PRESENCE OF A HYPERMODIFIED NUCLEOTIDE IN HELA CELL 18 S AND *SACCHAROMYCES CARLSBERGENSIS* 17 S RIBOSOMAL RNAs

B. E. H. MADEN and J. FORBES
Department of Biochemistry, University of Glasgow, Glasgow, Scotland

and

P. de JONGE and J. KLOOTWIJK
Biochemisch Laboratorium, Free University, Amsterdam, The Netherlands

Received 20 August 1975

1. Introduction

We have described, in separate reports, the methylated nucleotide sequences in HeLa cell [1,2] and yeast (*Saccharomyces carlsbergensis*) [3,4] rRNAs. The methylation patterns, though different in many details, also share several features in common. Among these is the presence of a hitherto unidentified methylated component in HeLa 18 S and yeast 17 S rRNAs.

Enger and Saponara [5] reported uptake of label from 1-^{14}C- or 2-^{14}C-labelled methionine into a single pancreatic ribonuclease digestion product of 18 S rRNA in Chinese hamster cells. After elaborate radiochemical analysis they tentatively identified a hypermodified nucleoside as 1-methyl-3-y-(α-amino-α-carboxypropyl)pseudouridine [6]. If this nucleoside were present in HeLa or yeast rRNAs, the methyl group should render it visible in fingerprints of the methyl-labelled RNAs. Here we show that the previously unidentified methyl-labelled component in HeLa 18 S and yeast 17 S rRNAs also incorporates label from [1-^{14}C]methionine, suggesting that it is the same compound as that described by Saponara and Enger [6]. A tentative sequence surrounding the yeast compound is presented. Data from other eukaryotes suggest that the product occurs in many eukaryotic 18 S RNAs.

2. Results and discussion

Figs 1 (a) and (b) show combined T_1 plus pancreatic ribonuclease fingerprints of HeLa cell 18 S rRNA, ^{32}P-labelled and [^{14}C]methyl-labelled respectively. As described previously [1,2,4], this fingerprinting system resolves many short, chemically modified sequences. All of the products have been characterized ([2], appendix II, and unpublished observations of B. E. H. M.) except for spot 50. This product is a dinucleotide which occurs approximately once per molecule of 18 S RNA (table 1); on alkaline hydrolysis it yields Cp and a characteristic 'triad' of components which are labelled with both ^{32}P and methyl label (fig.2). On hydrolysis with T_2 ribonuclease spot 50 yields Cp and a nucleotide which migrates slightly faster than Gp at pH 3.5 (fig.2). We shall refer to the latter nucleotide as Xp. We infer that the triad of methyl-labelled alkaline hydrolysis products results from instability of Xp in alkali. Yeast 17 S RNA yields an apparently identical T_1 plus pancreatic ribonuclease product in the same position of the fingerprint, also in approx. unimolar yield (spot 4, reference 4).

The distinctive electrophoretic mobility of XpCp, together with the instability of Xp to alkali, suggested an unusual modification. One possibility was the hypermodification described by Saponara and Enger [6]. Fig.1 (c) shows a combined T_1 plus pancreatic ribonuclease fingerprint of 18 S RNA from HeLa cells grown in the presence of [1-^{14}C]methionine. A single spot is present, with similar mobility to spot 50. When [1-^{14}C]-labelled and [^{14}C]methyl-labelled 18 S RNAs were mixed and fingerprinted, no extra spots appeared

