone from the chromatogram; there could be no doubt about the identity of the UpU-isonicotinoylhydrazone (Fig. 5*b*) but the sequence of the UpC-isonicotinoylhydrazone was confirmed when it was separated from ^{14}C-labelled CpU-isonicotinoylhydrazone. These are the only two sequences that could produce the -PypC and -PypU sequences predicted by pancreatic ribonuclease digestion. This result predicts the two sequences -pGpUpCpGpCpU and -pGpUpUpUpGpU. Because of the terminal dinucleoside monophosphate sequences the former must belong to 6S RNA and the latter to the 28S RNA. Each step is summarized in Table 3.

Table 3. *Summary of the fragments produced by terminal sequence analysis in Tables 2 and 4*

Stepwise degradation	Products from: Pancreatic ribonuclease	Products from: Ribonuclease T_1	Sequence
(*a*) 18 S RNA			
0	A	N_7p_6*	pGp(Np$_5$)PypA
1	U	N_6p_5	pGp(Np$_4$)PypUpA
2	ApU	N_5p_4	pGp(Np$_2$)PypApUpUpA
4	C	N_3p_2	pGp(Np)PypCpApUpUpA
5	$(Yp)_{5\text{ or }6}$GpApU†	ApU	pPy$(Yp)_{5\text{ or }6}$pGpApUpCpApUpUpA
(*b*) 28 S RNA			
0	GpU	U	pPypGpU
1	G	N_4p_3	pG(Np$_2$)PypGpU
2	U (or G)	N_3p_2	pG(Np)PypUpGpU
3	U (or C)	UpU	pGpUpUpUpGpU
(*c*) 6 S RNA			
0	U	CpU	pGpCpU
1	GpC	C	pPypGpCpU
2	G (or U)	N_3p_2	pGp(Np)PypGpCpU
3	C (or U)	UpC	pGpUpCpGpCpU

* N, nucleosides A, U or C.
† Y, nucleosides A or G.

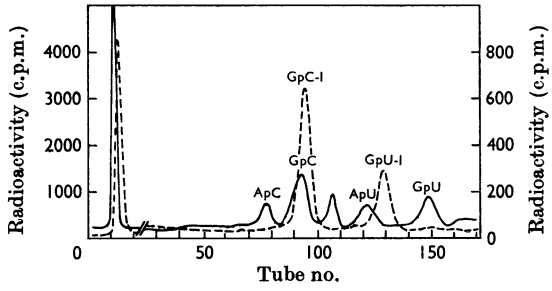

Fig. 3. Column chromatography on DEAE-cellulose of the pancreatic-ribonuclease-digestion products of [^{3}H]-isoniazid-labelled RNA from the large ribosomal subunit after one stepwise degradation. In this experiment the stepwise degradation was incomplete as shown by the presence of GpU-isonicotinoylhydrazone. ——, E_{260} of carrier pancreatic ribonuclease-digested RNA; ----, ^{3}H radioactivity. Elution conditions were as for Fig. 2, flow rate 39 ml/h (3.5 ml/tube). Only c.p.m. are shown on the ordinate. Where a break in the graph occurs the c.p.m. are those of the appropriate ordinate.

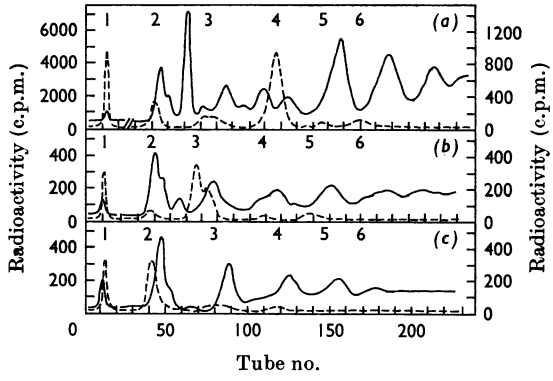

Fig. 4. Column chromatography on DEAE-Sephadex of the ribonuclease-T_1-digestion products of [^{3}H]isoniazid-labelled RNA from the large ribosomal subunit after stepwise degradation. (*a*) One stepwise degradation, (*b*) two cycles of stepwise degradation, (*c*) three cycles of stepwise degradation. Elution was by a linear gradient of 0.01 M–1.0 M sodium acetate buffer, pH 6.0, in 7 M-urea at 4°C. The numbers refer to the size of the oligomers eluted at these positions. ——, E_{260} of carrier ribonuclease T_1-digested RNA; ----, ^{3}H radioactivity. Flow rates: (*a*) and (*b*) 41 ml/h (3.5 ml/tube); (*c*) 38 ml/h (3.3 ml/tube). Only c.p.m. are shown on the ordinate. Where a break in the graph occurs the c.p.m. are those of the appropriate ordinate.

