

## Biosynthesis of a Hypermodified Nucleotide in *Saccharomyces carlsbergensis* 17S and HeLa-Cell 18S Ribosomal Ribonucleic Acid

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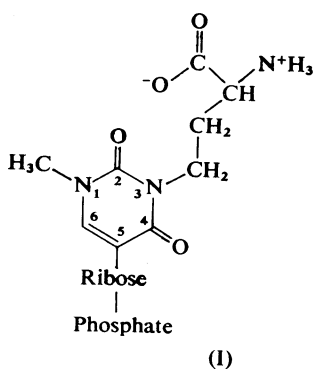
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The biosynthesis of a hypermodified nucleotide, similar to or identical with 3-(3-amino-3-carboxypropyl)-1-methylpseudouridine monophosphate, present in *Saccharomyces carlsbergensis* 17S and HeLa-cell 18S rRNA, was investigated with respect to the sequence of reactions required for synthesis and their timing in ribosome maturation. In both yeast and HeLa cells methylation precedes attachment of the 3-amino-3-carboxypropyl group. In yeast the methylated precursor nucleotide was tentatively characterized as 1-methylpseudouridine. This precursor nucleotide was demonstrated in both 37S and most of the cytoplasmic 18S pre-rRNA (rRNA precursor) molecules. The synthesis of the hypermodified nucleotide is completed just before the final cleavage of 18S pre-rRNA to give 17S rRNA, so that the final addition of the 3-amino-3-carboxypropyl group is a cytoplasmic event. Comparable experiments with HeLa cells indicated that formation of 1-methylpseudouridine occurs at the level of 45S RNA and addition of the 3-amino-3-carboxypropyl group occurs in the cytoplasm on newly synthesized 18S rRNA.

In a previous report we described the presence of a hypermodified nucleotide in HeLa-cell 18S and yeast (*Saccharomyces carlsbergensis*) 17S rRNA (Maden *et al.*, 1975). This nucleotide is referred to here as am $\Psi$ p and is similar to or identical with 3-(3-amino-3-carboxypropyl)-1-methylpseudouridine monophosphate (I),\* first characterized in Chinese-hamster 18S rRNA by Saponara & Enger (1974). The nucleotide is known to be present in several other eukaryotic 18S rRNA species as well (Khan & Maden, 1976).



\* We propose to refer to the hypermodified nucleoside described in this paper as 3-(3-amino-3-carboxypropyl)-1-methylpseudouridine instead of 1-methyl-3- $\gamma$ -( $\alpha$ -amino- $\alpha$ -carboxypropyl)pseudouridine used previously by Saponara & Enger (1974) and Maden *et al.* (1975). We propose to use am $\Psi$  as an abbreviation of this nomenclature.

Formation of am $\Psi$ p apparently requires three modification reactions: (a) pseudouridylation, (b) methylation and (c) attachment of the 3-amino-3-carboxypropyl group. Both the methyl group and the 3-amino-3-carboxypropyl group are transferred from methionine into this nucleotide (Enger & Saponara, 1968; Maden *et al.*, 1975). As part of our studies on modification of rRNA we wanted to establish (a) the sequence of the modification events required for synthesis of am $\Psi$ p and (b) the timing of these modifications within the process of ribosome maturation. We show here, for both yeast and HeLa cells, that pseudouridylation and  $N^1$ -methylation are early reactions, which occur on 37S and 45S pre-rRNA $\dagger$  respectively, and that the introduction of the 3-amino-3-carboxypropyl fragment into am $\Psi$ p is a late event in the ribosome maturation process. For both organisms this latter modification takes place in the cytoplasm. In yeast it occurs just before the final cleavage that gives rise to 17S rRNA.

### Experimental

#### Materials

Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. The following enzymes were from Calbiochem A.G., Lucerne, Switzerland: T<sub>1</sub> ribonuclease (EC 3.1.4.8), T<sub>2</sub> ribonuclease (EC 3.1.4.23) and U<sub>2</sub> ribonuclease (EC

$\dagger$  Abbreviations: pre-rRNA, ribosomal precursor RNA; U\*, an unknown derivative of uridine.

3.1.4.8). The following enzymes were from Worthington Biochemical Corp., Freehold, NJ, U.S.A.: pancreatic ribonuclease, RAF grade (EC 3.1.4.22); bacterial alkaline phosphatase, BAPF grade (EC 3.1.3.1); snake-venom phosphodiesterase, VPH grade (EC 3.1.4.1).

For chromatography uridine (Merck A.G., Darmstadt, Germany) and pseudouridine (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used as reference markers.

The scintillation fluid contained 4g of 2,5-diphenyl-oxazole, 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 30ml of Nuclear-Chicago Solubilizer in 1 litre of toluene.

#### Isolation of labelled RNA

<sup>32</sup>P-labelled 18S pre-rRNA and 17S rRNA were isolated from *Saccharomyces carlsbergensis* (N.C.Y.C. 74), as described by de Jonge *et al.* (1977) and Klootwijk *et al.* (1972) respectively.

Methyl-<sup>3</sup>H-labelled nuclear and cytoplasmic 18S pre-rRNA as well as 37S pre-rRNA were isolated from *S. carlsbergensis* as described elsewhere (Brand *et al.*, 1977).

