SECONDARY METHYLATION OF YEAST RIBOSOMAL RNA

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1. Introduction

The biosynthesis of ribosomal RNA (rRNA) in yeast starts with the transcription of a 42 S common precursor molecule which is subsequently converted in a number of steps into 26 S and 17 S rRNA [1,2]. The processing includes the stepwise removal of nonconserved RNA to produce an intermediate precursor RNA which is cleaved into two separate precursors, namely 29 S and 18 S RNA, that eventually are converted into 26 S and 17 S rRNA, respectively [2–5]. In this maturation process methylation of the rRNA precursors seems to play some, hitherto unknown, role. In a previous paper [6] it was shown that the attachment of methyl groups to predominantly ribose moieties occurs at the level of 42 S precursor RNA, immediately after or at the time of its transcription. However, we also obtained indirect evidence for an additional methylation of the heterocyclic bases at a later stage of rRNA formation.

In this communication we present a direct proof for the occurrence of a secondary base methylation of yeast rRNA in a final step of the maturation process. In an enzymatic digest of 17 S rRNA a methylated oligonucleotide, containing the sequence $m_2^6Apm_2^6Ap$, is found, which is lacking in the corresponding digest of 18 S RNA, the immediate precursor of 17 S rRNA. Thus, the final step in the formation of the small ribosomal subunit in yeast is accompanied by the introduction of at least four methyl groups into its RNA constituent.

2. Materials and methods

2.1. *Preparation of highly labeled [^{32}P] rRNA*

Labeling of rRNA with [^{32}P] orthophosphate (carrier-free, Philips-Duphar, Petten, The Netherlands) was achieved by growing cells of *Saccharomyces carlsbergensis* (Strain Sce/1/317) for several generations in a synthetic medium [3] containing [^{32}P] orthophosphate (0.2 mCi/ml of medium). RNA was extracted from ribosomes and purified as described previously [7]. The specific activity of rRNA thus prepared was approx. 1 μCi/μg.

2.2. *Digestion of RNA and sequence analysis of oligonucleotides*

RNA was digested with T_1-ribonuclease (Sankyo Co. Ltd., Tokyo, Japan) and alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J., USA) as described previously [4], and the digestion products were separated in two dimensions by electrophoresis according to Sanger et al. [8].

Oligonucleotides were identified by further enzymatic digestion with snake venom phosphodiesterase (Sigma Chemical Co., St. Louis, Mo., USA), or pancreatic ribonuclease (Sigma) occasionally preceded by reaction with a soluble carbodi-imide (CMCT) as described by Jeppesen [9]. Base compositions of oligonucleotides were determined after alkaline hydrolysis and separation of the nucleotides by electrophoresis on Whatman 3 MM paper at pH 3.5 [9]. All nucleotides were screened for possible modification by descending paper chromatography on Whatman no. 1, using isopropanol–conc. HCl–water (68:17:14.4, v/v) as solvent (solvent C). Other solvent systems used were: solvent D, isopropanol–conc. ammonia–water (7:1:2,

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Table 1
Amounts of [^{3}H]- and [^{14}C] radioactivity found in the spots of fig. 1.

Spot no.	^{14}C-dpm (17 S rRNA)	^{3}H-dpm (18 S RNA)	^{3}H/^{14}C ratio
y	118	141	1.2
1	116	129	1.1
2	130	142	1.1
3	125	111	0.9
4	134	172	1.3
5	148	282	1.9
6	494	17	0.0
7	267	296	1.1
8	48	68	1.4
9	122	135	1.1
10	133	148	1.1
11	138	57	0.4
12	126	140	1.1
13	156	249	1.6
14	135	171	1.3
15	98	80	0.8
16	170	219	1.3
17 + 18	288	203	0.7
19	69	118	1.7

v/v), and solvent E, ethylacetate–propanol-1–water (4:1:2, v/v) [10].

