The Preparation and Analysis of a New Trinucleotide from Yeast Ribonucleic Acid, Containing Two Adjacent 2'-O-Methylpentose Residues

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Quantities of the order of 10 mg of three alkali-stable trinucleotides were prepared from yeast ribonucleic acid. Analyses showed that they were of the type N1m-N2m-N3p, where m signifies 2'-O-methylation of the pentose residue. One, Am-Um-Gp, is newly described.

Nucleotides modified by 2'-O-methylation are known to occur in both transfer ribonucleic acids (tRNA) (see Hall, 1971) and ribosomal ribonucleic acids (rRNA) (Maden, 1971). Hydrolysis of RNA with alkali releases them as alkali-stable dinucleotides. If two consecutive nucleotides are modified by 2'-O-methylation, alkali-stable trinucleotides are released by such hydrolysis. The modified nucleotides occur non-randomly and are the result of enzymic methylation of precursor polynucleotides at definite stages in the maturation of the RNA species. They are much more abundant in rRNA species of the three eukaryotes, which have been studied, than in that of the one prokaryote, *Escherichia coli*, that has been investigated so far.

Materials and Methods

Preparation of trinucleotides

Details of this procedure have been given by Trim & Parker (1970a). Essentially, 150 g of yeast RNA was hydrolysed exhaustively with diethylamine at 55°C. The incompletely hydrolysed fraction of this hydrolysate was separated on columns of DEAE-cellulose by the procedure of Singh & Lane (1964). It included dinucleotides, trinucleotides and mononucleoside diphosphates. About 85% of the ultraviolet-absorbing material in this fraction was composed of di- and tri-nucleotides, which were separated as a single broad band from faster-moving nucleotides by electrophoresis on paper at pH 8.6. Electrophoresis on paper at pH 3 separated this band into seven components, each of which was further resolved by chromatography in solvent A: satd. (NH₄)₂SO₄–0.5 M-sodium acetate–propan-2-ol (79:19:2, by vol.) (Markham & Smith, 1951). Each product was desalted by the method of Rushizky & Sober (1962) and further purified by electrophoresis, first at pH 3 and then at pH 8.6. Analyses showed that each product was a single oligonucleotide species. The 14 dinucleotides obtained in this way have been described in detail by Trim & Parker (1970a), Howlett *et al.*

(1971) and Trim & Parker (1972). Three other products, one of which is described in detail in this paper, were trinucleotides, rendered alkali-stable by 2'-O-methylation of two of the constituent mononucleotide residues.

Determinations

Ultraviolet-absorption spectra. These were measured on the Cary model 14 recording spectrophotometer.

Phosphorus determination. Where necessary, nucleotide phosphate was converted into inorganic phosphate by the method of Bartlett (1959). Inorganic phosphate was measured by the method of Chen *et al.* (1956).

Purine-bound and pyrimidine-bound pentose. These were determined by the method of Trim & Parker (1970b).

Nucleotide sequences. These were determined by combination of results obtained by a procedure based on the enzymic sequencing method of Singh & Lane (1964) and the identification of the 5'-linked terminal nucleotide by a modification (Trim & Parker, 1972) of the method of Neu & Heppel (1964). The trinucleotide was dephosphorylated with *Escherichia coli* alkaline phosphatase and the enzyme was removed by chromatography in solvent B: propan-1-ol–aq. NH₃ soln. (sp.gr. 0.880)–water (10:2:7, by vol.) (Alexander *et al.*, 1961). One sample was used to identify the 5'-linked terminal nucleotide and the other sample was incubated with snake venom phosphodiesterase. The products of complete digestion with this enzyme were separated by electrophoresis on paper at pH 3 in 1 M-acetic acid for 1 h at 60–70 V/cm and identified spectrophotometrically.

2'-O-Methyl groups. Location and identification of these groups in the degradation products was done by converting them all into nucleosides and determining their mass spectra by the procedure of Howlett *et al.* (1971) by using an AEI MS9 mass spectrometer.

Nomenclature

Dinucleotides containing 2'-*O*-methylpentose are represented as N1m-N2p, according to convention (Trim & Parker, 1972), where N1 and N2 are purine or pyrimidine nucleosides and m indicates that N1 is a β -D-furanoside modified by 2'- or 3'-*O*-methylation. Similarly alkali-stable trinucleotides of the same type are represented as N1m-N2m-N3p, where N1, N2 and N3 are purine or pyrimidine nucleosides and m represents 2'- or 3'-*O*-methylation. To describe specific trinucleotides, the symbols A, G, C, U and ψ are used to represent adenosine, guanosine, cytidine, uridine and pseudouridine respectively.

Results and Discussion

In the richest known source of 2'-*O*-methyl nucleotides (Lane, 1965), sequences containing two consecutive modified nucleotides occur at the rate of about one per 1000 mononucleotide residues. Nine different alkali-stable trinucleotides have been isolated from rRNA species but much work remains to be done to detect, describe and estimate all such sequences, even in the rRNA samples of a single species. Am-Am-Up, Am-Gm-Cp and Am-Cm-Gp have been isolated from yeast rRNA (Gray & Lane, 1967) and Am-Gm-Cp, Gm-Am-Up, Gm-Um-Up, Cm-Cm-Cp, Cm-Um-Gp, Um-Gm-Up and Um-Gm-Up have been isolated from wheat-germ rRNA (Lane, 1965). In the present work a tenth alkali-stable trinucleotide, Am-Um-Gp, has been isolated from yeast RNA, but there is no direct evidence that it comes from the rRNA component of this material.