Yeast RNA pulse-labelled with [2(*n*)-<sup>3</sup>H]methionine was isolated to study the introduction of the 3-amino-3-carboxypropyl group into am $\Psi$ p. Conditions of growth, preparation of protoplasts and conditions of pulse-labelling were essentially the same as described previously (Brand & Planta, 1975), except that the labelling was carried out in 10 ml of the protoplast suspension ( $A_{550} = 0.8$ ) with 100  $\mu$ Ci of L-[2(*n*)-<sup>3</sup>H]methionine. Incorporation of label was stopped after 5 min and protoplasts were collected by centrifugation (Brand *et al.*, 1977). After lysis of the protoplasts extraction of RNA was carried out at 0°C by phenol/0.5% (w/v) sodium dodecyl sulphate as described by Retèl & Planta (1967). The <sup>3</sup>H-labelled RNA was analysed by electrophoresis on 2.6% polyacrylamide gels (Trapman *et al.*, 1975) with methyl-<sup>14</sup>C-labelled yeast rRNA (Klootwijk & Planta, 1973a) as a marker.

Experiments with HeLa-cell RNA were carried out after growth of cells in the presence of [methyl-<sup>14</sup>C]methionine or [1-<sup>14</sup>C]methionine. General procedures for radioactive labelling, isolation of RNA and fingerprinting analysis were described elsewhere (Maden & Salim, 1974; Maden *et al.*, 1975).

#### Isolation and analysis of the methylated precursor nucleotide of am $\Psi$ p from yeast

Methyl-<sup>3</sup>H-labelled 18S pre-rRNA, containing the methylated precursor nucleotide of am $\Psi$ p, was mixed with <sup>32</sup>P-labelled 18S pre-rRNA, digested with T<sub>1</sub> ribonuclease (Brownlee, 1972) and the products were separated by two-dimensional electrophoresis (Fellner, 1969). After radioautography the oligo-

nucleotide containing the precursor of am $\Psi$ p is represented by spot B (see the Results section). This <sup>32</sup>P- and methyl-<sup>3</sup>H-labelled oligonucleotide was eluted with 30% (v/v) triethylamine carbonate (pH10).

The methylated precursor nucleotide of am $\Psi$ p was characterized (*a*) as a nucleotide by electrophoresis and chromatography and (*b*) as a nucleoside by chromatography. To obtain the precursor nucleotide of am $\Psi$ p the oligonucleotide material of spot B was digested with pancreatic ribonuclease (Brownlee, 1972) and the products were fractionated for 3h at 30 V/cm on DEAE-cellulose paper in a 5% (v/v) acetic acid buffer adjusted to pH3.5 by addition of NH<sub>3</sub>. After radioautography the Up-like product (U\* $\Psi$ p), the precursor of am $\Psi$ p, was eluted and subjected to electrophoresis on Whatman 3MM paper at pH3.5 as described by Jeppesen (1971). <sup>32</sup>P-labelled rRNA, digested to completion with T<sub>2</sub> ribonuclease (Brand *et al.*, 1977) was used to provide reference nucleotides.

Alternatively, U\* $\Psi$ p was applied to a sheet of Whatman no. 1 paper and subjected to descending chromatography with solvent C, propan-2-ol/conc. HCl/water (340:85:72, by vol.), as described by Hall (1971). Up and  $\Psi$ p, recovered from a 'fingerprint' of a combined T<sub>1</sub>-plus-pancreatic-ribonuclease digest of <sup>32</sup>P-labelled rRNA (Klootwijk & Planta, 1973b), were used as reference compounds. The position of <sup>32</sup>P- and methyl-<sup>3</sup>H-labelled U\* $\Psi$ p was identified after radioautography.

To analyse the methyl-<sup>3</sup>H-labelled precursor nucleotide of am $\Psi$ p the oligonucleotide material of spot B (see above) was digested for 16h at 37°C with a mixture of 1.5  $\mu$ g of snake-venom phosphodiesterase and 0.5  $\mu$ g of bacterial alkaline phosphatase in 10  $\mu$ l of 50mM-Tris/HCl, pH8.9, containing 10mM-MgCl<sub>2</sub>. The digest was subjected to descending chromatography on Whatman no. 1 paper in solvent C (see above), as described by Hall (1971), and solvent A, 2-methylpropan-2-ol/ethyl methyl ketone/formic acid/water (8:6:3:3, by vol.), as described by Saponara & Enger (1974). The strip of the chromatogram was cut into pieces (1.5cm  $\times$  1.5cm) and assayed for <sup>3</sup>H radioactivity after solubilization with 3% (v/v) Nuclear-Chicago Solubilizer in a toluene-based scintillation fluid (37°C, overnight). Uridine and pseudouridine were used as references.

## Results

### *Saccharomyces carlsbergensis*

Formation of am $\Psi$ p has to be completed at a late stage of maturation of the small ribosomal subunit, for am $\Psi$ p is not present in 37S pre-rRNA (Brand *et al.*, 1977). Therefore to clarify the sequence of

events involved in the synthesis of am $\Psi$ p we first analysed 18S pre-rRNA, the direct precursor of yeast 17S rRNA, for the occurrence of am $\Psi$ p or any precursor of am $\Psi$ p. To this end  $^{32}\text{P}$ -labelled 18S pre-rRNA was compared with 17S rRNA by 'fingerprinting' of  $T_1$  ribonuclease digests as shown in Plate 1. Spot A, containing am $\Psi$ p in 17S rRNA (Maden *et al.*, 1975), is only weakly present in 18S pre-rRNA. This implies that part of the same oligonucleotide material should be recoverable from another position of the 'fingerprint', most probably owing to a different state of modification of am $\Psi$ p. giving rise to a different electrophoretic mobility. A likely candidate is the oligonucleotide of spot B of 18S pre-rRNA, which is not present in 17S rRNA. Sequence analysis of the oligonucleotides of spots A and B (cf. Table 1) strongly suggests that the same oligonucleotide is involved, but instead of am $\Psi$ p spot B contains another uridine-like nucleotide (U\* $\Psi$ p).