3. Results and discussion

A mixture of appropriate amounts of [^{14}C] methyl-labeled 17 S rRNA and [^{3}H] methyl-labeled 18 S precursor RNA was enzymatically digested as described before [4]. To permit identification of the digestion products the digest was fractionated in the second dimension on DEAE-cellulose paper [8], instead of applying homochromatography on DEAE-cellulose thin-layer plates as done before [4]. After radioautography (fig. 1), all [^{14}C] methyl containing spots were examined for the presence of ^{3}H-radioactivity derived from 18 S RNA. The results, summarized in table 1, are essentially the same as obtained before with another fingerprinting procedure [4]. The great majority of the spots contained both ^{3}H- and ^{14}C-radioactivity in a roughly constant ratio, indicating the close structural relationship between the two RNA species. However, spot no. 6 (corresponding to spot no. 4 in fig. 3 of [4] representing an oligonucleotide apparently containing four methyl groups (cf. table 1), which is present in the 17 S rRNA digest, is absolutely lacking in the 18 S RNA digest. The structure of this oligonucleotide was analyzed in detail as described below.

To elucidate the methylation sites in this oligonucleotide, a "fingerprint" was made of 100 μg [^{14}C] methyl-labeled 17 S rRNA, and the oligonucleotide corresponding to spot no. 6 was eluted and analyzed as described in the first part of table 2. It appeared that N^6, N^6-dimethyladenosine (m_2^6A) was the only methylated nucleoside present in this oligonucleotide as could be verified by paper chromatographic analysis with solvents C, D and E using the authentic compound (purchased from Cyclo Chemical Co., Los Angeles, Cal., U.S.A.) as a marker. After partial alkaline hydrolysis a product was found which, according to its alkali-resistance and electrophoretic mobility, must be identical to $m_2^6Apm_2^6Ap$ [11, 12]. From the data it could be concluded that $m_2^6Apm_2^6Ap$ is located at the 5′-end of the oligonucleotide.

Sequence analysis of the oligonucleotide was performed with ^{32}P-labeled material. To detect the position of the methylated oligonucleotide concerned on the much more complicated fingerprint of ^{32}P-labeled 17 S rRNA, a mixture of separately prepared [^{14}C] methyl-labeled 17 S rRNA and ^{32}P-labeled 17 S rRNA was fingerprinted (fig. 2). All the ^{32}P-products were excised and counted for ^{14}C-radioactivity. The amounts of ^{32}P-labeled RNA employed were such as to afford a very low degree of spill-over of ^{32}P-counts in the ^{14}C-channel, compared with the ^{14}C-counts present in any of the methylated products. The ^{14}C-containing spots in fig. 2 are numbered in accordance with fig. 1.

Next a preparative fingerprint of 40 μg ^{32}P-labeled 17 S rRNA was made. Spot no. 6 was excised, and the oligonucleotide material eluted and purified by re-electrophoresis on DEAE-cellulose paper at pH 3.5. The products arising upon hydrolysis of this oligonucleotide with alkali or with enzymes are listed in the lower half of table 2. From these data it is possible to deduce the complete sequence of the oligonucleotide as being:

$m_2^6Apm_2^6ApCpUpCpG$.

Fig. 1. Fingerprint of the products obtained by digestion of a mixture of [^{14}C]methyl-labeled 17 S rRNA and [^{3}H]methyl-labeled 18 S RNA with T_1-ribonuclease and alkaline phosphatase. [^{3}H]Methyl-labeled 18 S RNA and [^{14}C]methyl-labeled 17 S rRNA were isolated as described previously [4]. A mixture of 90 μg (5,200 ^{3}H-dpm) 18 S RNA and 2 μg (5,000 ^{14}C-dpm) 17 S rRNA was digested with T_1-ribonuclease and alkaline phosphatase, and the products were separated on cellulose acetate (pH 3.5, 7 M urea) and DEAE-cellulose paper (7% formic acid) according to Sanger et al. [8]. All ^{14}C-spots, visualized by radioautography, were assayed for both ^{3}H- and ^{14}C-radioactivity after solubilization with Nuclear Chicago Solubilizer (37°, overnight) and addition of toluene based scintillation mixture (see table 1). A correction was made for spill-over of ^{14}C-counts in the ^{3}H-channel.