Terminal sequence determination of RNA from the small ribosomal subunit (*Table* 4). Preliminary studies by paper electrophoresis (as well as molecular-weight determination after various treatments; Table 1) indicated that the 18 S RNA from the small ribosomal subunit had a single -PypA terminus and mol.wt. 0.67×10^6. Chromatography of the ribonuclease T_1 products on DEAE-Sephadex in 7 M-urea revealed a heptanucleoside hexaphosphate isonicotinoylhydrazone (Fig. 6*a* and Table 4) predicting the sequence -pGpNpNpNpNpNpPypA, where N can be any nucleoside except guanosine.

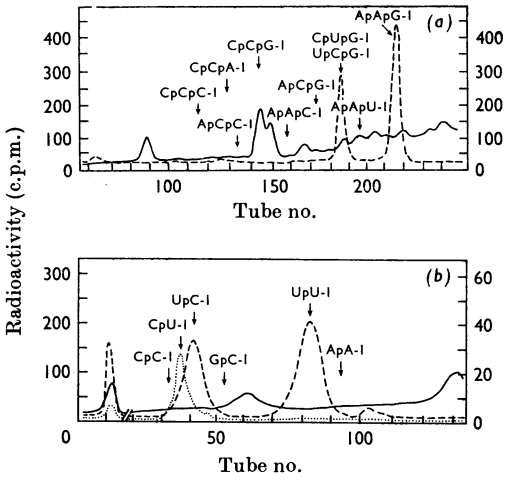

Fig. 5. Column chromatography on DEAE-cellulose of the products of ribonuclease T_1 digestion of [^{3}H]isoniazid-labelled RNA from the large ribosomal subunit after stepwise degradation. (*a*) Two cycles of stepwise degradation. CpCpC-I and CpCpA-I etc. mark the approximate positions of marker CpCpC-isonicotinoylhydrazone etc. determined relative to the carrier digest. ——, E_{260} of carrier ribonuclease T_1-digested RNA; ----, ^{3}H radioactivity. (*b*) Three cycles of stepwise degradation. Carrier ^{14}C-labelled CpU-isonicotinoylhydrazone was added to differentiate between UpC- and CpU-isonicotinoylhydrazones as shown by arrows. Elution conditions were as for Fig. 2. ——, E_{260} of carrier ribonuclease T_1-digested RNA; ----, ^{3}H radioactivity; ...., ^{14}C radioactivity. Flow rate: (*a*) 37 ml/h, (3.5 ml/tube); (*b*) 38 ml/h (3.75 ml/tube). Efficiency of counting in double-label experiment was 7% for ^{3}H and 50% for ^{14}C. Only c.p.m. are shown on the ordinate. Where a break occurs in the graph the c.p.m. are those of the appropriate ordinate.

Sequence after one stepwise degradation. Pancreatic ribonuclease released a nucleoside isonicotinoylhydrazone that was determined as uridine isonicotinoylhydrazone by paper electrophoresis, predicting the sequence -pGpNpNpNpNpPypUpA. The presence of GpC- and GpU-isonicotinoylhydrazone in the DEAE-cellulose chromatogram of this digest (Table 4) indicates some contamination by the RNA from the large ribosomal subunit in this preparation. The absence of adenosine isonicotinoylhydrazone determined by paper electrophoresis indicated that the stepwise degradation was essentially complete. Ribonuclease T_1 released the expected hexanucleoside pentaphosphate isonicotinoylhydrazone (Fig. 6*b*) although its yield was low, even taking into account the contamination by the RNA from the large ribosomal subunit.