To establish the nature of U\* $\Psi$ p we first determined whether this nucleotide is methylated. A mixture of

*methyl*- $^3\text{H}$ - and  $^{32}\text{P}$ -labelled 18S pre-rRNA was digested with  $T_1$  ribonuclease and analysed as described in Plate 1. Spot B contains a significant amount of *methyl*- $^3\text{H}$ -label and this label remains associated with U\* $\Psi$ p after subsequent digestion of the oligonucleotide of spot B with pancreatic ribonuclease. Therefore U\* $\Psi$ p is a methylated uridine derivative and the sensitivity to pancreatic ribonuclease indicates the presence of a base methyl group. Further characterization of this compound was obtained by electrophoretic and chromatographic analysis. The data summarized in Table 2 strongly suggest the presence of m $^1\Psi$ p in spot B. This is inferred from the intermediate mobility of U\* $\Psi$ p between those of Up and  $\Psi$ p in solvent C and from the  $R_F$  value in solvent A compared with literature data (Saponara & Enger, 1974). The finding of m $^1\Psi$  as a precursor of am $\Psi$ p indicates the following sequence of modifications: (a) formation of  $\Psi$ p, (b) methylation at the N-1 position thus made available and (c) introduction of the 3-amino-3-carboxypropyl group.

Table 1. *Sequence analysis of the oligonucleotide of spot B in a 'fingerprint' of a  $T_1$ -ribonuclease digest of yeast 18S pre-rRNA* Yeast  $^{32}\text{P}$ -labelled 18S pre-rRNA was 'fingerprinted' as described in Plate 1. The oligonucleotide material of spot B was eluted and digested with either pancreatic or  $U_2$  ribonuclease under the same conditions as described by Brownlee (1972). The products were fractionated by electrophoresis for 3 h at 30V/cm on DEAE-cellulose paper in a 5% (v/v) acetic acid buffer adjusted to pH 3.5 by addition of  $\text{NH}_3$ . After radioautography  $^{32}\text{P}$  radioactivity in all spots was counted (Clausen, 1968). Base compositions of the oligonucleotide products were determined after digestion to completion with  $T_2$  ribonuclease and separation of the nucleotides by electrophoresis on Whatman 3MM paper at pH 3.5 (Brand *et al.*, 1977). Alternatively the longest  $U_2$ -ribonuclease digestion product was analysed after digestion to completion with snake-venom phosphodiesterase (de Jonge *et al.*, 1977). Molar yields of products relative to Gp are given in parentheses. Data for spot A are taken from Maden *et al.* (1975).

Ribonuclease for digestion	Spot	RNA species	Products
Pancreatic	A	17S	A-A-Cp (1.0), A-Cp (2.0), Gp (1.0), am $\Psi$ -Cp (0.8)
Pancreatic	B	18S	A-A-Cp (1.1), A-Cp (2.0), Gp (1.0), U* $\Psi$ p (0.7)*, Cp (1.3)
$U_2$	A	17S	C-am $\Psi$ -C-Ap (0.8), C-Ap (0.9), C-Gp (1.0), Ap (2.3)
$U_2$	B	18S	C(U*,C)Ap (1.0), C-Ap (1.2), C-Gp (1.0), Ap (1.9)

\* U\* $\Psi$ p moves slightly ahead of Up on electrophoresis on DEAE-cellulose paper at pH 3.5 (see also Table 2).

Table 2. *Characterization of the methylated precursor nucleotide of am $\Psi$ p*

The methylated precursor nucleotide of am $\Psi$ p was isolated from a mixture of *methyl*- $^3\text{H}$ - and  $^{32}\text{P}$ -labelled yeast 18S pre-rRNA and characterized as described in the Experimental section.  $R_U$  is the mobility of the component relative to that of uridine 3'-phosphate.

	Precursor compound (U* $\Psi$ p)		References				
	Nucleotide	Nucleoside	Up	$\Psi$ p	U	$\Psi$	m $^1\Psi$
$R_U$ values for electrophoresis at pH 3.5 on:							
DEAE-cellulose	1.07	—	1.00	1.00	—	—	—
Whatman 3MM	0.95	—	1.00	1.00	—	—	—
$R_F$ values for chromatography with solvents:							
Propan-2-ol/conc. HCl/water (340:85:72 by vol.; solvent C)	0.68	0.56	0.72	0.58	0.59	0.43	—
2-Methylpropan-2-ol/ethyl methyl ketone/formic acid/water (8:6:3:3 by vol.; solvent A)	—	0.31	—	—	0.34	0.17	0.32*

\* See Saponara & Enger (1974).

To establish the timing of the various modification steps during ribosome maturation we analysed the molar yield of  $m^1\Psi p$  and  $am\Psi p$  in 37S pre-rRNA, the predominant common precursor of yeast 26S and 17S rRNA, and in nuclear and cytoplasmic 18S pre-rRNA. Both fractions of 18S pre-rRNA can be distinguished, because in yeast, unlike in other eukaryotes, the final maturation of 18S pre-rRNA occurs in the cytoplasm (Trapman & Planta, 1976). Table 3 shows that practically all 37S pre-rRNA molecules contain  $m^1\Psi p$ . Therefore both formation of  $\Psi p$  and methylation are early modifications, as they take place before or just after the formation of 37S pre-rRNA. The attachment of the 3-amino-3-carboxypropyl group, on the other hand, seems to be a late event at the level of cytoplasmic 18S pre-rRNA, as can be inferred from the very low molar yield of the completed nucleotide ( $am\Psi p$ ) in cytoplasmic 18S pre-rRNA (Table 3). This low value, however,

could also have arisen from some 17S rRNA contamination. To establish clearly whether the completion of synthesis of  $am\Psi p$  occurs before or after cleavage of 18S pre-rRNA, pulse-labelling of yeast protoplasts was performed with L-[2(*n*)- $^3H$ ]-methionine. Gel-electrophoretic analysis (Fig. 1*a*) showed  $^3H$  label in 17S rRNA, as expected, and in 18S pre-rRNA. In the top of the gel (fraction nos. 20–40) a relatively large peak of  $^3H$  radioactivity was seen, which was caused most probably by contamination with protein labelled in methionine, because this  $^3H$ -labelled material is resistant to incubation with pancreatic ribonuclease (Fig. 1*b*) and sensitive to incubation with Proteinase-K (Merck, Darmstadt, Germany) (result not shown). We therefore conclude that introduction of the 3-amino-3-carboxypropyl group into  $am\Psi p$  takes place before the final maturation of 18S pre-rRNA into 17S rRNA.