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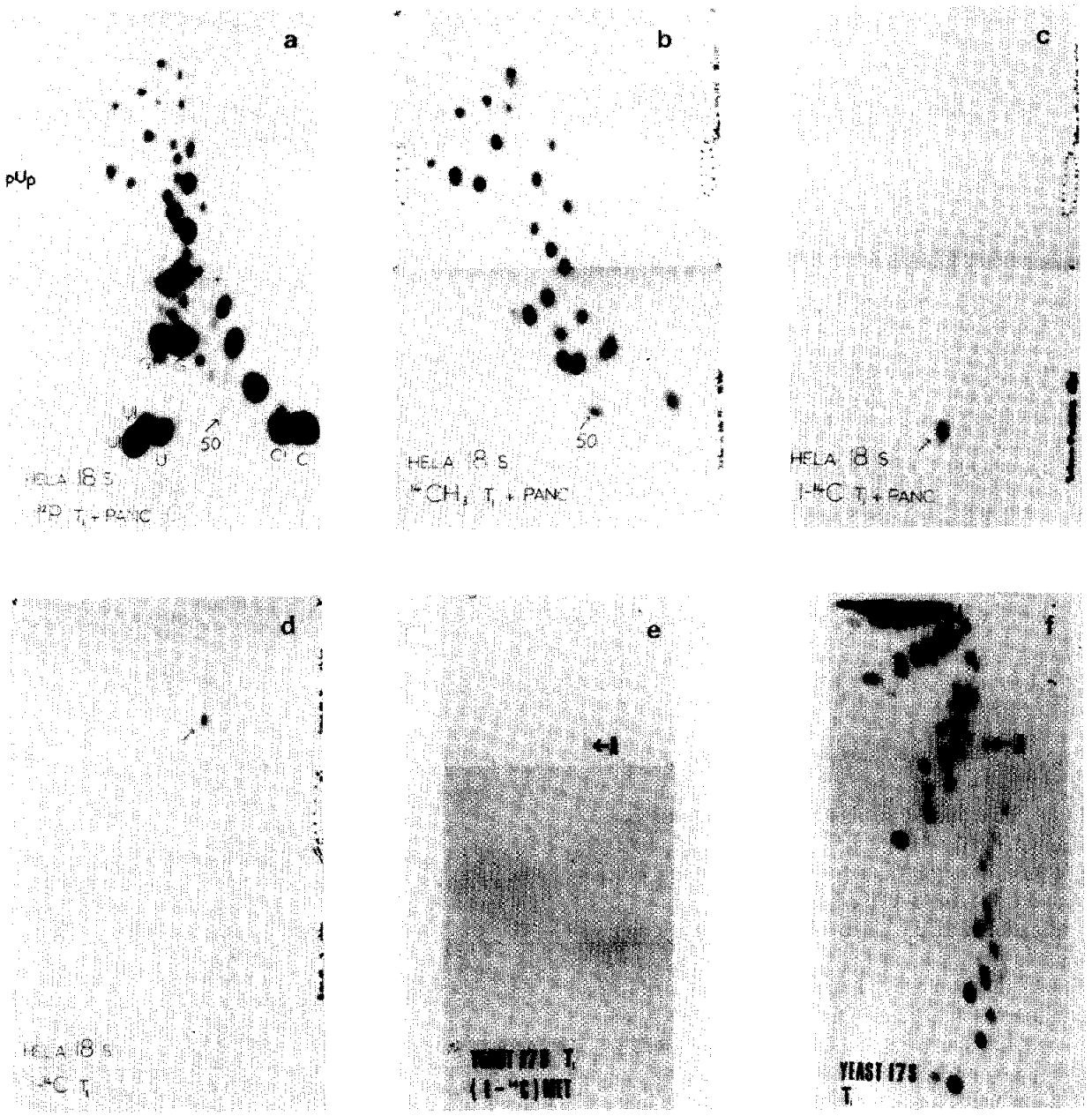

Fig. 1. Fingerprints of HeLa cell 18 S and yeast (*Saccharomyces carlsbergensis*) 17 S rRNAs. The ribosomal RNAs were labelled, purified and fingerprinted using conditions which were described in detail previously [2,3]. Labelling with [1-^{14}C]methionine (Radiochemical Centre, Amersham) was performed using similar conditions of cell growth, specific activity and quantity of methionine to those described previously for $^{14}CH_3$-labelled methionine. In the fingerprints the first dimension (cellulose acetate) is from right to left and the second dimension (DEAE paper) from top to bottom. In (a) mononucleotides and cyclic mononucleotides (!) are indicated. These products occur in high yield. By comparison spot 50 is faint but is present, for example, in roughly equivalent amount to pUp. Details on quantitation are given in table 1. (b) In [^{14}C]methyl fingerprints spot 50 was sometimes rather 'flattened', as shown here. For details on other methyl spots see ref. 2. (c) to (f), see text for further details.

