STANDARD AND NON STANDARD PRODUCTS IN COMBINED T_1 PLUS PANCREATIC RNAase FINGERPRINTS OF HeLa CELL rRNA AND ITS PRECURSORS

B.E.H. MADEN and J. FORBES

Department of Biochemistry, University of Glasgow, Glasgow, G12 8QQ, U.K.

Received 23 October 1972

1. Introduction

Birnboim [1, 2] has used combined T_1 plus pancreatic RNAase digestion in a study of HeLa cell rRNA and its precursors. By column and gel separation procedures he identified several oligonucleotides of the type AnX, where X is U, G or C, as well as some methylated products which were not fully characterized. Brownlee and Cartwright [3] also used combined T_1 plus pancreatic RNAase digestion, followed by two dimensional fingerprinting [4], in a study of *E. coli* 16 S rRNA and its precursor.

We show here that when ^{32}P-labelled HeLa cell rRNA or its precursors are digested and fingerprinted in this way, not only are all the standard (AnX) products separated, but also several non standard products as well. The latter include pseudouridylic acid (ψ)-containing products, an array of methylated products and pUp from 18 S RNA. The procedure should therefore prove useful for examining high molecular weight RNA's generally, and particularly other eukaryotic rRNA's for the presence of such distinctive products.

2. Methods

Ribosomal and nucleolar RNA were labelled for 16 hr with $^{32}PO_4$ as will be described elsewhere (Salim and Maden, in preparation) and isolated by cell fractionation and sucrose gradient centrifugation [5, 6]. 32 S RNA was obtained free from possible contamination by partly degraded 45 S RNA by means of an "actinomycin chase" [6]. Aliquots containing 10–20 μg and 0.5–5 $\times 10^6$ cpm were lyophilized and digested with a mixture of T_1 and pancreatic ribonuclease (1:10 enzyme to substrate ratio, 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA, 45–60 min at 37°). The products were separated by electrophoresis on cellulose acetate at pH 3.5 (2.5 hr, 4.5 KV) followed by DEAE paper in 7% formic acid (16 hr, 1 KV). The region from the origin to the first pink marker dye was transferred from cellulose acetate to DEAE paper. Vigorous digestion was important. Under milder conditions a faint array of incomplete digestion products was sometimes seen, interfering with recognition of genuine enzyme resistant "minor" products.

3. Results and discussion

Fingerprints are shown in fig. 1. Standard products (unshaded in diagrams) were identified by base composition analysis. Products 20X, 22X, 23X and 26X (grey in diagrams) were characterized as ψ, $A_2\psi$, $A_3\psi$ and $A_6\psi$, respectively, by alkaline hydrolysis followed by chromatography of the "U-like" product in isopropanol:HCl:H_2O [7]. Nearly all of the remaining minor products (black in diagrams) are methylated, including the highly characteristic 28 S products 1–11 and 18 S products 2–9′. These products can be selectively labelled with [^{14}C-CH_3]methionine, and are further described in the following paper [8]. 18 S RNA also yields one non-methylated product (1′) with a very rapid mobility at pH 3.5. Treatment of this product with alkaline phosphatase converted all label to P_i. The product was characterized as pUp, and is present to the extent of approx. 0.7 mole per mole

