THE PSEUDOURIDINE CONTENT OF HeLa CELL RIBOSOMAL RNA

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1. Introduction

Pseudouridine occurs in ribosomal RNAs [1–6] as well as in tRNA. Like methylation, it is more abundant in eukaryotic [1–5] than in prokaryotic [4,6] rRNAs. The detailed information now available on methylation of eukaryotic rRNAs [7–10] made it seem desirable to undertake a comparable investigation for pseudouridine. Here we determine the pseudouridine content of HeLa cell rRNA and report on the sequence specificity of this type of modification. The findings extend those of an earlier report [5] on pseudouridine in HeLa rRNA. The numbers of pseudouridines were found to be rather similar to the numbers of 2′-*O*-methyl groups, as pointed out by Lane for other rRNAs [3,4].

2. Methods

HeLa cells were labelled with $^{32}PO_4$ and rRNA was prepared as described previously [7,11]. 28 S RNA was 5.8 S-free [11]. Fingerprinting analysis confirmed that the rRNAs were free from tRNA. RNA samples containing several hundred thousand cpm in a few micrograms were subjected to hydrolysis by alkali or T_2 ribonuclease [12]. In the T_2 method only 3′ phosphates are liberated, resulting in sharper separations after chromatography. It was essential to use only a few micrograms of substrate to ensure complete digestion when using the T_2 method.

The products were first separated by electrophoresis on Whatman paper (46 × 57 cm) in 5% acetic acid/pyridine buffer at pH 3.5 (ref. [12]) and were located by autoradiography (fig.1a). Usually four samples of each RNA were run in parallel. Two were used for base composition analysis, by scintillation counting of the four major bands, and two for pseudouridine (Ψp) determination.

Ψp is not significantly resolved from Up by electrophoresis at pH 3.5. Therefore the area containing Up and Ψp was excised and stitched to a second sheet of Whatman 52 paper (46 × 57 cm) 15 cm from one end. Descending chromatography was carried out for 40 h in isopropanol/HCl/H_2O (68:17.6:14.4 by vol. [12,13]) (fig.1b). Ψp displays an 'R_U' of 0.8 by this method [12], but because of the large excess of Up, some of which trailed during chromatography, separation of Ψp remained incomplete. Therefore the area shown in fig.1b was excised and stitched to another sheet of Whatman 52 paper. Descending chromatography was repeated for 40 h at right angles to the first dimension, using the same solvent. Ψp was now completely separated from Up (fig.1c). The regions containing Up and Ψp were divided from each other by visual inspection. Each region was then cut into 2 × 2 cm squares for scintillation counting, including the 'trailing arms' of Up. Previously purified Up, when rerun, gave practically no spillover into the Ψp area (see legend to table 1).

3. Results and discussion

The base compositions obtained from the electrophoretic separations (fig.1a) closely resembled published data [5,14]. Therefore only the combined Up plus Ψp values are shown (first column of table 1). From estimates of the chain lengths of the rRNAs, and allowing for small quantities of material released as alkali-stable dinucleotides (legend to table 1), the

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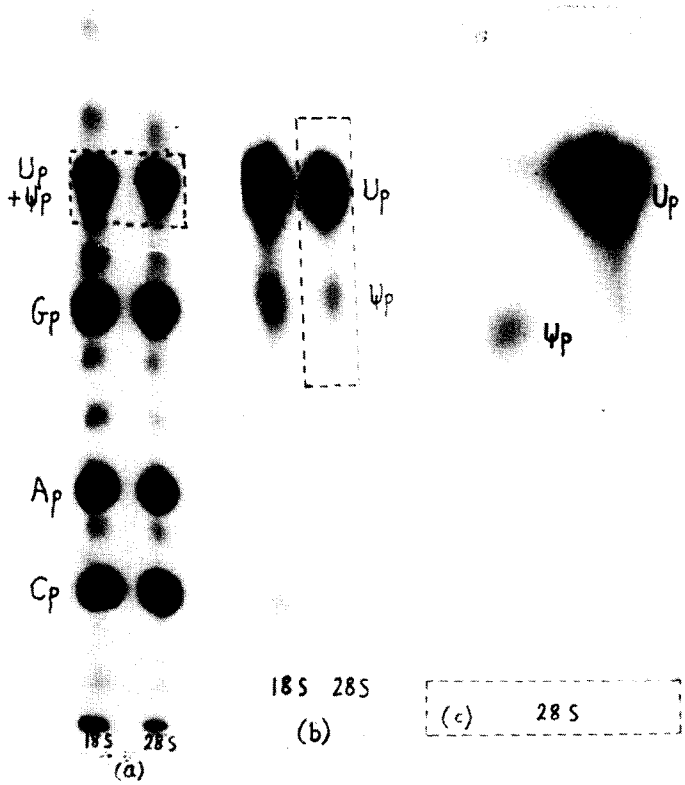

Fig.1. Consecutive stages in the separation of Ψp as described in Methods: (a) electrophoresis, (b) chromatography, first dimension, (c) chromatography, second dimension. In (b) there is a trace of Gm–Gp near the origin; this sometimes overlaps Ψp on electrophoresis but migrates slowly on chromatography.