Table 3. Molar yields of  $m^1\Psi p$  and  $am\Psi p$  in 37S, 18S and 17S RNA from yeast

A mixture of  $^{32}P$ - and *methyl*- $^3H$ -labelled 18S pre-rRNA was digested with  $T_1$  ribonuclease and the products were separated as shown in Fig. 1(*a*). The amount of  $^3H$  radioactivity in spot B was assayed in a liquid-scintillation counter after solubilization with 3% (v/v) Nuclear-Chicago Solubilizer (37°C, overnight) and divided by the mean value of  $^3H$  radioactivity (d.p.m.) per methyl group of 18S pre-rRNA. The latter value was derived from the amount of  $^3H$  d.p.m. of the fully methylated oligonucleotides, whose position was inferred from previous work (Brand *et al.*, 1977). *methyl*- $^3H$ -labelled 37S pre-rRNA was mixed with *methyl*- $^{14}C$ -labelled 26S and 17S rRNA and 'fingerprinted' as described elsewhere (Brand *et al.*, 1977). The position of the *methyl*- $^3H$ -labelled oligonucleotide containing  $m^1\Psi p$  was determined by using the characteristic separation pattern of the *methyl*- $^{14}C$ -labelled oligonucleotides as a reference (Klootwijk & Planta, 1973*a*). To this end the relevant part of the electrophoretogram was cut into squares (1.5 cm  $\times$  1.5 cm) and assayed for  $^3H$  radioactivity as described above. The amount of  $^3H$  d.p.m. in the precursor nucleotide ( $m^1\Psi p$ ) was divided by the mean value of  $^3H$  d.p.m. per methyl group of 37S pre-rRNA. This latter value was derived in the same way as described above for *methyl*- $^3H$ -labelled 18S pre-rRNA. n.d., Not detectable.

RNA species	Molar yield	
	$m^1\Psi p$	$am\Psi p$
37S	0.9	n.d.*
18S nuclear	—	n.d.
18S cytoplasmic	0.7	0.2
17S	n.d.†	1†

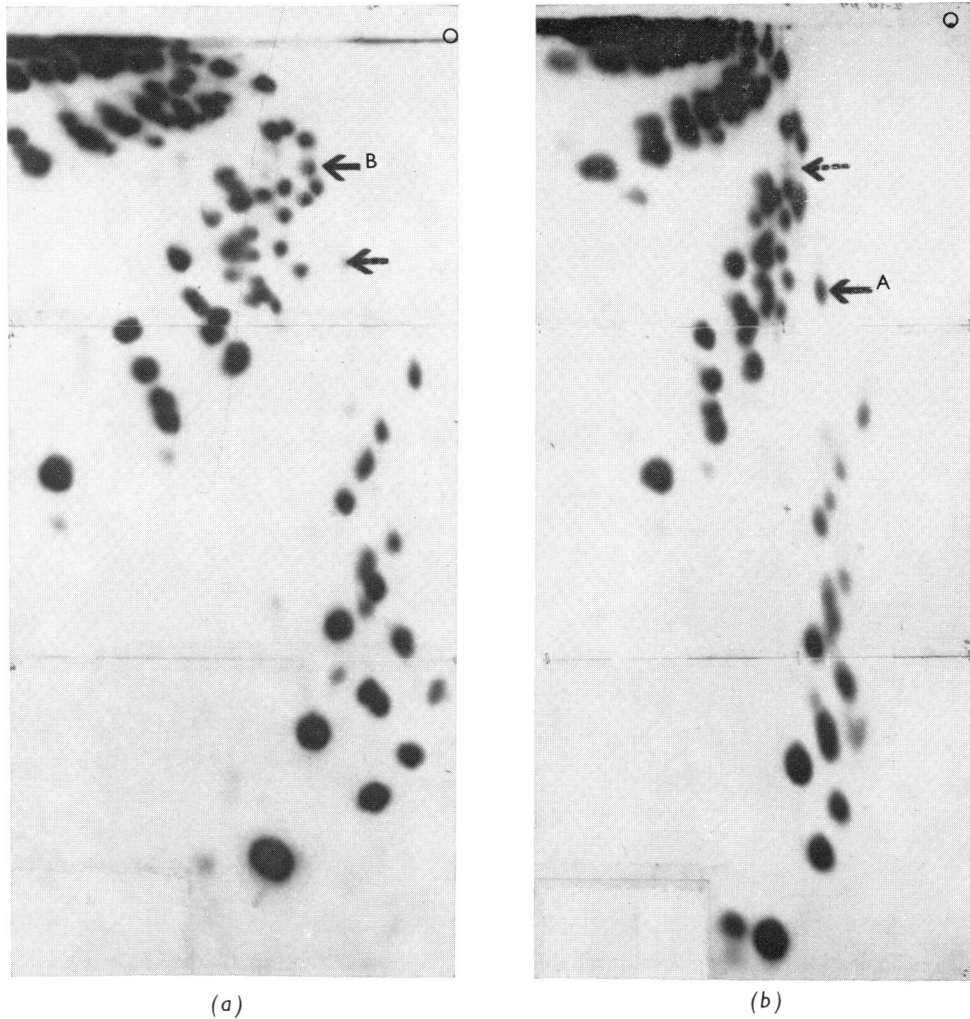
\* See Brand *et al.* (1977).