Fingerprints of the products of pancreatic RNAase digestion of [^{14}C]methyl-labeled 17 S rRNA allow the analysis of the sequence connected to the 5′-terminal part (m_2^6Apm$_2^6$ApCp) of this oligonucleotide. These studies (to be published) show the presence of at least one Gp-residue adjacent to m_2^6Ap. This implies that in 17 S rRNA the sequence Gpm$_2^6$Apm$_2^6$ApCpUpCpG is present. Even if this sequence is connected with a non-conserved terminal section in 18 S RNA, the position of the two G-residues will warrant the presence of an oligonucleotide with the base sequence ApApCpUpCpG(p) in a T_1-ribonuclease digest of 18 S RNA. Therefore, it must be concluded that this oligonucleotide is not yet methylated in 18 S RNA, and so is not detected in a fingerprint of methyl-labeled 18 S RNA. This means that the transition from 18 S RNA to 17 S rRNA must be preceded by the introduction of at least four methyl groups in the precursor molecule, in accord with the suggestion of Retèl et al. [6] that additional base methylation occurs at a late stage of rRNA formation.

It is interesting that a nearly identical oligonucleotide (Gpm$_2^6$Apm$_2^6$ApCpCpUpG) was found to be present in 16 S rRNA from *Escherichia coli* [12,13]. This sequence was located not far from the 3′-end of the molecule. The immediate precursor of *E. coli* 16 S rRNA, which is about 40 nucleotides longer than the mature molecule, lacks the methylated nucleotides –m$_2^6$Apm$_2^6$A– [14]. The relevant methyl groups are also lacking in 16 S rRNA from a kasugamycin-resistant strain of *E. coli* [15]. It seems likely, that a similar situation exists for rRNA of HeLa cells. N^6, N^6-Dimethyladenosine is exclusively found in the 18 S rRNA and not in the 45 S precursor RNA [16]. This is confirmed by the results obtained by Maden et al. [17]. The fingerprint of [^{14}C]methyl-labeled 18 S rRNA contains a very intense, m_2^6A containing spot