In this work three alkali-stable trinucleotides containing 95% of the calculated P were isolated in quantities of the order of 10mg. They were separated from each other and from the accompanying dinucleotides (Trim & Parker, 1970a) by taking advantage of differences in their electrophoretic

mobilities at pH3 and their mobilities in solvent A (Table 1).

The novel trinucleotide, separated cleanly from dinucleotides by electrophoresis at pH3, was found to run as a single component in the highly resolving chromatographic solvent A. Values for total P and 3'-terminal phosphate, recorded in Table 2, pentose (not recorded), electrophoretic mobility and mobility in solvent A (Table 1) are compatible with its proposed nucleotide composition, which is supported by ultraviolet-absorption spectra measured at pH2, 7 and 12.

The corresponding trinucleoside diphosphate, obtained by dephosphorylation with alkaline phosphomonoesterase, was degraded by the method of Neu & Heppel (1964). This permitted the identification and estimation of the 3'-terminal nucleotide, as the corresponding base, and the residual dinucleotide (Table 2). The latter was recovered and analysed. The product of dephosphorylation by phosphomonoesterase was resistant to further degradation by the method of Neu & Heppel (1964). This is consistent with the proposed modification of the 2'-hydroxy position in each nucleotide, which was further confirmed by pentose estimation.

The full nucleotide sequence of the trinucleotide was then established by complete degradation of the corresponding trinucleoside diphosphate with snake venom phosphodiesterase thus:

pN3 was already identified by yielding the base obtained in the periodate degradation of the parent trinucleotide. N1m is the only nucleoside produced by diesterase treatment so that the remaining mononucleotide product must be pN2m.

Where necessary, the mononucleotides produced by the diesterase treatment were dephosphorylated. Mass spectra of the resulting nucleosides showed that N1m and N2m contained 2'-*O*-methylpentose, leaving the pentose configuration uncertain, and N3 was shown to be a riboside.

Table 1. *Electrophoretic and chromatographic mobilities of trinucleotides*

Electrophoretic mobilities were measured relative to Up on a flat-plate apparatus in 0.1M-ammonium formate buffer, pH3.0 (see Trim & Parker, 1970a), with Whatman no. 3MM paper. Chromatographic mobilities, relative to Up, were measured by ascending chromatography in solvent A on Whatman no. 1 paper.

Trinucleotide	Electrophoretic mobility (at pH3 relative to Up)		Mobility in solvent A (relative to Up)
	Measured	Calculated	
(1) Am-Gm-Cp	0.49	0.49	0.15
(2) Am-Am-Up	0.72	0.63	0.09
(3) Am-Um-Gp	0.83	0.94	0.15

Table 2. *Nucleotide sequence of trinucleotide*

A small quantity ($0.33\mu\text{mol}$) of the trinucleotide was degraded. (1) By the method of Neu & Heppel (1964), under conditions described by Trim & Parker (1972). The products were identified and estimated and the 3'-terminal P of the dinucleotide products was removed and measured. Purine-bound and pyrimidine-bound pentose in these dinucleotides were measured by the method of Trim & Parker (1970b). (2) By the method of Singh & Lane (1964). The products of phosphodiesterase action were separated by electrophoresis on paper at pH3 in 0.5M-acetic acid and eluted with water. All products were identified by spectrophotometry. N1m products were suitable for mass spectrometry. pN2m and pN3 products were dephosphorylated with alkaline phosphomonoesterase and purified for mass spectrometry by chromatography on paper in solvent B followed by electrophoresis on paper at pH8.6 in NH_4HCO_3 .

Trinucleotide	P (%)	Products of degradation					
		By method of Neu & Heppel (1964)					
		By phosphatase (μmol of P _i)	By phosphodiesterase		Terminal base (μmol)	Dinucleotide (μmol)	
			N1m	pN2m			pN3
Am-Um-Gp	8.17	0.34	Guanine, 0.24	AmpUmp, 0.24	Am	pUm	pG

Thus the identity of the trinucleotide was established by stepwise degradation and the identification and estimation, with high recovery, of each degradation product.

These measurements were also made on the trinucleotides 1 and 2 (Table 1) to confirm and amplify the identifications made by Gray & Lane (1967).

References

- Alexander, M., Heppel, L. A. & Hurwitz, J. (1961) *J. Biol. Chem.* **236**, 3014-3019
 Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466-471
 Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756-1758
 Gray, M. W. & Lane, B. G. (1967) *Biochim. Biophys. Acta* **134**, 243-257
 Hall, R. H. (1971) *The Modified Nucleosides in Nucleic Acids*, pp. 1-451, Columbia University Press, New York and London
 Howlett, H. A., Johnson, M. W., Trim, A. R., Eagles, J. & Self, R. (1971) *Anal. Biochem.* **39**, 429-440
 Lane, B. G. (1965) *Biochemistry* **4**, 212-219
 Maden, B. E. H. (1971) *Progr. Biophys. Mol. Biol.* **22**, 127-177
 Markham, R. & Smith, J. D. (1951) *Biochem. J.* **49**, 401-406
 Neu, H. C. & Heppel, L. A. (1964) *J. Biol. Chem.* **239**, 2927-2934
 Rushizky, G. W. & Sober, H. A. (1962) *Biochim. Biophys. Acta* **55**, 217
 Singh, H. & Lane, B. G. (1964) *Can. J. Biochem.* **42**, 87-93
 Trim, A. R. & Parker, J. E. (1970a) *Biochem. J.* **116**, 589-598
 Trim, A. R. & Parker, J. E. (1970b) *Anal. Biochem.* **35**, 475-479
 Trim, A. R. & Parker, J. E. (1972) *Anal. Biochem.* **46**, 482-488