† See Klootwijk & Planta (1973*a*).

#### HeLa cells

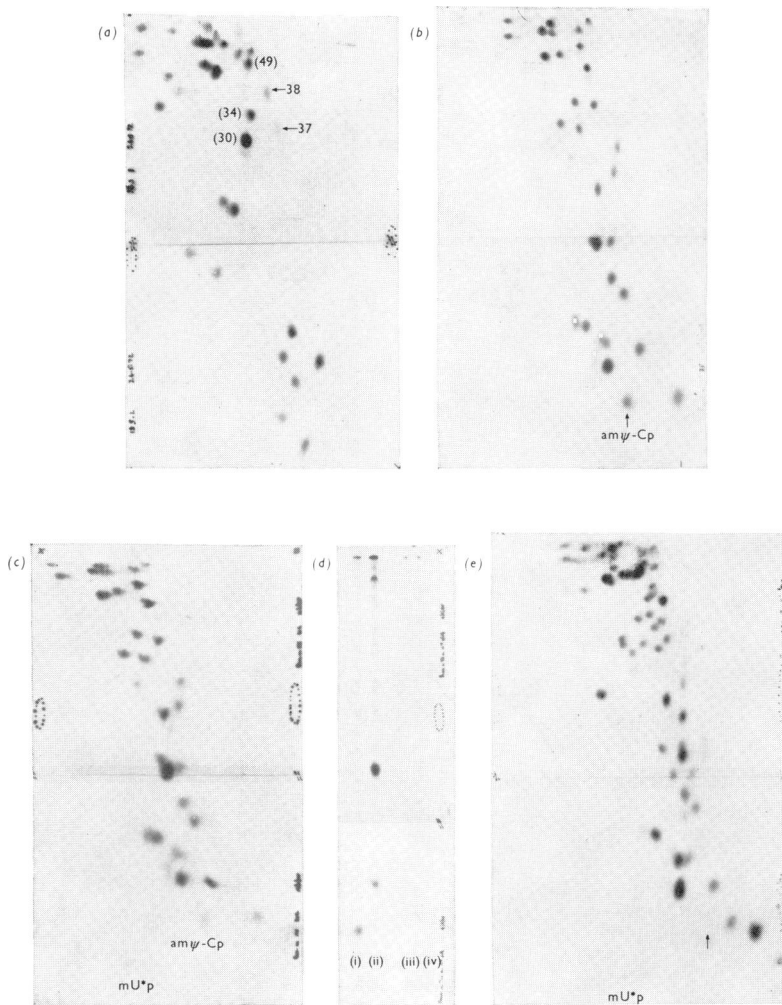
'Fingerprints' of  $T_1$ -ribonuclease digests of methyl-labelled RNA. Plate 2(*a*) shows a 'fingerprint' of a  $T_1$ -ribonuclease digest of HeLa-cell 18S rRNA, prepared after labelling cells for 90 min with [*methyl*- $^{14}C$ ]methionine: 90 min is a relatively short labelling period for HeLa cells, comprising about 7% of the generation time and about three times the minimum period required for emergence of newly labelled 18S RNA into the cytoplasm (Penman *et al.*, 1966). The 'fingerprint' differs from that of 18S RNA prepared after 48 h labelling periods (Maden & Salim, 1974) in the presence of an extra spot, no. 38. The hypermodified nucleotide itself,  $am\Psi p$ , occurs in spot 37 (Maden *et al.* 1975). (Spots 30, 34 and 49 are indicated for orientation and are the base-methylated sequences that are modified late during the maturation of 18S RNA; Maden & Salim, 1974.)

Spot 38 was eluted with 30% triethylamine carbonate, digested with  $T_2$  ribonuclease and subjected to electrophoresis on Whatman no. 52 paper at pH 3.5. A single methyl-labelled component was released, which co-migrated with Up. The approximate molar recoveries of spots 38 and 37 are given in the top two lines of Table 4. The yield of spot 37, containing  $am\Psi p$ , is lower in pulse-labelled samples in which spot 38 is present, than in samples labelled for 48 h. These findings suggest a precursor-product relationship between the methylated nucleotides in spots 38 and 37. However, the molar recovery of spot 37 itself is poor in 'fingerprints' of  $T_1$ -ribonuclease digests of HeLa-cell rRNA, probably owing to streaking of this long oligonucleotide in the second dimension of the 'fingerprint' and/or incomplete transfer from the first dimension. We therefore used other 'fingerprinting' systems to pursue the analysis of the biosynthesis of  $am\Psi p$ .



EXPLANATION OF PLATE I

*Two-dimensional separation of a  $T_1$ -ribonuclease digest of  $^{32}P$ -labelled yeast 17S rRNA (b) and 18S pre-rRNA (a)*  
Yeast 17S rRNA and 18S pre-rRNA, labelled and purified as described in the Experimental section, were digested with  $T_1$  ribonuclease (Brownlee, 1972) and the products were separated by two-dimensional electrophoresis (Fellner, 1969). The separation pattern was detected by radioautography (medical X-ray film, Sakura, type QH). First dimension is from right to left and the second dimensions downwards; O, origin.