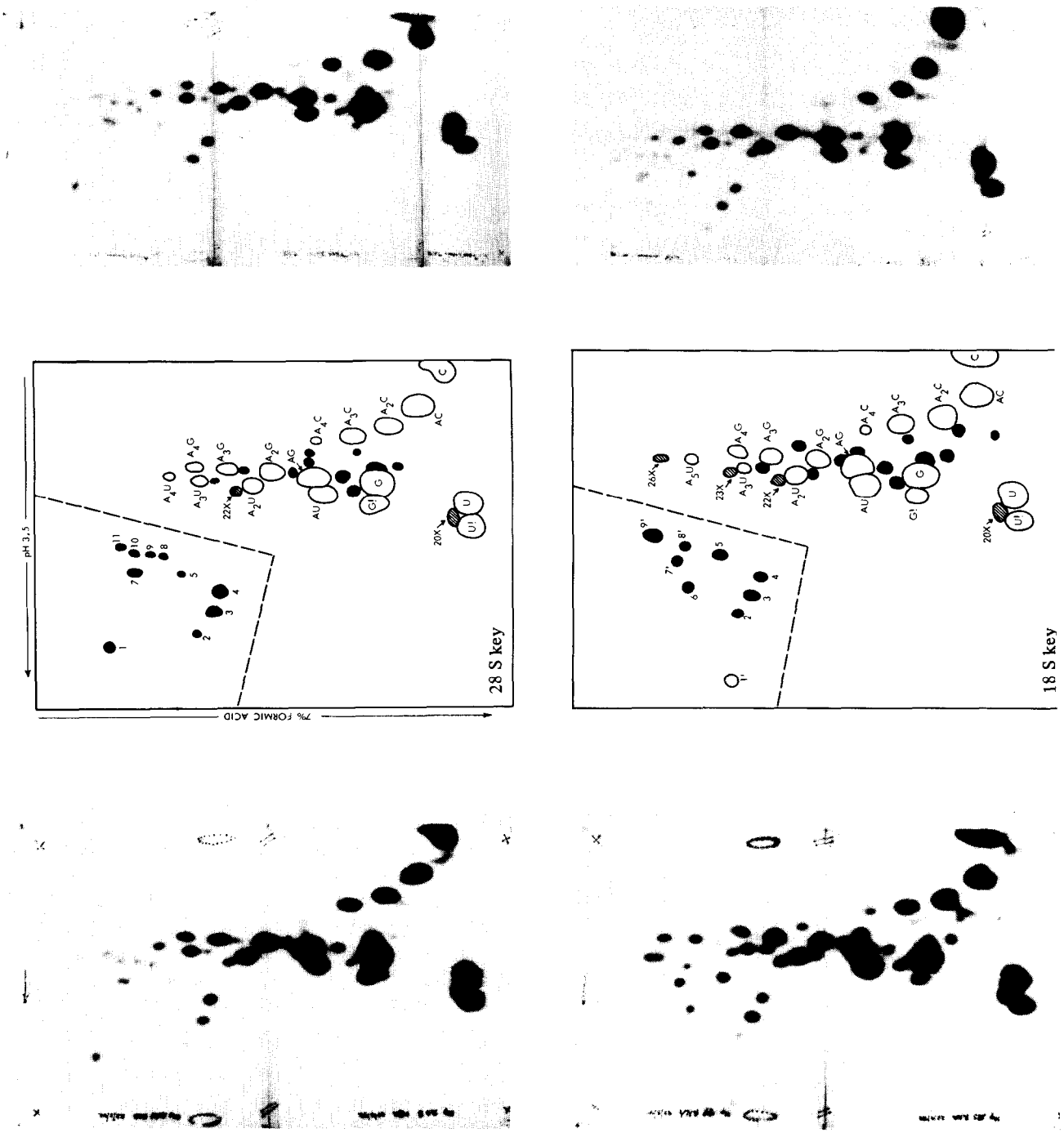

Fig. 1. Combined T_1 plus pancreatic RNAase fingerprints of HeLa cell ^{32}P-labelled rRNA and precursors. In the diagrams products 1–11 are offset to the left to facilitate lettering. U!, G!: 2′3′-cyclic mononucleotides. ψ-Containing products are shaded, and all methylated products which are clearly resolved in the fingerprints are marked black. Primed numbers refer to certain 18 S unique products.

Table 1
Molar yields of A rich products and ψ-containing products from rRNA and precursors.

	18 S	28 S	32 S	45 S
A_5U	0.73 (1)	–	–	1.59 (2)
A_4U	0.18 (–)	1.79 (2)	1.98 (2–3)	4–5
A_5G	–	–	–	0.6 (1?)
A_4G	1.4 (2)*	3.5 (4)	3.6 (4)	5.6 (6–7)
A_5C	–	–	–	–
A_4C	0.8 (1)	0.8 (1)	1.2 (1–2)	2.4 (3)
$A_6\psi$	0.6 (1)	–	–	0.81 (1)
$A_3\psi$	0.9 (1)	–	–	1.29 (1–1.5)
$A_2\psi$	2.5 (2–3)	3	3	6.3 (6)
ψ	++	++	++	++

From fingerprints of uniformly labelled RNA containing ~ 0.5×10^6 cpm all spots were cut out for scintillation counting. The sum of the counts was divided by the number of nucleotides in the respective RNA species, estimated from their molecular weights [10]. From the quotients, and knowledge of the sequences and cpm of the individual products, molar abundances were derived. Suggested integers are in parentheses.