Table 1
Pseudouridine content of HeLa cell rRNA

	Up + Ψp (%) total mononucleotides	Approx numbers per molecule	$\frac{\Psi p}{Up + \Psi p}$ (%)	Ψp mononucleotides in digest	'Extra' (see text)	Ψp total	2′-*O*-methyl groups
18 S	21.5	420	8.59 ± 0.14	36 ± 1	1	37 ± 1	∿ 38
28 S	15.9	795	7.29 ± 0.23	58 ± 2	2	60 ± 2	∿ 64

In column 1 the 'Up plus Ψp' values are the means of 9 determinations from alkaline and T_2 hydrolysates, the two methods giving good agreement with each other. Only the cpm in the four mononucleotide bands were included in the calculations, the cpm in separate, 2′-*O*-methyl dinucleotide bands being omitted, as was also the case in other 'base composition' data [5,14]. The numbers in column 2 were derived assuming that 18 S RNA, M_R 0.67 × 10^6 [17,18] ∿ 2000 nucleotides, of which ∿ 1950 migrate in the four mononucleotide bands, and 28 S RNA, M_R 1.70 × 10^6 (mean of 1.64 × 10^6 [17] and 1.76 × 10^6 [18]) ∿ 5100 nucleotides, of which ∿ 5000 migrate in the mononucleotide bands. (Gp and Ap each comigrate with some alkali-stable dinucleotides, the remaining dinucleotides migrating as fully resolved bands.) The numbers in column 3 were from 5 determinations on T_2 hydrolysates. These gave good reproducibility with a 'blank' value of 0.13% when pure Up was rerun. This 'blank' has been subtracted here. Alkaline hydrolysates gave slightly less good reproducibility, possibly due to the presence of both 2′ and 3′ phosphates, and the alkaline hydrolysate results are therefore not included.

approximate molar amounts of Up plus Ψp in the hydrolysates per mole of RNA were calculated (table 1, column 2). The molar amount of Ψp per RNA, released as free mononucleotide, was then determined from the chromatographic separation (table 1, columns 3 and 4). Three pseudouridines are not liberated as the simple mononucleotide (column 5) but were identified separately: a hypermodified nucleotide in 18 S RNA [15,16] and alkali-stable compounds, Ψm–Gp and Um–Gm–Ψp in 28 S RNA [7]. No other pseudouridine-containing, alkali-stable compounds have been discovered. Column 6 gives the appropriately corrected estimates for the pseudouridine content. Finally the 2′-*O*-methyl content of HeLa cell rRNA is given in column 7. The values are essentially from ref. [7], but have been refined in the light of additional data on the molar yields of a few methylated sequences (Khan and Maden, in preparation). As noted by Lane for other rRNAs [3,4], there is rather a close numerical correspondence between the total pseudouridine content and the 2′-*O*-methyl content of HeLa cell rRNA.

Most or all methylation sites in rRNA possess different primary sequences from each other. Nevertheless methylation is highly specific in that it is confined to particular locations in the polynucleotide chains [7,8,19]. It is known that in 5.8 S rRNA this is also true for pseudouridylation, this modification occurring at two specific points in the mammalian sequence [19,11]. In high molecular weight rRNAs certain methylated sequences contain Ψp in unimolar amounts [3,7,8]. Non-random frequencies of Ψp in several short oligonucleotides, derived by pancreatic RNAase digestion of rRNA, also suggested that this modification is confined to particular sites in the rRNA sequences [5].

A systematic analysis of the sequence specificity of pseudouridylation has therefore been commenced as follows: T_1 ribonuclease fingerprints, and T_1 ribonuclease plus alkaline phosphatase fingerprints, were prepared from 18 S and 28 S RNA to separate as many as possible of the uracil-containing oligonucleotides. These were screened for the presence of Ψp by alkaline or T_2 ribonuclease hydrolysis, followed by electrophoresis at pH 3.5 and chromatography in one dimension as described under Methods. Figure 2 shows the 18 S fingerprints, with keys. Most of the longer, unique oligonucleotides lack Ψp, but several contain it in good yield. In addition, as expected, several short 'high yield' spots such as U–Gp contain the comigrating analogue such as Ψ–Gp. Qualitatively similar results were obtained for 28 S RNA [20].

Lack of a specific isotopic label renders quantitation of Ψp difficult in individual oligonucleotides, especially when these oligonucleotides overlap each other in a fingerprint. However, all the results described or cited here indicate that Ψp occurs at specific locations in the rRNA sequences, and that, like the methylation sites, these are quantitatively relatively abundant in HeLa and other eukaryotic rRNAs. Further structural studies may provide clues as to the biological significance of this type of modification and to whether there is any causal connection between pseudouridylation and 2′-*O*-methylation in rRNA.

