MINOR NUCLEOTIDES IN THE RIBOSOMAL RNA OF *THERMOMYCES LANUGINOSUS*

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ABSTRACT

^{32}P labelled nucleotides of rRNA isolated from the thermophilic fungus, *Thermomyces lanuginosus*, were analysed for ribose-methylated dinucleotides and other modified nucleotides. The study revealed the presence of all the sixteen possible dinucleotides, pseudouridylate and a few other unidentified nucleotides. The total number of ribose methylations in the rRNA of this organism is higher than that for other non-vertebrates. The most abundant modified nucleotide is pseudouridylate and its molar proportion is higher than that reported for any other organism.

INTRODUCTION

RIBOSOMAL RNA from diversified sources has been shown to contain modified nucleotides mainly pseudouridylic acid and methylated derivatives[1–4]. Unlike in transfer RNA, the vast majority of methylations in rRNA are ribose methylations[5,6]. Conversion of certain uridine residues to pseudouridine and methylation of ribose moiety and bases at specific sites, occur during the maturation of rRNA[4,6,7]. Comparative studies have shown that certain site specific modifications are characteristic of rRNA from different sources[8–14]. The sequences and the number of these modified nucleotides are conserved during evolution of rRNA[10,14–16]. Eukaryotic rRNA contains relatively more modifications than that from prokaryotic species[3,4,8]. It has been demonstrated that the presence of the hyper-modified dinucleotide $m_2^6Am_2^6A$, in the sequence $m_2^6Am_2^6ACCUG$ at the 3′ end of 16S rRNA of *E. coli* makes the bacterium sensitive to the antibiotic, Kasugamycin[17]. Although extensive work has been carried out on the rRNA from various sources there has been no report on the modified nucleotides in the rRNA of multicellular fungi. Thermophilic organisms, in general, are of special interest in biochemical studies in that they reveal the extremes to which evolution has been pushed successfully to carry out the complete life cycle of the organism.

MATERIALS AND METHODS

Thermomyces lanuginosus used in the present studies was gift from Dr. Ramesh Maheswari of this Department. RNase T_2 was from Sigma Chemical Co., St. Louis and carrier-free ^{32}P-orthophosphate was from Bhabha Atomic Research Centre, Bombay.

Isolation of RNA

The organism was grown in a synthetic medium[18]. For radiolabelling of RNA, 20–40 microcuries of ^{32}P-orthophosphate per ml and KCl were added instead of K_2HPO_4. Cells were grown at 50° C for 24 hr and the total RNA was isolated by the phenol extraction procedure[19]. High molecular weight rRNA was separated by precipitation with high salt[20,21] or by gel filtration on a Sephadex G–200 column.

Preparation of Nucleotides

Conversion of ^{32}P-labelled RNA to nucleotides was performed by digestion of RNA with RNase T_2 in pH 4·5 buffer at 37° C for 16 hr. Alkali digestion was carried out with 0·3M KOH for 18 hr at 37° C.

Separation of Nucleotides

High voltage electrophoresis was carried out on Whatman No. 1 paper in 0·5% pyridine-acetate buffer, pH 3·5, containing 0·005M Na_2EDTA at 60V/cm. Two-dimensional thin-layer chromatography was done on cellulose plates according to the method of Nishimura[22].

Spots were detected by autoradiography. They were cut out/scrapped and the radioactivity was measured in a LS–100 Liquid scintillation counter.

Chromatography on Dowex-I-X-8 column was carried out according to the procedure of Madison and Holley[23] with minor modifications.

RESULTS AND DISCUSSION

The purity of the ^{32}P-labelled rRNA prepared by high salt precipitation was checked by gel filtration on a Sephadex G–150 column. The radioactivity was eluted from the column just after the void volume as a single peak showing that the rRNA was free from tRNA. RNase T_2 and alkali digests of the rRNA were subjected to high voltage paper electrophoresis. The overall patterns obtained in the two cases were essentially the same, although minor differences could be noted. Alkali is known to modify certain minor nucleotides[22]. Partial separation of a number of spots into 2′ and 3′ phosphate derivatives could be observed in the alkali digested sample. The presence of at least 12 minor spots, in addition to the four major species Cp, Ap, Gp and Up, could be noted in all the autoradiograms (Fig. 1). This pattern was highly reproducible. Each of the major and the minor spots (1 to 12) was cut out, the radioactivity

measured and the molar percentage of each was calculated (Table I). The GC content of the RNA is approximately equal to the AU content. In this respect *T. lanuginosus* resembles most other fungi[24]. Radioactivity in the minor spots 1 to 12 amounted to approximately 2·9% of the total counts. This is the minimum as some of the spots are likely to be masked by the large spots.