Sequence after two stepwise degradations. DEAE-Sephadex chromatography in 7 M-urea identified a dinucleoside monophosphate isonicotinoylhydrazone from the pancreatic ribonuclease digest, and the expected pentanucleoside tetraphosphate isonicotinoylhydrazone from the ribonuclease T_1 digest (Fig. 6*c*), both in good yields (Table 4). The pancreatic ribonuclease product was determined as ApU-isonicotinoylhydrazone by chromatography on DEAE-cellulose, predicting the sequence -pGpNpNpPypApUpUpA.

Sequence after four stepwise degradations. Because of the discovery of the ApU sequence after two stepwise degradations no results were collected after three stepwise degradations. After four stepwise degradations pancreatic ribonuclease released cytosine isonicotinoylhydrazone in reasonable yield, predicting the sequence -pGpNpPypCpApUpUpA. The expected trinucleoside diphosphate isonicotinoylhydrazone was found on DEAE-Sephadex–7 M-urea chromatography of the ribonuclease T_1 digest (Fig. 6*d*). DEAE-cellulose chromatography of this digest revealed a trinucleoside diphosphate isonicotinoylhydrazone that ran ahead of UpCpG- but behind ApCpG-, ApApC-, ApCpC- and CpCpA-isonicotinoylhydrazones, predicting from this and the results from pancreatic ribonuclease digestion a sequence of UpUpC-, UpCpC- or ApUpC-isonicotinoylhydrazone.

Sequence after five stepwise degradations. Pancreatic ribonuclease produced a low yield of nonanucleoside octaphosphate- or octanucleoside heptaphosphate-isonicotinoylhydrazone, predicting the sequence -Pyp(Yp)$_{5\text{ or }6}$GpApUpCpApUpUpA, when Y is A or G. Ribonuclease T_1 confirmed the predicted GpApU- sequence by releasing a dinucleoside monophosphate isonicotinoylhydrazone identified as ApU-isonicotinoylhydrazone by DEAE-cellulose chromatography (Fig. 6*e*).

DISCUSSION

In extensive use of previously untried techniques, or combinations of proven techniques in new ways, it is important to be able to assess the accuracy of the method. The terminal sequences of several RNA species have been determined before, sometimes incidentally to complete sequence determination or sometimes as a single experiment using one technique. The combination of end-group labelling with stepwise degradation has not been previously exploited. This combination of techniques can answer two questions: first, how much of the sequence determined is unique, and second, how efficient is the stepwise degradation of the RNA?

In the above experiments, after only one or two cycles of stepwise degradation, the apparent molecular weight of the RNA whose sequence is being determined remains constant, but is lower

Table 4. *Digestion products of isoniazid-labelled RNA from the* 40S *ribosomal subunit*

Details are as described in Table 2.

Treatment of RNA before oxidation and condensation with isoniazid	Enzyme used	Fractionation method (%): 7M-Urea–DEAE, Sephadex column		DEAE-cellulose column		Paper electrophoresis	
None	Pancreatic ribonuclease	Not run		Not run		A	65*
						C	12
						Origin	7
	Ribonuclease T_1	Mono	21	Not run		Oligo	75*
		Di	6			Origin	15
		Tri	9				
		Tetra	5				
		Penta	6				
		Hexa	3				
		Hepta	50				
		(96% recovery)					
One stepwise degradation	Pancreatic ribonuclease	Mono	74	Mono	80	U	50
		Di	14	ApC	2	A	2
		Tri	6	GpC	4	C	3
		Tetra	3	ApA and other (di-)	6	Oligo	9
		Penta	1				
		Residue	2	GpU	3	Origin	22
		(66% recovery)		Other (tri)	5		
				(69% recovery)			
	Ribonuclease T_1	Mono	29	Not run		Not run	
		Di	8				
		Tri	13				
		Tetra	6				
		Penta	5				
		Hexa	34				
		Hepta	1				
		Residue	3				
		(81% recovery)					
Two stepwise degradations	Pancreatic ribonuclease	Mono	26	Mono	29	U	1
		Di	67	ApU	65	Oligo and origin	91
		Tri	4	Residue	6		
		Residue	3	(70% recovery)			
		(64% recovery)					
	Ribonuclease T_1	Mono	14	Not run		Oligo-1	9
		Di	4			Oligo-2	33
		Tri	8			Origin	38
		Tetra	3				
		Penta	63				
		Hexa	4				
		Residue	5				
		(76% recovery)					
Four stepwise degradations	Pancreatic ribonuclease	Mono	72	Not run		U	5
		Di	14			A	3
		Tri	10			C	24
		Tetra	5			Origin	48
		(59% recovery)					
	Ribonuclease T_1	Mono	26	Mono	32	Oligo	63
		Di	12	ApUpC	47	C	4
		Tri	51	Residue	22		
		Tetra	9	(54% recovery)			
		Residue	2				
		(67% recovery)					
Five stepwise degradations	Pancreatic ribonuclease	Mono	42	Not run		C	7
		Di	14			Origin	78
		Tri	9				
		Octa or nona	34				
		(46% recovery)					
	Ribonuclease T_1	Mono	23 19*	Mono	25	Origin	73 (25)*
		Di	56 52	ApU	52	Oligo	— (45)
		Tri	16 15	ApUpC	8		
		Residue	5 9	Residue	15		
		(48% recovery)		(46% recovery)			
				(73% recovery)			

