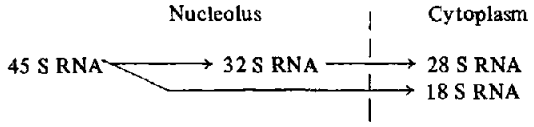
# METHYLATED OLIGONUCLEOTIDES FROM HeLa CELL RIBOSOMAL AND NUCLEOLAR RNA

M. SALIM*, R.WILLIAMSON** and B.E.H.MADEN*
*Department of Biochemistry, Glasgow University, Glasgow, W.2, Scotland
**Beatson Institute for Cancer Research, Hill Street, Glasgow, C.3, Scotland

Received 29 October 1970

## 1. Introduction

A variety of kinetic and chemical lines of evidence support the following model for ribosomal RNA (rRNA) production in mammalian cells [1–6]:




45 S RNA is the primary nucleolar transcription product which is believed to contain the polynucleotide sequences of both 28 S and 18 S rRNA. 32 S RNA is a kinetic intermediate which appears by various criteria to be the direct precursor to 28 S rRNA.

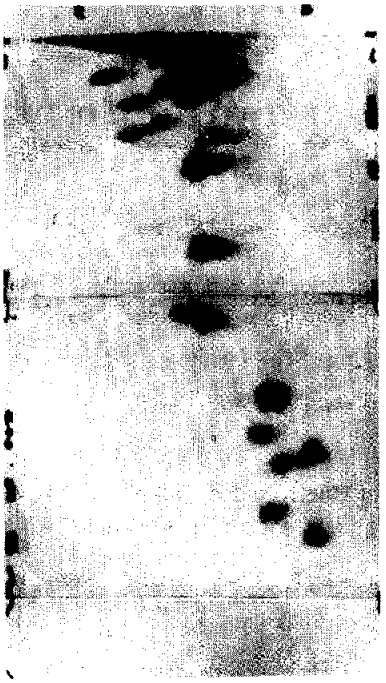
One of the lines of evidence supporting this model for rRNA production in HeLa cells derives from the fact that both rRNA and its presumed nucleolar precursors are methylated. Methylation is normally closely coupled to transcription of 45 S RNA [3, 4], and there is correspondence in distribution of methyl groups between alkali resistant (2′-*O*-methyl) dinucleotides of rRNA and of the respective nucleolar RNA species, as shown by column chromatography of radioactive 2′-*O*-methyl dinucleotides [5]. (Results of a related study for rat Novikoff hepatoma cells have also recently been reported, [7].)

We present here some preliminary findings of a further analysis of the methylation patterns of HeLa cell rRNA and nucleolar RNA, using fingerprinting techniques developed by Sanger and co-workers [8] and applied by Fellner to methyl-labelled *E. coli* rRNA [9, 10]. The results are consistent with the above for rRNA production in HeLa cells, and give some preliminary indication of the degree of complexity of the methylation pattern of rRNA in these cells.

## 2. Methods

To obtain $^{14}$C-methyl-labelled RNA of sufficiently high specific activity for fingerprinting by the method of Sanger et al. [8] the following techniques were adopted. For cytoplasmic rRNA about $10^7$ $S_3$ HeLa cells were resuspended in 50 ml of methionine-free Eagle's medium [11] with 7% calf serum, to which 200 $\mu$Ci of $^{14}$C-methyl-labelled methionine (Radiochemical Centre, Amersham, 60 $\mu$Ci/$\mu$mole) were added. $4 \times 10^{-5}$ M adenosine and guanosine were also added to suppress uptake of methyl label into the purine ring system. The cells were allowed to grow for 36 hr (1½ generations), by which time most of the label had been taken up from the medium. For nucleolar RNA about $2 \times 10^8$ cells were resuspended in 100 ml of methionine-free medium with 100 $\mu$Ci $^{14}$C-methionine and the same concentrations of dialyzed serum and purine nucleosides. Labelling was allowed to proceed for about 2 hr. rRNA was prepared from cytoplasm by precipitation of ribosomes with $Mg^{2+}$ [12], treatment of the ribosomal pellet with 1% SDS and centrifugation of the released RNA on sucrose gradients under standard conditions [13]. Nucleoli were prepared according to Penman et al. [14] and the RNA released by SDS and centrifuged through sucrose gradients. 95–99% of the radioactivity in the region of rRNA, 32 S and 45 S RNA in