whole area containing Up and the minor nucleotide was cut out from the paper, the radioactivity was eluted[25] and subjected to two-dimensional thin layer chromatography[22]. The minor spot moved to the standardised position of pseudouridine-3 -phosphate (ψ_p). The radioactivity in this spot accounted to about 10% of that in Up. It could also be detected by two-dimensional thin layer chromatography of the total digest

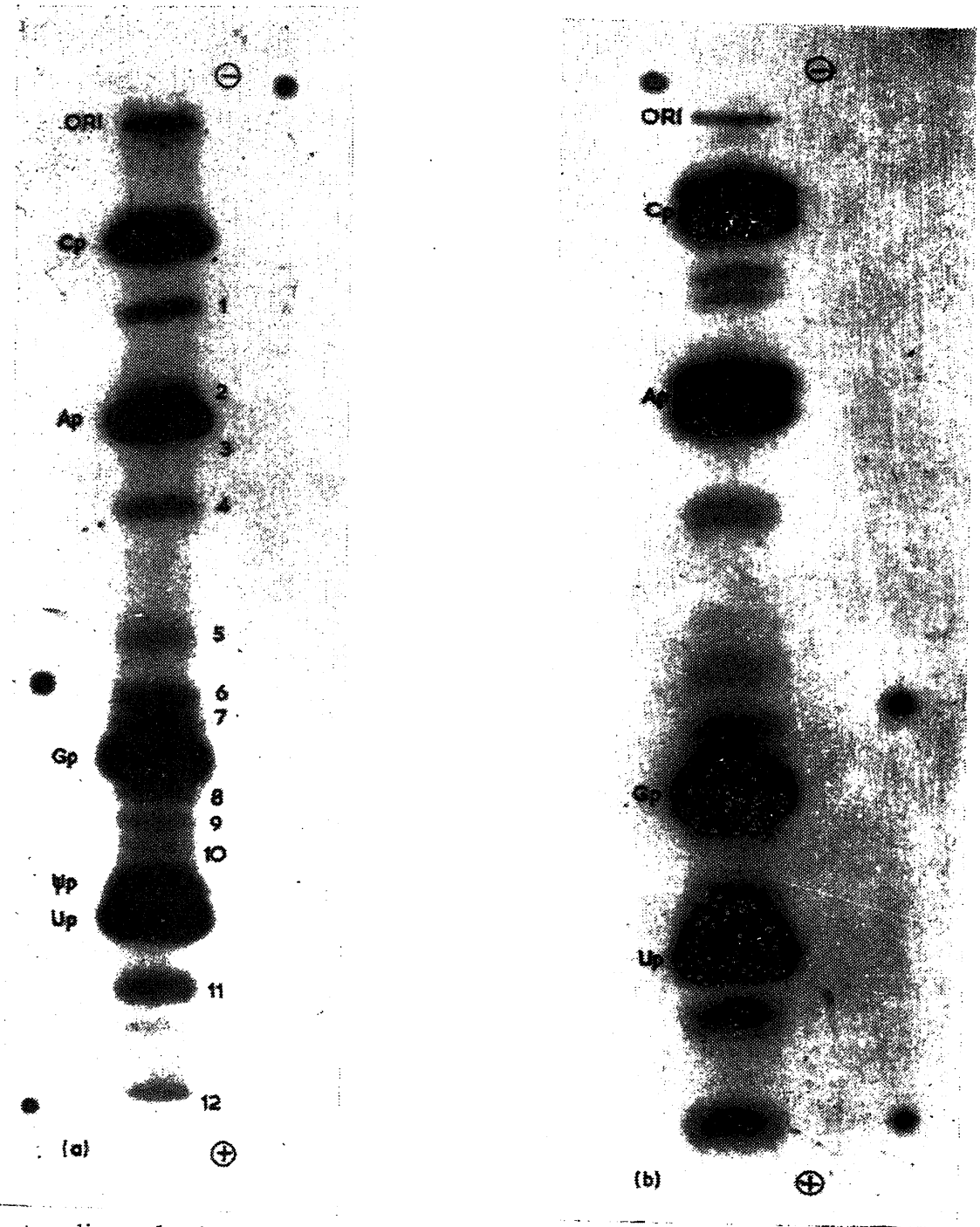


FIG. 1. Autoradiograph of the separation of nucleotides by high voltage electrophoresis. ^{32}P-nucleotide prepared by RNase T_2 digestion (*a*) and alkali digestion (*b*) of combined rRNA of *T. lanuginosus* were separated on a Whatman No. 1 paper at pH 3·5 (60V/cm).

The radioactive spot due to Up was contaminated with a minor spot whose relative proportion was much higher than that of any other minor spot. The

of the rRNA as the most prominent spot, apart from the major nucleotide spots (Fig. 2*a*). It amounted to 2·25% of the total, about 10% of Up.

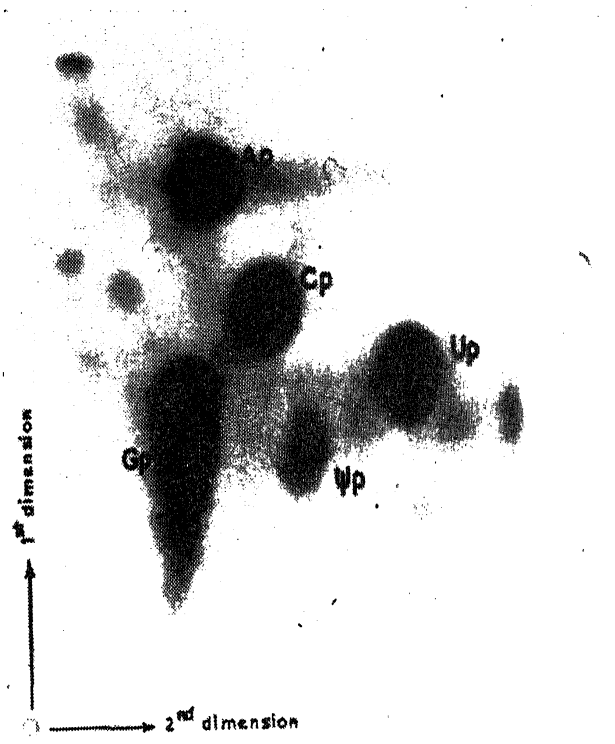
Separation of ψ_p from the total digest was achieved by chromatography on a Dowex-I-X-8 column[23]. A small peak of radioactivity could be observed in between the positions of Cp and Up (Fig. 3). The radioactivity in this peak was approximately 10% of that in Up, thus confirming the results of the thin layer chromatography experiment (Table II). These fractions were pooled, the solution