* Isoniazid-labelled RNA was purified by sucrose-density-gradient centrifugation before enzymic digestion.

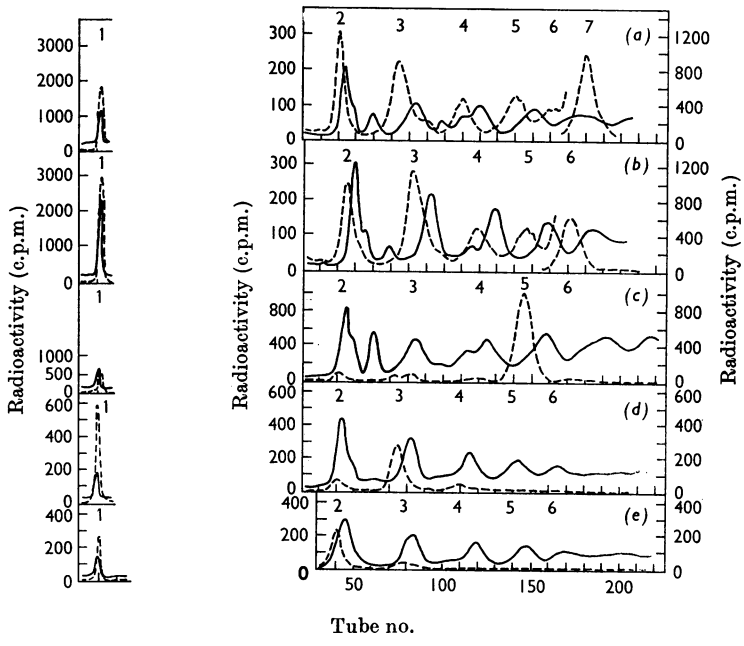

Fig. 6. Column chromatography on DEAE-Sephadex of the ribonuclease T_1 digestion products of [^{3}H]isoniazid-labelled RNA from the small ribosomal subunit before and after stepwise degradation. The numbers refer to the size of the oligomers eluted at these positions. (*a*) Untreated RNA, (*b*) one stepwise degradation, (*c*) two cycles of stepwise degradation, (*d*) four cycles of stepwise degradation, (*e*) five cycles of stepwise degradation. Elution and other conditions were as for Fig. 4. ——, E_{260} of the carrier ribonuclease T_1-digested RNA; ----, ^{3}H radioactivity. Flow rates: (*a*), (*b*) and (*c*) 41ml/h (3.5ml/tube); (*d*) 38ml/h (3.3ml/tube); (*e*) 36ml/h (3.3ml/tube). Only c.p.m. are shown on the ordinate. Where a break occurs on the graph the c.p.m. are those of the appropriate ordinate.