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Fig. 2. (a) T_1 ribonuclease fingerprint of ^{32}P-labelled 18 S RNA. (b) T_1 ribonuclease plus alkaline phosphatase fingerprint. Digestion conditions according to ref. [12]. Separations were on cellogel in the first dimension (3.5 h, 4.8 kV) and DEAE paper (7% formic acid) in the second dimension (40 h, 1.2 kV). These conditions maximize resolution of products in the 'one uridylate' (a) and 'two uridylate' (b) graticules. (c) and (d) are keys. Products above and to the right of the interrupted lines are penta- and larger oligo-nucleotides, most of which occur once per molecule. Not all the unique products are resolved from each other, however. Open circles, no Ψp. Closed circles, Ψp content suggests (at least) one pseudouridine in an unique sequence. Cross-hatched circles, multiple yield products or incompletely resolved mixtures with some Ψp present.

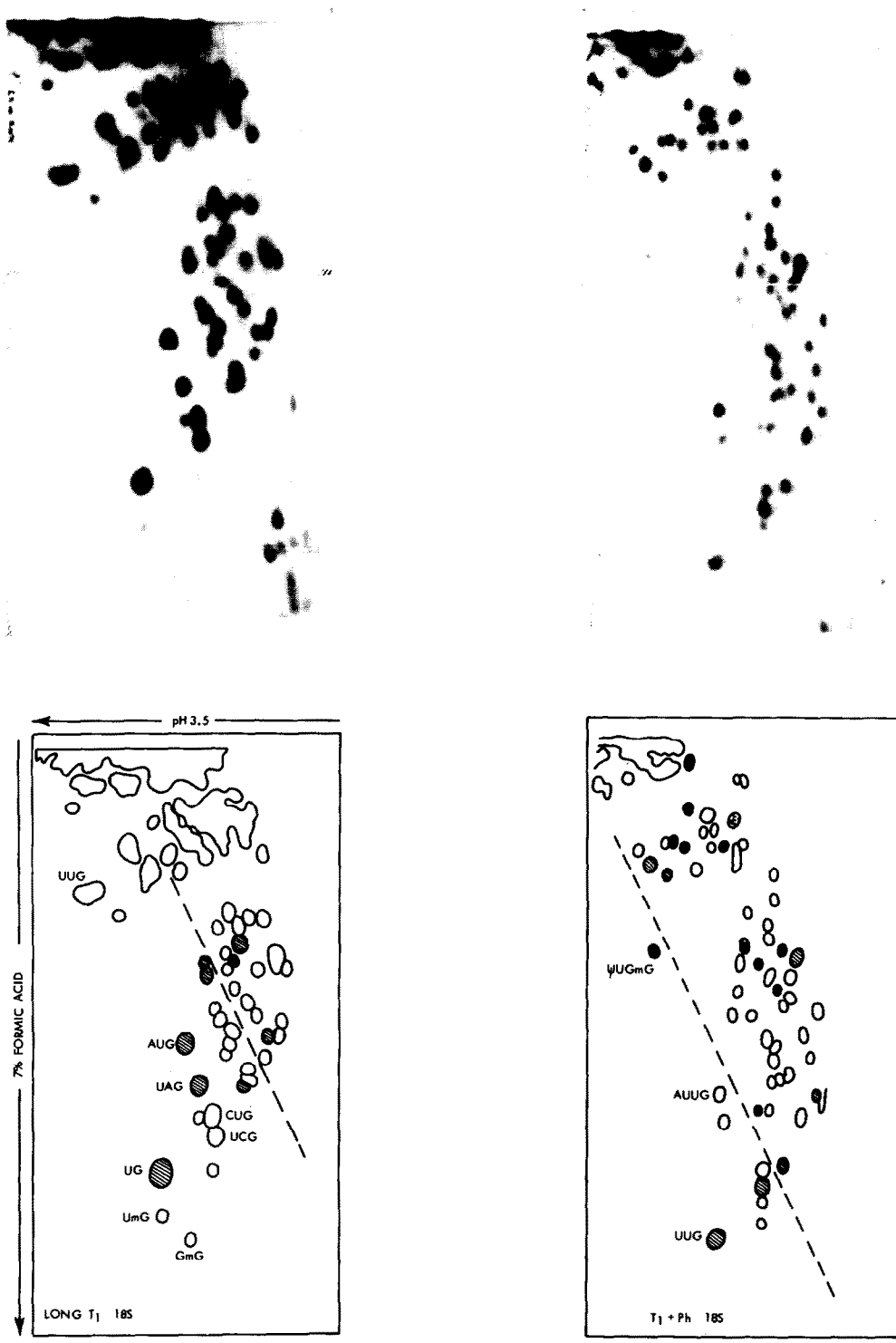
pH 3.5
7% FORMIC ACID
UUG
AUG
UAG
CUG
UCG
UG
UmG
GmG
LONG T1 18S
ψUGmG
AUUG
UUG
T1 + Ph 18S

Fig. 2

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