concentrated and it was subjected to high voltage paper electrophoresis using Up as marker. The radioactivity moved slightly slower than Up, confirming further that the minor peak eluting between Cp and Up in Dowex-I-X-8 column was ψ_p. The molar percentage of ψ_p (2·25%) in the rRNA of *T. lanuginosus*, obtained in the present studies, is higher than the previously reported high percentages for this nucleotide in other species, 1·9% in wheat germ[1], 1·6% in germinating wheat embryo[11] and 1·2% in He La cells[26].

TABLE I

Nucleotide composition of the combined rRNA of T. lanuginosus

Spot No.	CPM	Molar % of nucleotides	Number of nucleotides in 5800	Number of methylated nucleotides	
				Calculated	Nearest integer
Cp	862380	21·57	1251	..	..
1	14950	0·37	21·7	10·8	11
2	2710	0·07	3·9	2·0	2
Ap	952940	23·84	1383	..	..
3	2675	0·07	3·9	1·9	2
4	13270	0·33	19·3	9·7	9–10
5	4685	0·12	6·8	3·4	3–4
6 + 7	20030	0·50	29·1	14·6	14–15
Gp	1143520	28·60	1659	..	..
8+9+10	20090	0·50	29·2	14·6	14–15
Up + ψ_p	924250	23·12	1341	..	..
11	19285	0·48	28·0	14·0	14
12	16820	0·42	24·4	12·2	12

Each of the spots (Fig. 1*a*) was cut out and counted. The values given are the averages of three experiments. The number of nucleotides was calculated assuming a molecular weight of $2·1 \times 10^6$ (5800 nucleotides) for the combined rRNA. Spots 1–12 are assumed to be dinucleotides for purposes of calculation. $(G + C)/(A + U) = 1·07$ and $(A + G)/(C + U) = 1·17$.