than expected unless further purified by sucrose-density-gradient centrifugation (Hunt, 1970*b*). However, the sucrose-density-gradient profile does change after several stepwise degradations from a main peak at 28S to 16–18S and from 18S to 8–9S for RNA from the large and small subunits respectively. If this change in sedimentation constant is due to polynucleotide chain breakage it would be expected that new end groups would appear and the apparent molecular weight of the RNA would decrease. That this does not occur would indicate that any chain breakage of the RNA is probably non-enzymic and yields a 2′:3′-cyclic phosphate terminus not attacked by alkaline phosphatase, but the change in sedimentation constant may be explained alternatively by a change in shape of the RNA molecules. Tables 2 and 3 show that the yield of recovered radioactivity from column chromatograms in 7M-urea on DEAE-Sephadex decreases as the number of stepwise degradations increases. No good explanation for this phenomenon can be proposed except to note that when the small-ribosomal-subunit RNA after five stepwise degradations was purified by sucrose gradient centrifugation the yield of radioactivity from the 7M-urea–DEAE-Sephadex column after ribonuclease T_1 digestion increased from 48% to 73% without markedly altering the profile of the chromatogram (Table 4). Such a purification also decreased the amount of material remaining at the origin in paper electrophoresis. Examination of the results from Tables 2, 3 and 4 also shows that no inconsistencies in sequences are found in subsequent stepwise degradations. The profiles and quantitative results from successive stepwise degradations, especially of the RNA from the small ribosomal subunit (Fig. 6 and Table 4), indicate that the stepwise degradation technique can be used for at least five cycles, and possibly more, with high yields of the expected sequences. This is in contrast with the experience of Steinschneider & Fraenkel-Conrat (1966) who found that after four stepwise degradations their results were difficult to interpret when they examined the released nucleosides alone.

However, Weith & Gilham (1969) were able to use the stepwise degradation method alone to determine the sequence of ten nucleotides of the 3′-terminal oligonucleotide of Qβ RNA.

The ability to determine the sequences of both 28 S and 6 S RNA together depends on being able to couple together the sequences determined from each step (Tables 2 and 4). After two stepwise degradations two trinucleoside diphosphate isonicotinoylhydrazones were found. The one belonging to the 6 S RNA must have a 3′-terminal guanine, and the first-eluted trinucleoside diphosphate isonicotinoylhydrazone was tentatively identified as UpCpG-isonicotinoylhydrazone. To confirm this observation RNA from the large ribosomal subunit was subjected to two stepwise degradations and then treated for 5min at 60°C with 0.5% sodium dodecyl sulphate before or after reaction with isoniazid (Hunt, 1970*b*). In this way the 6 S RNA was released from the 28 S RNA, which now ran as a broad peak close to 18 S on a sucrose density gradient. The '28 S' isoniazid RNA was digested with ribonuclease T_1 and chromatographed on DEAE-cellulose. The UpCpG-isonicotinoylhydrazone peak was decreased from 21% of the total to 4%, and the UpUpU-isonicotinoylhydrazone peak increased from 36 to 55%, hence proving the assignment of the sequence UpCpG to the 6 S RNA.

The determination of the sequences described leaves little doubt that the majority of each of the RNA molecules classified as 28 S, 18 S and 6 S RNA from reticulocyte ribosomes have unique terminal sequences. It is true that the part of the molecules being examined is relatively small, but the finding of unique sequences encourages the prediction that the rest of the molecules are likely to be similarly unique. This would indicate that the majority of the duplicated ribosomal genes produce RNA of similar sequence, but cannot help to determine whether there is gene duplication or a 'master–slave' arrangement in the DNA.

Comparison with sequences in other rRNA molecules from other species should determine if this is a general phenomenon. The 16 S RNA from *E. coli* has been shown to have a major unique sequence with very little heterogeneity by the elegant work of Fellner *et al.* (1970), who also indicate that the 23 S RNA from *E. coli* has a major unique sequence. The 3′-terminal sequence of *E. coli* 16 S RNA from their studies, -pGp[ApApApUp,$(ApUp)_2$,-$(ApCp)_2Cp_3Up$]A, compared with the 18 S RNA sequence from reticulocytes, -pPyp$(Np)_{5\text{ or }6}$-pGpApUpCpApUpUpA reveals a common -PypA terminus with the possibility of further homology, but also some large differences. The 5 S RNA from *E. coli* has two unique sequences (Brownlee, Sanger & Barrell, 1967) and 5 S RNA from KB cells, rabbit and mouse have identical sequences (Forget & Weissman, 1967; Labrie & Sanger, 1969; Williamson & Brownlee, 1969) and have 3′-terminal sequences of -pGpCpCpApGpGpCpApU from *E. coli* and -pGpUpApGpGpCpUpUp(U) from KB cells. There is no common sequence between the 5 S RNA and the 18 S rRNA or the 28 S and 6 S RNA or any common sequence between the 28 S, 18 S and 6 S RNA that have been examined here.