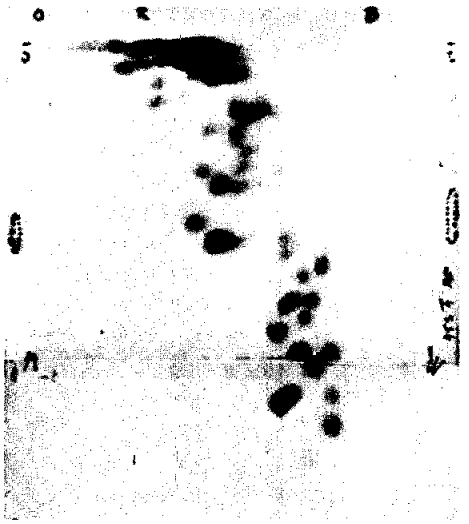
(a) 28 S RNA.



(b) 18 S RNA.

Fig. 1. Fingerprints of $^{14}$C-methyl-labelled HeLa cell ribosomal and molecular RNA. Labelling of the cells, RNA extraction and fingerprinting were carried out as described under methods. The first dimension is from right to left; the second from top to bottom.

(c) 32 S RNA.



(d) 45 S RNA.

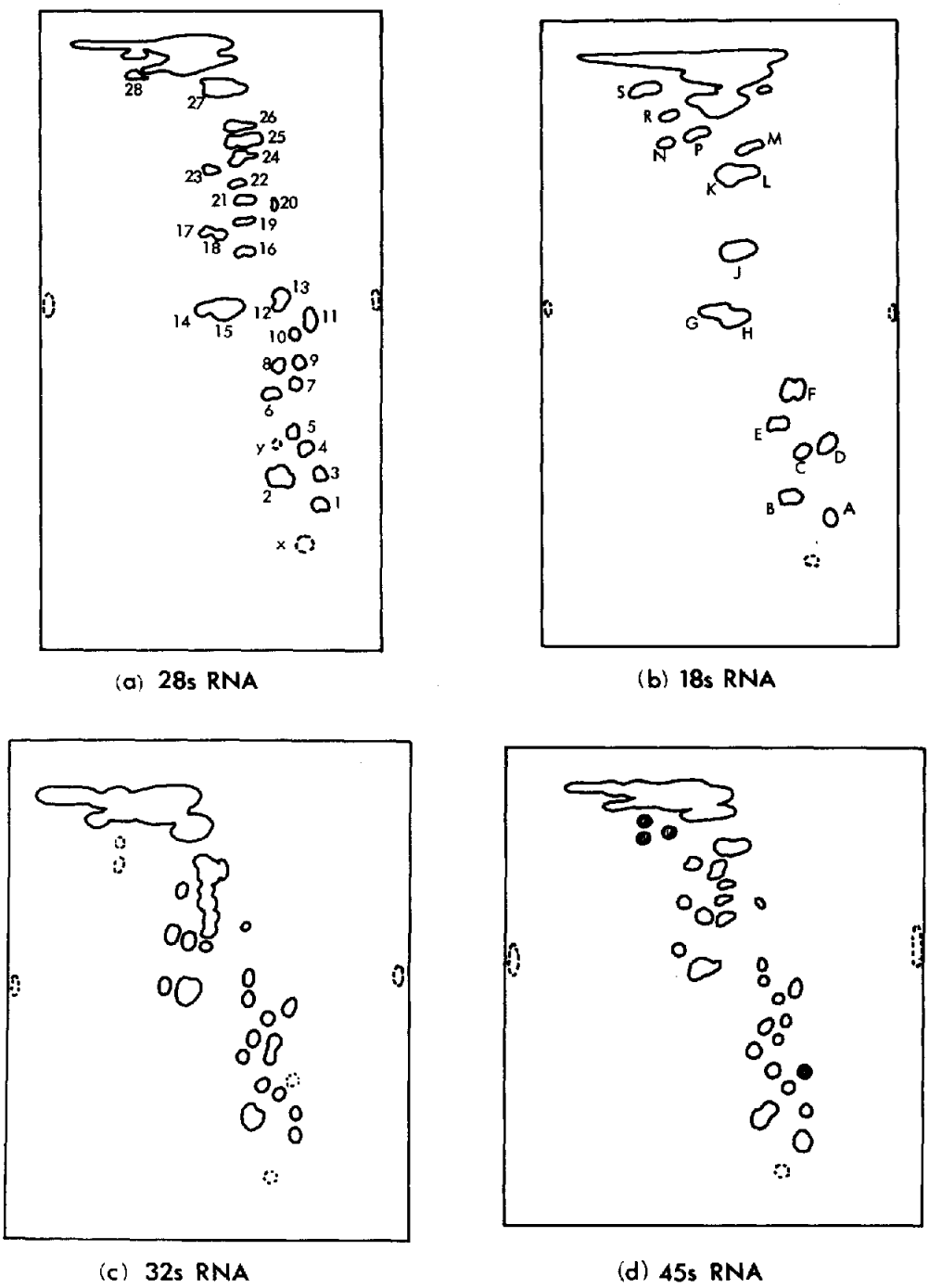



Fig. 2. Key to the fingerprints of HeLa cell ribosomal and nucleolar RNA shown in fig. 1. The dotted circles represent faint spots. The dotted ovals at the lateral edges indicate the position reached by the blue dye at the end of the second dimension run. For further comments see text.

the region of rRNA, 32 S and 45 S RNA in the gradients was acid soluble after treatment with 0.3 N KOH for 18 hr at 37°.

Appropriately pooled fractions, containing 40–60 μg RNA and (in the various preparations) 50–180,000 dpm, were freed of SDS by repeated ethanol precipitation, dried *in vacuo* and finally redissolved in 10 μl of 0.01 M tris, 0.001 M EDTA, pH 7.5, containing $T_1$ ribonuclease to give a 1:20 enzyme:RNA ratio. After digestion (30 min, 37°) the products were separated by electrophoresis using cellulose acetate strips (Oxoid Ltd., London) at pH 3.5 in the first dimension and DEAE paper with 7% formic acid in the second dimension. The products were located by autoradiography, using Kodirex X-ray film.

## 3. Results and discussion

Fig. 1a and b show autoradiographs of fingerprints of methyl-labelled 28 S and 18 S HeLa cell rRNA. Fig. 1c and d show fingerprints of methyl-labelled 32 S and 45 S nucleolar RNA. The position of the marker dyes is shown for reference in each case. A key to the autoradiographs is shown in fig. 2.

A number of points are evident from inspection of fig. 1a and b:

(i) Both 28 S and 18 S RNA yield a fairly large number of well resolved spots, and also some incompletely resolved material near the origin of the second dimension. The fingerprints are more complex than those of methyl-labelled *E. coli* rRNA [9, 10]. This is not surprising in view of the relatively high level of methylation of HeLa cell rRNA (reported to be about 12 methyl groups per 1000 nucleotides in 28 S RNA and 18 methyl groups per 1000 nucleotides in 18 S RNA [5, 15], and further suggests that a relatively large number of distinct sequences are methylated in HeLa cell rRNA.

(ii) The methylation patterns of 28 S and 18 S RNA show clear qualitative differences. Many spots are unique to 28 S RNA, including numbers 3, 10–13, 19 and 21. Spots D, N, P and R are unique to 18 S RNA. Other spots from 28 S and 18 S RNA display similar to overlapping mobilities (e.g. 1 with A, 2 with B, 4 or 5 with C, probably 6 and 8 with E and F, several of the spots in the region 14–28 with corresponding spots in the region G–M, and 28 with S). It is probable that some of these spots will prove to represent oligonucleotides common to both 28 S and 18 S RNA.

(iii) There are (reproducible) intensity differences between different spots in the same autoradiograph. For example in fingerprints of 28 S RNA spots 2 and 15 are always the densest ones. Quantitative analysis of these intensity differences is in progress.

(iv) There are two faint spots in 28 S RNA (x and y) and one in 18 S RNA (of similar mobility to x). These are much more heavily labelled when purine nucleosides are omitted from the labelling medium. Their mobilities correspond to those of G and AG in digests of $^{32}$P-labelled RNA. They therefore probably represent faint residual labelling of the purine ring system. That the remaining spots do not represent ring-labelled products is strongly suggested by two considerations: (a) all of these major spots are much more heavily labelled than the presumptive G, whereas G is the most heavily labelled spot in digests of $^{32}$P-labelled rRNA; (b) in $^{32}$P, $^{3}$H-*methyl* double label fingerprints several of the strongest methyl-labelled spots are only weakly labelled with $^{32}$P (unpublished observations). We therefore believe that the fingerprints in fig. 1 a and b truely represent methylated material from HeLa cell rRNA.