* A_4G from 18 S RNA contains roughly equimolar amounts of the unmethylated product and the methylated derivative $(A_2,AmA)G$.

18 S RNA. It is the only end group which has so far been positively identified in this work. pUp was previously identified as the major 5′ end group of L cell 18 S RNA [9].

The distribution of the longest standard products and the ψ-containing products between rRNA and the precursors is summarized in table 1. 28 S RNA, and also pure 32 S RNA, yield A_4U, A_4G and A_4C but no longer runs of A residues. By contrast 18 S RNA yields A_5U, as also reported by Birnboim [2], but almost no A_4U. The modified sequences $A_3\psi$ and $A_6\psi$ are also unique to 18 S RNA, whereas $A_2\psi$ and free ψ are common to both RNA species. Unlike several methylated products [8], A-rich products were recovered in significantly submolar yields. While this might conceivably imply sequence heterogeneity, it seems more likely to signify overdigestion with pancreatic RNAase. It is difficult to obtain complete digestion by this enzyme without introducing some cleavages at A residues [11]. Trace amounts of A_4U in 18 S fingerprints might also have arisen in this way.

The number of sequences in 45 S RNA in which $A \geqslant 4$ is somewhat larger than the number of such sequences in 28 S + 18 S RNA (nearest integers ~ 17 and 12, respectively). This implies that the non conserved regions of 45 S RNA contain a few, but only a few, runs of 4 or more A residues. Yields of the shorter standard products were also checked and the combined yields agreed approximately with the overall base compositions of the RNA molecules [12–14].

Perhaps the most striking results of this analysis are the confirmation of ψ as a modified nucleotide in HeLa rRNA and its precursors, and the complete separation of an array of 2′-*O*-methylated products. ψ was first identified in HeLa rRNA and precursors by Attardi and co-workers [13, 14]. The present results, like theirs, show that this type of modification takes place in the nucleolus at the level of 45 S RNA. Qualitatively the distribution of ψ-containing sequences in the precursors resembles that in the respective mature rRNA species. Quantitation, particularly of free ψ†, has so far been insufficiently precise to permit a definite conclusion as to wheter ψ is entirely resti restricted to the ribosomal parts of the precursors. The pure methylated products 1–11 have been characterized in some detail [8]. Ready detection of such products in digests of ^{32}P-labelled RNA offers a means for examining some aspects of RNA methylation in situations where methyl labeling may be difficult or impractical.

† Product 20X may be the cyclic form of ψ.

Acknowledgements

We thank Dr. H.C. Birnboim for communicating his results to us before publication. pUp was identified by C.D. Lees. This work was supported by a grant from the Cancer Research Campaign to the late Professor J.N. Davidson, F.R.S. and Professor R.M.S. Smellie.

References

[1] H.C. Birnboim and B.V. Coakley, Biochem. Biophys. Res. Commun. 42 (1971) 1169.
[2] H.C. Birnboim, Canadian J. Biochem. 50 (1972) 993.
[3] G.G. Brownlee and E. Cartwright, Nature New Biol. 232 (1971) 50.
[4] F. Sanger, G.G. Brownlee and B.G. Barrell, J. Mol. Biol. 13 (1965) 373.
[5] S. Penman, in: Fundamental Techniques in Virology, Chap. 5, eds. K. Habel and N.P. Salzman (Academic Press, New York, 1969) see especially p. 39, last par.
[6] B.E.H. Maden, M. Salim and D.F. Summers, Nature New Biol. 237 (1972) 5.
[7] P. Fellner, European J. Biochem. 11 (1969) 12.
[8] B.E.H. Maden, C.D. Lees and M. Salim, FEBS Letters 28 (1972) 293.
[9] B.G. Lane and T. Tamaoki, J. Mol. 27 (1967) 335.
[10] B.E.H. Maden, Progr. Biophys. Mol. Biol. 22 (1971) 146 (see table 6).
[11] G. Beck, P. Fellner and J.P. Ebel, FEBS Letters 7 (1970) 51.
[12] M. Willems, E. Wagner, R. Laing and S. Penman, J. Mol. Biol. 32 (1968) 211.
[13] F. Amaldi and G. Attardi, J. Mol. Biol. 33 (1968) 737.
[14] Ph. Jeanteur, F. Amaldi and G. Attardi, J. Mol. Biol. 33 (1968) 757.