Most evidence from biosynthetic studies of mammalian rRNA indicates that it is synthesized as a larger precursor containing sequences of both the 18 S and 28 S components. This does not appear to be true for bacteria although both the 23 S and 16 S RNA molecules appear to have slightly larger precursors (Adesnik & Levinthal, 1969). Little is known about the origin of the '6 S' RNA. Whatever the mechanism of synthesis, the breakdown from the large precursor to the final rRNA must be highly specific, since it is possible to obtain unique terminal sequences. No evidence was found of overlapping sequences being produced. There is nothing in common between the three sequences described here or apparently between them and the '5 S' mammalian RNA. There may be some sequence identity between the 16 S *E. coli* RNA and 18 S rabbit RNA, but this must await further studies on the sequence determination of the large ribonuclease T_1 oligonucleotide isolated by Fellner *et al.* (1970). To obtain such specific cleavage is remarkable and may well depend on the sequences close to the 3′-terminus, but these sequences could equally well be on the 3′-side of the cleavage site as the 5′-side and are not determinable. However, results from the 5′-end of *E. coli* 23 S and 16 S RNA would indicate no sequences in common between them or the 5′-end of 5 S RNA, although *E. coli* 5 S RNA produced in the presence of chloramphenicol shows a notable extension of nucleotides at the 5′-end, which could be due to a deficiency in the specificity of transcribing the DNA or in scission by an enzyme (Forget & Jordan, 1970). More results should become available when terminal sequences of the 45 S ribosomal RNA precursor from rabbit are determined.

The method described above has demonstrated its great usefulness in determining sequences from RNA molecules that cannot be labelled to a high specific radioactivity with ^{32}P. It has its limitations in that it can only be readily used for terminal sequence analysis and that the isonicotinoylhydrazones are not as stable as one would wish. It may well be possible to modify the approach by the use of ^{3}H-labelled borohydride to reduce periodate-oxidized RNA, especially in view of its higher specific radioactivity. Randerath, Flood & Randerath (1969) have shown periodate oxidation and boro[^{3}H]hydride reduction to be feasible for labelling nucleosides and 'mapping' procedures may

well be utilized for ^{3}H-labelled 3'-termini and polynucleotide kinase ^{32}P-phosphorylated 5'-termini of oligonucleotides produced by enzymic digestion of RNA (Székely & Sanger, 1969). However, while this may allow 'Sanger' techniques to be used on unlabelled RNA, it does rely on the purity of the RNA being high. The main point of the combination method using isoniazid or possibly borohydride after periodate oxidation of the 3'-end is that one can detect impurities relatively simply, such as the mixture of 6S with 28S RNA, and yet still determine sequences in the presence of the impurities, as has been well demonstrated here. The combination of stepwise degradation and end-group labelling with the use of different enzymes has made it relatively easy to determine sequences with double checks as the sequence progresses. This is a great advantage over the use of stepwise degradation and detection of the released base alone since partial reaction and multiple sequences can easily be accounted for in the combination method but only serve to confuse the interpretation of results from stepwise degradation alone.

This method has been devised to make it relatively easy to determine sequences on the 3'-end of all unlabelled RNA, but especially from mRNA. Since the genetic code has been well established, it would seem unnecessary to obtain a complete sequence of mRNA, but in terms of control, initiation and termination the terminal sequences would seem to be the most important. It is clear that the method described allows determination of sequences of the terminal ten or more nucleotides at the 3'-terminus, which correspond to the peptide-chain-termination end, and does not rely on the fortuitous positioning of any nucleotides. It is not clear, lacking a good stepwise degradation process, that the same kind of sequence determination could be obtained at the 5'-end except perhaps by the use of an exonuclease such as spleen diesterase (EC 3.1.4.1) since endonuclease digestion may or may not produce long oligonucleotide fragments.

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