Table 1
Molar yield of HeLa T_1 plus pancreatic ribonuclease spot 50

Isotope	Yield	Number of Determinations
^{32}P	0.81	4
$^{14}CH_3$	0.94	4
$^{14}CH_3$ + 1-^{14}C	1.85	1

In ^{32}P determinations the total radioactivity in all spots (fig.1 (a)) was determined by scintillation counting, large spots being cut into several small pieces for counting in successive vials. Molar yields were calculated from the radioactivity in the individual spots as a fraction of the total radioactivity, assuming that mammalian 18 S RNA (0.65×10^{-6} daltons, [11] contains 2000 nucleotides. Spot 50 was assumed to be a dinucleotide. The ^{32}P yield of this spot may be a slight underestimate due to slight streaking. $^{14}CH_3$ determinations are relative to the distribution of methyl label in all other spots in fig.1 (b), assuming that 18 S RNA contains 45 methyl groups [1,2]. In the $^{14}CH_3$ + 1-^{14}C determination equal A_{260} quantities of 18 S RNA were mixed and fingerprinted after labelling of two parallel cultures under identical conditions of growth and specific activity, with $^{14}CH_3$ and [1-^{14}C] methionine respectively. The result is expressed relative to distribution of methyl label in all other methyl labelled spots. The degree of augmentation of spot 50 is consistent with Saponara's and Enger's conclusion that the product contains one carboxyl substituent [6], (rather than two, as suggested earlier [5]).

in the fingerprint, but labelling of spot 50 was augmented, confirming that it is indeed spot 50, and not some other previously unrecognized spot, which contains the carboxyl label (table 1).

Uptake of carboxyl label from methionine suggests that Xp is similar to the nucleotide described by Saponara and Enger [6]. Two further observations are consistent with this inference: (i) Susceptibility of the phosphodiester bond linking Xp and Cp, to hydrolysis by both alkali and T_2 ribonuclease, indicates that the methyl group in Xp is located on the base, and not on the 2'-O-ribose position of the nucleotide. (ii) It was confirmed, in agreement with Enger and Saponara [5], that ^{35}S label is not incorporated from methionine into Xp, nor into rRNA generally. This indicates that the whole methionine skeleton is *not* transferred to form Xp, and that methyl and carboxyl carbon fragments are transferred by separate reactions. Kinetic experiments on the biosynthetic sequence of the modification reactions are in progress (Klootwijk and Maden, unpublished observations).

After T_1 ribonuclease digestion of either HeLa or yeast rRNA XpCp is present within a long oligonucleotide. Figs 1 (d) and (e) show the mobility of the carboxyl-labelled oligonucleotide in the respective T_1