EXPLANATION OF PLATE 2

*Separations of enzymic digests of <sup>14</sup>C-labelled 18S rRNA and 45S pre-rRNA from HeLa cells*

(a) 'Fingerprint' of a T<sub>1</sub>-ribonuclease digest of 18S rRNA, from cells labelled for 90 min with [*methyl*-<sup>14</sup>C]methionine. Approx. 300 μg was digested. First dimension (right to left) was on Cellogel in 7 M-urea, pH 3.5, second dimension (downwards) was on DEAE-paper, in 7% formic acid. For further details see Maden & Salim (1974). (b) 'Fingerprint' of a pancreatic-ribonuclease digest of 18S rRNA, from cells labelled for 48 h with [*methyl*-<sup>14</sup>C]methionine. Approx. 20 μg was digested with 2 μg of enzyme in 5 μl of 0.01 M-EDTA/0.01 M-Tris/HCl, pH 7.4. First dimension was on cellulose acetate, pH 3.5; second dimension was on DEAE-paper, in 7% formic acid (Fellner, 1969). (c) 'Fingerprint' of a pancreatic-ribonuclease digest of 18S RNA, labelled for 90 min with [*methyl*-<sup>14</sup>C]methionine. Approx. 300 μg was used; first dimension was on Cellogel, pH 3.5, second dimension was as above. (d) Tracks (i) and (iii): cytoplasmic 18S RNA from 10<sup>8</sup> cells was labelled for 75 min with [<sup>14</sup>C]methionine or [*methyl*-<sup>14</sup>C]methionine, commencing 5 min after addition of actinomycin D (4 μg/ml). In both cases regular Eagle's medium (Eagle, 1959) was replaced by methionine-free medium immediately before addition of actinomycin, and 80 μCi of labelled methionine (50–60 mCi/mmol) was added 5 min later. Pancreatic-ribonuclease digests of the RNA species were applied directly to DEAE-paper and run in 7% formic acid for similar periods to (b) and (c) (16 h, 1 kV). Tracks (iii) and (iv) show the results for nucleolar 20S RNA from the same cells. (e) 'Fingerprint' of a pancreatic ribonuclease digest of 45S RNA from cells labelled for 90 min with [*methyl*-<sup>14</sup>C]methionine. Arrow indicates position to which amΨp-Cp would run, if present. [All products seen here, but not in (c), are attributable to the 28S RNA sequence; B. E. H. Maden, unpublished work.]

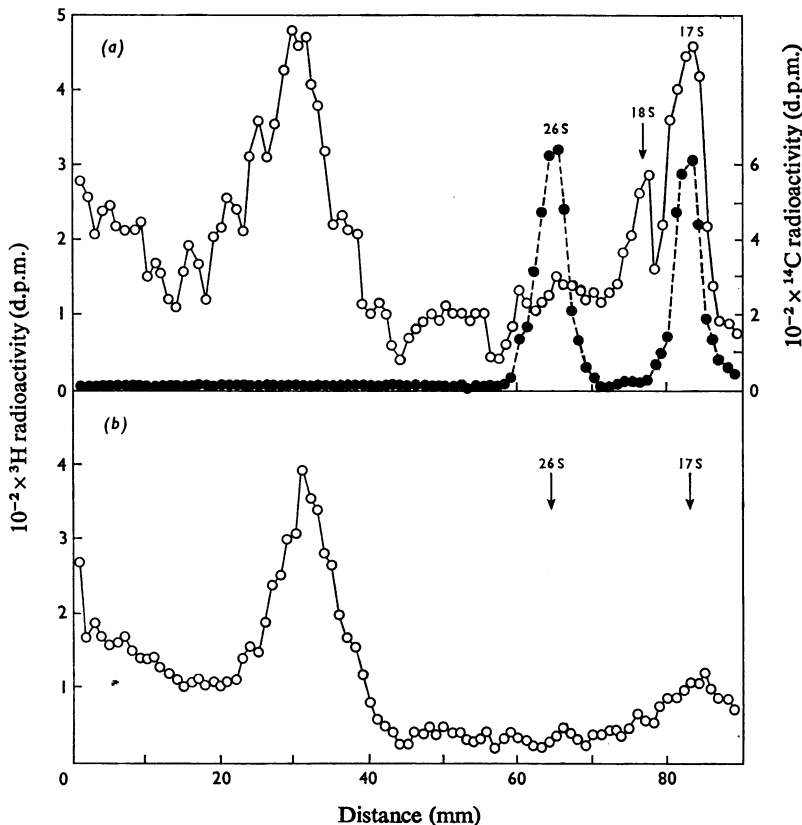


Fig. 1. Polyacrylamide-gel electrophoresis of yeast RNA pulse-labelled with  $[2(n)-^3\text{H}]$ methionine

Yeast protoplasts were pulse-labelled for 5 min at 15°C with  $L-[2(n)-^3\text{H}]$ methionine, RNA was extracted and analysed on 2.6% polyacrylamide gels as described in the Experimental section. methyl- $^{14}\text{C}$ -labelled yeast 26S and 17S rRNA were used as markers. Electrophoresis was from left to right. In (a) 114  $\mu\text{g}$  of  $^3\text{H}$ -labelled RNA was electrophoresed for 6 h at 3 mA/gel; in (b) 114  $\mu\text{g}$  of  $^3\text{H}$ -labelled RNA was preincubated with 57  $\mu\text{l}$  of pancreatic ribonuclease (0.2 mg/ml) for 30 min at 37°C, freeze-dried and subjected to electrophoresis under the same conditions as in (a).  $\circ$ ,  $^3\text{H}$ ;  $\bullet$ ,  $^{14}\text{C}$ .