Turning to fig. 1c and d the following points are evident:

(i) The fingerprint of 32 S RNA is similar to that of 28 S RNA. There is good qualitative and (on inspection) rough quantitative correspondence between all the well resolved spots in the two fingerprints. Correspondence is clear for spots 1–15 in 28 S RNA and a similar array of spots in the 32 S fingerprint. Moreover there is strong similarity in outline of the region in the 32 S fingerprint corresponding to spots 16–27 in 28 S RNA, although resolution of the spots is incomplete for 32 S RNA.

(ii) The fingerprint of 45 S RNA contains all of the spots present in 32 S and 28 S RNA, together with four additional well resolved spots, shaded in fig. 2d. The four shaded spots display similar mobilities, relative to the marker dyes, as the four "unique" 18 S spots, D, N, P and R. (Spots corresponding to D, N and R also show very faintly in the 32 S RNA fingerprint.)

(iii) There are no clearly resolved spots in 32 S or 45 S RNA which are not also present either in 28 S or in 18 S RNA.

These results therefore indicate that 45 S RNA contains all of the methylated oligonucleotides which are resolved in 28 S fingerprints, together with the four distinctive 18 S oligonucleotides. The 32 S RNA fingerprint corresponds well with that of 28 S RNA, with faint 45 S (or 18 S) contamination. There is no evidence for distinctive methylated sequences of *non*-ribosomal type in the nucleolar RNA species, at least among the well resolved spots. This is consistent with other data which indicate that, although there is net loss of 40–50% material in conversion of 45 S to ribosomal RNA, there is little or no loss of methyl groups [1, 2, 5, 6, 16].

In summary, the fingerprints of methyl labelled rRNA and nucleolar RNA presented here are consistent with the accepted model for rRNA production in HeLa cells. Further work may permit more rigorous comparison of the methylated material in the respective RNA species, by accurate quantitation, further separation of incompletely resolved material, and sequence analyses. Meanwhile the present results constitute a useful visual display confirming the general nature of the relationship between rRNA and nucleolar RNA in HeLa cells.

## Acknowledgements

This work was carried out with the aid of grants from the Cancer Research Campaign, The Wellcome Trust and The Medical Research Council to Professor J.N.Davidson, F.R.S. and Professor R.M.S.Smellie; and grants from the Cancer Research Campaign and the Medical Research Council to the Beatson Institute for Cancer Research. Technical assistance from Mrs. Theresa McShane is gratefully acknowledged.

## References

[1] J.E.Darnell, Bacteriol. Rev. 32 (1968) 262.
[2] B.E.H.Maden, Progr. Biophys. Mol. Biol. 22 (1971) in press (review).
[3] H.Greenberg and S.Penman, J. Mol. Biol. 21 (1966) 527.
[4] E.F.Zimmerman and B.W.Holler, J. Mol. Biol. 23 (1967) 149.
[5] E.S.Wagner, S.Penman and V.I.Ingram, J. Mol. Biol. 29 (1967) 371.
[6] P.Jeanteur and G.Attardi, J. Mol. Biol. 45 (1969) 305.
[7] Y.C.Choi and H.Busch, J. Biol. Chem. 245 (1970) 1954.
[8] F.Sanger, G.G.Brownlee and B.G.Barrell, J. Mol. Biol. 13 (1965) 373.
[9] P.Fellner and F.Sanger, Nature 219 (1968) 236.
[10] P.Fellner, European J. Biochem. 11 (1969) 12.
[11] H.Eagle, Science 130 (1959) 432.
[12] J.R.Warner, J. Mol. Biol. 19 (1966) 383.
[13] B.E.H.Maden, M.H.Vaughan, J.R.Warner and J.E.Darnell, J. Mol. Biol. 45 (1969) 265.
[14] S.Penman, I.Smith and E.Holtzman, Science 154 (1966) 786.
[15] M.H.Vaughan, R.Soeiro, J.R.Warner and J.E.Darnell, Proc. Natl. Acad. Sci. U.S. 58 (1967) 1527.
[16] R.A.Weinberg and S.Penman, J. Mol. Biol. 47 (1970) 169.