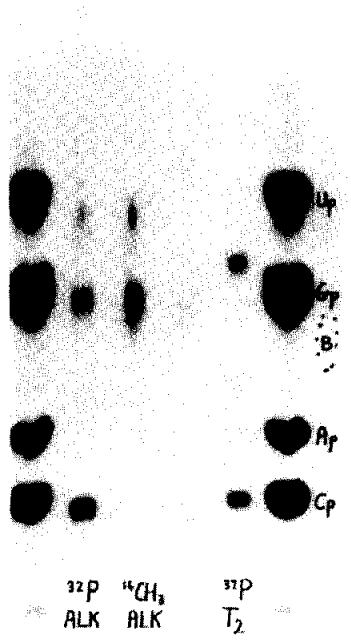

Fig.2. Degradation of T_1 plus pancreatic ribonuclease spot 50 from HeLa cell 18 S RNA, followed by electrophoresis on Whatman 52 paper at pH 3.5 (pyridine-acetic acid, 5%; 4 KV 45 min). The dense spots on either side are marker mononucleotides from an alkaline hydrolysate of 28 S rRNA. B signifies the position of the blue marker dye. '^{32}P alk': The ^{32}P-labelled spot 50 was subjected to alkaline hydrolysis (0.2 N NaOH, 18 h, 37°C). The products were Cp and a characteristic triad of products migrating in the region from Gp to Up. The quantity of label in the triad was roughly equivalent to that in Cp, though there may also be a trace of label running far ahead of Up, in the approximate position of Pi. '$^{14}CH_3$ alk': The same characteristic triad of products is seen containing methyl label. '^{32}P T_2': Spot 50 was subjected to hydrolysis with T_2 ribonuclease as in ref. 10. Cp and the presumably intact Xp are seen, the latter migrating just ahead of Gp. (Between '$^{14}CH_3$ alk' and '^{32}P T_2' an alkaline hydrolysate of the 1-^{14}C labelled spot 50 was run. A faint band is seen, co-migrating with the slowest $^{14}CH_3$ band. However the quantity of material was too small to judge whether the other components of the triad also contain 1-^{14}C label).

Table 2
Sequence data of spot A in T_1 ribonuclease fingerprint of yeast 17 S rRNA

Digestion	Products (molar yields relative to Gp)
panc RNAse	A-A-Cp(1.0), A-Cp(2.0), X-Cp(0.8), Gp(1.0)
U2RNAse	C-X-C-Ap(0.8), C-Ap(0.9), C-Gp(1.0), Ap(2.3)

Digestion products were separated by electrophoresis on DEAE paper at pH 3.5 and after radioautography ^{32}P radioactivity in all spots was determined by scintillation counting. Base compositions were analysed as described previously [4].

fingerprints. The position corresponds to that of the hitherto uncharacterized HeLa T_1 spot 37 [2] and yeast T_1 spot 8 [3]. HeLa spot 37 was recovered in submolar yield [2], but the essentially unimolar recovery of the corresponding T_1 plus pancreatic product 50 (table 1) indicates that the low yield of the T_1 product was due to streaking or instability of this long oligonucleotide, probably in the second dimension (7% formic acid) of the fingerprint.

The yeast T_1 product is well resolved in ^{32}P fingerprints (fig.1 (f)), as was confirmed in a double label fingerprint of ^{32}P plus carboxyl-labelled RNAs. The ^{32}P-labelled oligonucleotide was subjected to sequence analysis. The data in table 2 indicate that the product is a decanucleotide. These and further data, from partial digestion with spleen and venom phosphodiesterases, lead to proposal of the following tentative sequence:-

A–A–C–X–C–A–C–A–C–Gp

The HeLa sequence has not been determined, but from the electrophoretic mobility of the T_1 product it is evident that this also contains several Cp and Ap residues, together with Xp and Gp. Therefore it is likely that the sequence is related to that in yeast.

T_1 plus pancreatic ribonuclease product 50 occurs in fingerprints of chick and *Xenopus laevis* 18 S RNAs (^{32}P- and [^{14}C] methyl-labelled; Khan and Maden, unpublished observations) and in *Drosophila melanogaster* 18 S RNA (^{32}P-labelled; evident but not specifically noted, in plate IV of ref.7). In each case the alkaline degradation products were similar to those in fig.2. No such component has been reported in *E. coli* 16 S RNA [8,9].

In summary, a hypermodified nucleotide, which is similar or identical to that described by Saponara and Enger [6], is present in the 17-18 S rRNAs of several distantly related eukaryotes. It seems reasonable to infer that this nucleotide serves an important function within the eukaryotic small ribosomal subunit.

Acknowledgements

The work was supported by grants from the Medical Research Council and from the Netherlands Organization for the Advancement of Pure Research (Z. W. O.). The authors are grateful to Miss I. Klein for skillful technical assistance.

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