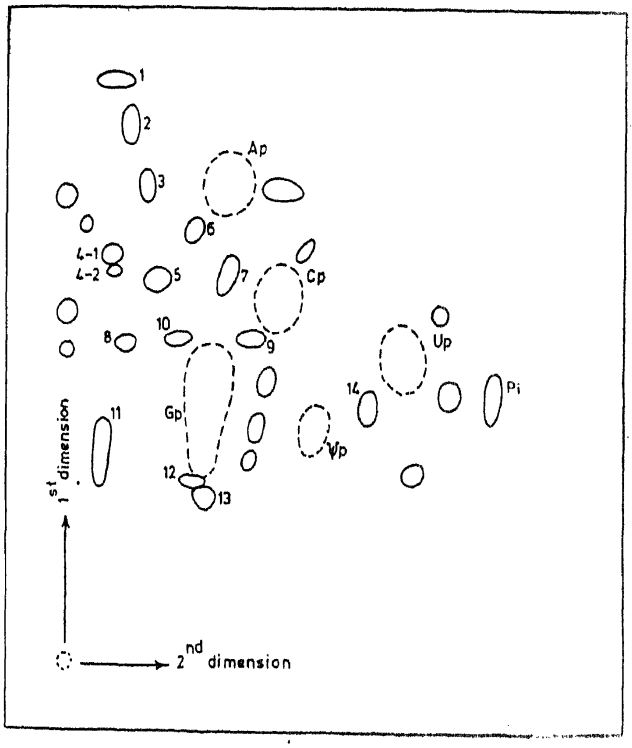


FIG. 2. Two-dimensional thin layer chromatography of RNase T_2 digest. RNase T_2 digest of ^{32}P-labelled combined rRNA of *T. lanuginosus* was subjected to two-dimensional thin layer chromatography. Solvent systems used were : First dimension, isobutyric acid : 0·5 M NH_4OH (5 : 3 v/v) and second dimension, isopropanol : Con. HCl : H_2O (70 : 15 : 15 v/v/v) (*a*) autoradiograph of the separation, (*b*) representation of the various spots numbered according to that in ref. 13.

As expected most of the minor spots in the autoradiogram (Fig 1*a*) were found to be dinucleotides or oligo-

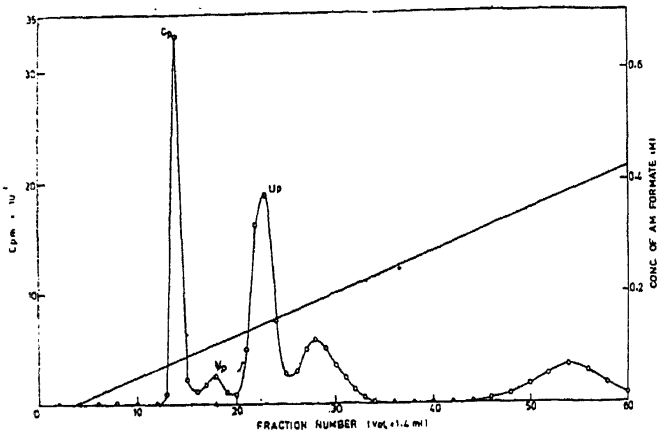


FIG. 3. Separation of nucleotides on Dowex-I-X-8 column. RNase T_2 digest (3·2 lakhs CPM) was chromatographed on a Dowex-I-X-8 column (0·5 × 32 cm) with a linear gradient 0·005 M to 1·0 M ammonium formate, pH 3·9. Fractions of 1·4 ml were collected at a flow rate of 3·5 ml/hr. 1 ml samples were counted.