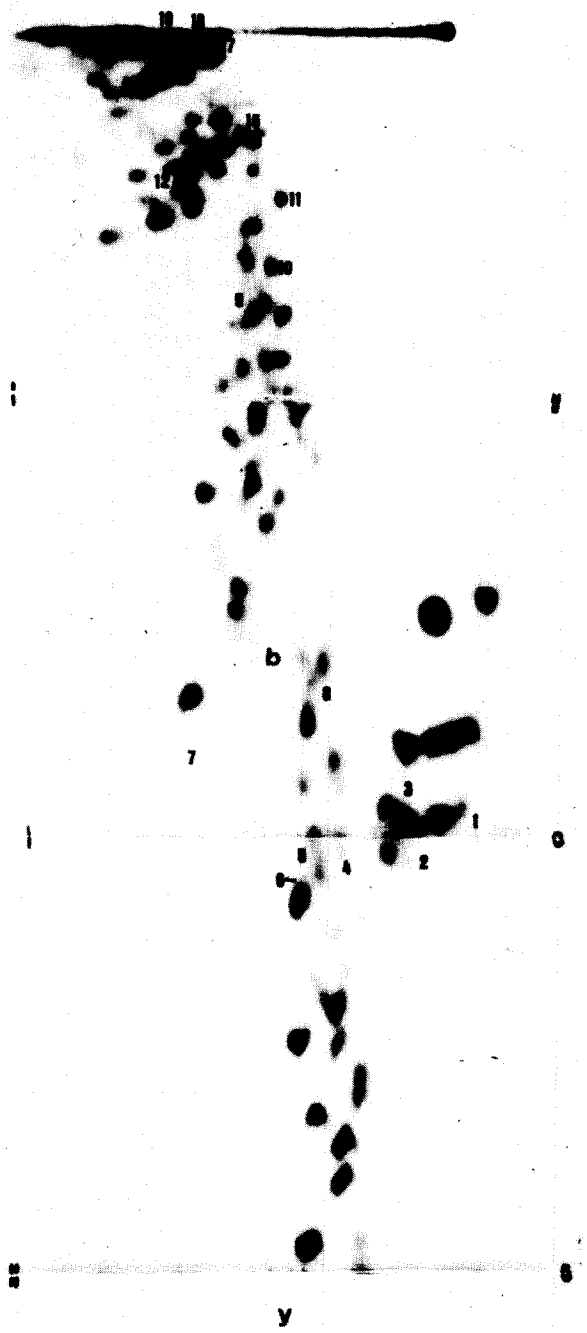

Fig. 2. Fingerprint of the products obtained by digestion of a mixture of [^{14}C] methyl-labeled 17 S rRNA and ^{32}P-labeled 17 S rRNA with T_1-ribonuclease and alkaline phosphatase. ^{32}P-Labeled 17 S rRNA was obtained as described in the Methods section. A mixture of 90 μg (230,000 dpm) [^{14}C] methyl-labeled 17 S rRNA and 1 μg (2,550,000 cpm) ^{32}P-labeled 17 S rRNA was digested, and the digestion products were separated as described under fig. 1. After radioautography, all spots were excised and counted directly for both ^{14}C- and ^{32}P-radioactivities according to the procedure of Fellner [12]. The numbers of the [^{14}C] methyl containing spots correspond to those in fig. 1.

Table 2
Sequence analysis of the methylated oligonucleotide corresponding to spot no. 6.

Label	Digestion with	Products (radioactivity ratios)
^{14}C-methyl	Venom phosphodiesterase and alkaline phosphatase	m_2^6A
^{14}C-methyl	Venom phosphodiesterase	m_2^6A (1.0), pm_2^6A (1.1)
^{14}C-methyl	Alkali (0.3 N, 16 hr, 37°)	m_2^6Ap, $m_2^6Apm_2^6Ap$
^{14}C-methyl	Alkali (1 N, 90 hr, 37°)	m_2^6Ap
^{32}P	Alkali (0.3 N, 16 hr, 37°)	Up (1.0), Cp (2.1), m_2^6Ap + $m_2^6Apm_2^6Ap$ (2.0)
^{32}P	Venom phosphodiesterase	pG (1.0), pC (2.0), pU (1.1), pm_2^6A (1.0)
^{32}P	Pancreatic RNAase	$m_2^6Apm_2^6ApCp$ (3.0), Cp (1.1), Up (1.0)
^{32}P	Pancreatic RNAase after CMCT treatment	$m_2^6Apm_2^6ApCp$ (2.9), UpCp (2.0)

which is not found in the fingerprint of any nucleolar precursor of 18 S rRNA.

Therefore, secondary methylation producing m_2^6A-residues may be a universal phenomenon accompanying the maturation of the small ribosomal subunit. These additional methylated nucleotides may form part of the site recognized by the enzyme responsible for the final maturation step, or be involved in the binding site of one of the ribosomal proteins, as already proposed by Ehresmann et al. [13].

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References

[1] J. Retèl and R.J. Planta, European J. Biochem. 3 (1967) 248.
[2] J. Retèl and R.J. Planta, Biochim. Biophys. Acta 224 (1970) 458.
[3] R.C. van den Bos and R.J. Planta, Biochim. Biophys. Acta 247 (1971) 175.
[4] R.C. van den Bos, J. Klootwijk and R.J. Planta, FEBS Letters 24 (1972) 93.
[5] R.C. van den Bos, Ph.D. Thesis, Vrije Universiteit, Amsterdam, 1972.
[6] J. Retèl, R.C. van den Bos and R.J. Planta, Biochim. Biophys. Acta 195 (1969) 370.
[7] J. Retèl and R.J. Planta, Biochim. Biophys. Acta 169 (1968) 416.
[8] F. Sanger, G.G. Brownlee and B.G. Barrell, J. Mol. Biol. 13 (1965) 373.
[9] P.G.N. Jeppesen, Biochem. J. 124 (1971) 357.
[10] R.H. Hall, The Modified Nucleosides in Nucleic Acids (Columbia University Press, New York and London, 1971).
[11] J.L. Nichols and B.G. Lane, Biochim. Biophys. Acta 119 (1966) 649.
[12] P. Fellner, European J. Biochem. 11 (1969) 12.
[13] C. Ehresmann, P. Fellner and J.P. Ebel, FEBS Letters 13 (1971) 325.
[14] F. Hayes, D. Hayes, P. Fellner and C. Ehresmann, Nature New Biology 232 (1971) 54.
[15] T.L. Helser, J.E. Davies and J.E. Dahlberg, Nature New Biology 233 (1971) 12.
[16] E.F. Zimmerman, Biochemistry 7 (1968) 3156.
[17] B.E.H. Maden, M. Salim and D.F. Summers, Nature New Biology 237 (1972) 5.