#### 'Fingerprints' of pancreatic-ribonuclease digests

Plate 2(b) shows a 'fingerprint' of pancreatic-ribonuclease-digested methyl-labelled 18S RNA, prepared after a 48 h labelling period. am $\Psi$ p is released within the dinucleotide am $\Psi$ -Cp (as in the  $T_1$ -plus-pancreatic-ribonuclease digest previously described: Maden *et al.*, 1975), and is recovered in almost unimolar yield (third line of Table 4). Plate 2(c) shows a 'fingerprint' of 18S rRNA prepared after a 90 min labelling period. am $\Psi$ p-Cp occurs in lower yield (fourth line of Table 4), and two very faint spots, indicated as mU\*p, are present. These occur in approximately the same positions as Up and 2':3'-cyclic Up, and co-migrate with Up on electrophoresis on Whatman 52 paper at pH 3.5. The approximate molar yield of mU\*p is shown in line 4 of Table 4.

These findings suggest the following explanation: the final step in the biosynthesis of am $\Psi$ p is addition of the 3-amino-3-carboxypropyl group. This occurs in the cytoplasm as in yeast. mU\*p is the incompletely modified compound 1-methyl pseudouridine (Saponara & Enger, 1974). This is released as the mononucleotide in 'fingerprints' of pancreatic-ribonuclease digests (and also in 'fingerprints' of combined  $T_1$ -plus-pancreatic-ribonuclease digests) and within oligonucleotide 37 in 'fingerprints' of  $T_1$ -ribonuclease digests. The fully modified nucleotide am $\Psi$ p is not a substrate for pancreatic ribonuclease. Hence this compound is released as the dinucleotide am $\Psi$ -Cp in 'fingerprints' of pancreatic ribonuclease digests.

*Experiments with actinomycin D.* The 3-amino-3-carboxypropyl group in am $\Psi$ p can be selectively labelled by  $[1-^{14}\text{C}]$ methionine (Saponara & Enger,

Table 4. Molar yields of  $m^1\Psi p$  and  $am\Psi p$  in 18S rRNA and 45S pre-rRNA from HeLa cells

The molar yields are from 'fingerprints' of methyl- $^{14}C$ -labelled RNA, and are expressed relative to a value of unity for the mean of several oligonucleotides that are known to occur approximately once per RNA molecule (Maden & Salim, 1974). The presumed  $m^1\Psi p$  is liberated within spot 38 in 'fingerprints' of  $T_1$ -ribonuclease (RNAase) digests and as the mononucleotide in 'fingerprints' of pancreatic-ribonuclease digests.  $am\Psi p$  is liberated within spot 37, which is recovered in low yield (see the text), in 'fingerprints' of  $T_1$ -ribonuclease digests, and within the dinucleotide  $am\Psi-Cp$  in 'fingerprints' of pancreatic ribonuclease digests. 'Pulse' signifies a labelling period of 90–150 min. 'Pancreatic ribonuclease' signifies data obtained principally from 'fingerprints' of pancreatic-ribonuclease digests and also from combined 'fingerprints' of  $T_1$ -plus-pancreatic-ribonuclease digests, in which  $m^1\Psi p$  and  $am\Psi-Cp$  are also liberated as such. Comparability of molar yields in the latter two 'fingerprinting' systems is indicated as follows: the mean value for  $am\Psi-Cp$  from five 'fingerprints' of  $T_1$ -plus-pancreatic-ribonuclease digests of 18S RNA, labelled for 48 h was 0.94 (Maden *et al.*, 1975), compared with 0.87 for four 'fingerprints' of pancreatic-ribonuclease digests (line 3 of this Table). n.d., Not detectable.

RNA species and 'fingerprinting' system	Molar yield		No. of determinations
	$m^1\Psi p$	$am\Psi p$	
18S, 48 h, $T_1$ ribonuclease	n.d.	( $\geq 0.50$ )	5
18S, pulse, $T_1$ ribonuclease	0.32	( $\geq 0.37$ )	3
18S, 48 h, pancreatic ribonuclease	n.d.	0.87	4
18S, pulse, pancreatic ribonuclease	0.24	0.53	2
45S, pulse, pancreatic ribonuclease	0.32	n.d.	4

1974; Maden *et al.*, 1975). If there is a substantial delay between transcription and addition of this group one would expect to observe continued uptake of label from [ $^{14}C$ ]methionine into  $am\Psi p$  after inhibition of transcription by actinomycin D. Track (i) of Plate 2(d) shows the result of such an experiment. Carboxyl label is clearly evident in a pancreatic-ribonuclease-digestion product which co-migrates with  $am\Psi-Cp$ . Track (ii) shows an analogous methyl-labelling experiment. Methyl label is seen principally in three products. These are the oligonucleotides obtained after pancreatic ribonuclease digestion of the three known 'late-methylated' sites in 18S rRNA (Maden & Salim, 1974; B. E. H. Maden, unpublished work). There is also a trace of methyl label in  $am\Psi-Cp$ , though this result was variable. The carboxyl-labelling result is consistent with a substantial delay between transcription and addition of the 3-amino-3-carboxypropyl group. The methyl-labelling result might be consistent with a very minor delay in addition of the methyl group, as discussed below.

**45S RNA.** Plate 2(e) shows a 'fingerprint' of a pancreatic-ribonuclease digest of methyl-labelled 45S RNA.  $am\Psi-Cp$  is completely absent; the arrow indicates the position to which this product would migrate if present.  $mU^*p$  is present in low yield (it may be difficult to see after photographic reproduction of the 'fingerprint'). The molar recovery is shown in line 5 of Table 4. Absence of  $am\Psi p$  from 45S-RNA 'fingerprints' shows clearly that addition of the 3-amino-3-carboxypropyl group occurs after cleavage of the 18S sequence from 45S RNA. The low yield of  $mU^*p$  suggests that there is a minor delay in this methylation as compared with other methylations on 45S RNA. Possibly this lag

period is required for prior pseudouridine formation in HeLa cells.