TABLE II

Pseudouridine-3′-phosphate content of rRNA of T. lanuginosus *determined by different methods*

Separation method	Radioactivity (CPM)			ψ_p per cent
	Total digest	Up	ψ_p	
Two-dimensional TLC of total digest	219800	..	5040	2·29
Two-dimensional TLC of Up + ψ_p	..	106215	11330	10·67
Dowex-I-X-8 column chromatography	..	51340	5610	10·93

The separation methods employed are given in the text The results presented in each are of one of the representative experiments.

nucleotides, ribose methylation being the predominant modification. After transfer from Whatman No. 1 paper to DEAE-cellulose paper and electrophoresis in the second dimension at pH 3·5, most of the minor spots moved well behind the major nucleotides (data not presented). Identification of the dinucleotide spots was done by comparison of the two-dimensional thin layer chromatographic pattern (Fig. 2*b*) with that for dinucleotides obtained by Hashimoto *et al.* for mouse hepatoma rRNA[13]. All the sixteen dinucleotide spots could be identified by examination of a number of autoradiographs from their position. These are numbered according to the numbering followed by the above investigators (Fig. 2*b*). The identity of each of the dinucleotides is shown in Table III. It is assumed that spot No. 9 contains both CmpUp and UmpCp. Thus all the 16 possible dinucleotides are present in the rRNA of *Thermomyces lanuginosus*. The unidentified spots may represent mono, tri or tetranucleotides. Two prominent minor nucleotide spots found in the enzymatic digest are 1 and 4 (Fig. 1*a*). These have been identified as CmpCp and AmpCp respectively (data not presented). The corresponding spots in the two-dimensional thin layer pattern are spot numbers 5 and 2 (Fig. 2*b*). These are comparatively intense spots as in the electrophoretic pattern. It is interesting that although the percentage of Cp is the lowest among the major nucleotides the proportion of CmpCp among dinucleotides is relatively more (Table I). Further work on the determination of the molar quantities of dinucleotides and characterisation of each of the other nucleotides is in progress.

TABLE III

Identification of dinucleotides by two-dimensional thin layer chromatography

Spot No.	Dinucleotide	Spot. No.	Dinucleotide
1	AmpAp	8	CmpGp
2	AmpCp	9	CmpUp
3	CmpAp		UmpCp
4–1	AmpGp	10	GmpCp
4–2	GmpAp	11	GmpGp
5	CmpCp	12	GmpUp
6	AmpUp	13	UmpGp
7	UmpAp	14	UmpUp

Spot numbers indicated are those shown in Fig. 2*b*. The numbering has been done according to that followed by Hashimoto *et al.*[13].

The total number of ribose methylations of rRNA varies from organism to organism. Vertebrate rRNA has the highest number and it is significantly lower in other eukaryotes. The rRNA of vertebrates has a combined molecular weight ranging from $2·2 \times 10^6$ for amphibians to $2·4 \times 10^6$ for mammals, but all of them contain 95 or more ribose methylations[12,27]. The combined molecular weight of the rRNA of non-vertebrates such as *Drosophila* and yeast is only slightly smaller (approximately $2·1 \times 10^6$). The former contains 59 to 64 ribose methylated nucleotides[27] while the latter contains only 55 to 59 such modifications[10,11,27]. Assuming that only 85% of the radioactivity in the minor spots observed in the

electrophoretic pattern for the rRNA of *T. lanuginosus* is due to ribose methylated nucleotides and the combined molecular weight of the rRNA is about $2{\cdot}1\times10^6$, the same as that for *Drosophila* and yeast, the number of ribose methylation in the present case is at least 70. This is more than that found in the rRNA of non-vertebrates like *Drosophila* and yeast.

Pseudouridine-3′-phosphate content of the combined rRNA obtained in the present studies is the highest reported for any organism. It is, at present, unknown whether the high content of ψ_p and ribose methylated nucleotides in the rRNA of *T. lanuginosus* is in any way related to its thermophilicity.

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INSA AWARDS

Dr. D. P. Antia, Industrial and Management Consultant, Calcutta, has been awarded the S. H. Zaheer Medal for 1980 for his contribution in the field of non-ferrous metal technology. Dr. A. G. Dutta, Indian Institute of Experimental Medicine, Calcutta, has been awarded the Bashambar Nath Chopra Memorial Lectureship for 1980 for his contribution to Biological Oxidation Mechanism.