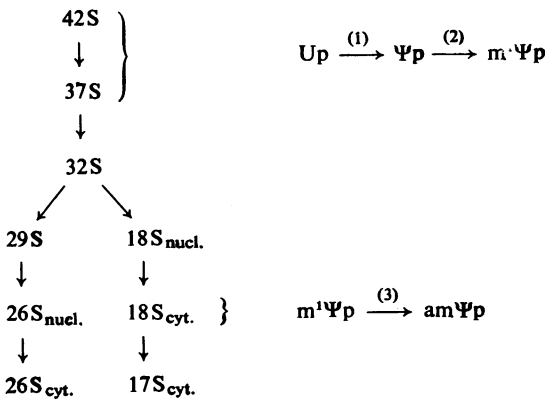
**20S RNA.** In the experiments in Plate 2(d) small quantities of nucleolar 20S RNA, the immediate precursor to cytoplasmic 18S RNA, were also examined. No actinomycin-resistant uptake of label was found by using either [ $^{14}C$ ]methionine or methyl- $^{14}C$ ]methionine. These findings further support the conclusion that the final step in  $am\Psi p$  formation occurs in the cytoplasm, and also indicate that the three principal late methylation events occur in the cytoplasm.

## Discussion

The results presented here for the synthesis of  $am\Psi p$  in yeast 17S rRNA and HeLa-cell 18S rRNA clearly show that the methylation of  $am\Psi p$  is an early modification, as this methyl group can be found shortly after transcription in both yeast and HeLa cells. Since this methylation is supposed to occur at the *N*-1 position of the pyrimidine it must have been preceded by a conversion of the uridine into pseudo-uridine. On the other hand, the attachment of the 3-amino-3-carboxypropyl chain is a late event in both eukaryotes.

The timing of the three modifications required for the formation of  $am\Psi p$  in yeast is summarized in Scheme 1. The precursor nucleotide ( $m^1\Psi p$ ) was demonstrated in both 37S and in most of the 18S pre-rRNA molecules in the cytoplasm (Table 3). As introduction of the 3-amino-3-carboxypropyl fragment into  $am\Psi p$  takes place just before the final cleavage of 18S pre-rRNA into 17S rRNA (Fig. 1), this modification in yeast is a cytoplasmic event.





Scheme 1. Biosynthesis of  $\text{am}\Psi\text{p}$  in yeast 17S rRNA  
Abbreviations: nucl., nuclear; cyt., cytoplasmic.

The findings in HeLa cells are essentially similar to those in yeast, differing only insofar as there is an appreciable lag in introduction of the methyl group to the *N*-1 position of pseudouridine in HeLa-cell 45S RNA, and that introduction of the 3-amino-3-carboxypropyl group appears to occur on mature 18S RNA rather than on the 20S precursor (the molecule which is analogous to the 18S precursor in yeast). Apart from these minor differences the synthesis of  $\text{am}\Psi\text{p}$  proceeds by a basically similar pathway in the two distantly related eukaryotes.

The finding of  $\text{am}\Psi\text{p}$  in all 18S RNA species for which its presence has been examined (Saponara & Enger, 1974; Khan & Maden, 1976) suggests that this hypermodified nucleotide plays an important role.  $\text{am}\Psi\text{p}$  might be essential either for some late stage in maturation and/or for the function of the small ribosomal subunit.

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## References

- Brand, R. C. & Planta, R. J. (1975) *Mol. Biol. Rep.* **2**, 321-325
- Brand, R. C., Klootwijk, J., Van Steenberghe, T. J. M., De Kok, A. J. & Planta, R. J. (1977) *Eur. J. Biochem.* **75**, 311-318
- Brownlee, G. G. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), vol. 3, part I, pp. 1-265, North-Holland, Amsterdam and London
- Clausen, T. (1968) *Anal. Biochem.* **22**, 70-73
- de Jonge, P., Klootwijk, J. & Planta, R. J. (1977) *Eur. J. Biochem.* **72**, 361-369
- Eagle, H. (1959) *Science* **130**, 432-437
- Enger, M. D. & Saponara, A. G. (1968) *J. Mol. Biol.* **33**, 319-322
- Fellner, P. (1969) *Eur. J. Biochem.* **11**, 12-27
- Hall, R. H. (1971) *The Modified Nucleosides in Nucleic Acids*, 1st edn., p. 411, Columbia University Press, New York
- Jeppesen, P. G. N. (1971) *Biochem. J.* **124**, 357-366
- Khan, M. S. N. & Maden, B. E. H. (1976) *J. Mol. Biol.* **101**, 235-254
- Klootwijk, J. & Planta, R. J. (1973a) *Eur. J. Biochem.* **39**, 325-333
- Klootwijk, J. & Planta, R. J. (1973b) *Mol. Biol. Rep.* **1**, 187-191
- Klootwijk, J., Van den Bos, R. C. & Planta, R. J. (1972) *FEBS Lett.* **27**, 102-106
- Maden, B. E. H. & Salim, M. (1974) *J. Mol. Biol.* **88**, 133-164
- Maden, B. E. H., Forbes, J., De Jonge, P. & Klootwijk, J. (1975) *FEBS Lett.* **59**, 60-63
- Penman, S., Smith, I. & Holtzman, E. (1966) *Science* **154**, 786-789
- Retèl, J. & Planta, R. J. (1967) *Eur. J. Biochem.* **3**, 248-258
- Saponara, A. G. & Enger, M. D. (1974) *Biochim. Biophys. Acta* **349**, 61-77
- Trapman, J. & Planta, R. J. (1976) *Biochim. Biophys. Acta* **442**, 265-274
- Trapman, J., Retèl, J. & Planta, R. J. (1975) *Exp. Cell Res.* **90**, 95-104