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Extensive Homologies between the Methylated Nucleotide Sequences in Several Vertebrate Ribosomal Ribonucleic Acids

By M. SHAH N. KHAN, MOHAMAD SALIM* and B. EDWARD H. MADEN
Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

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The methylated nucleotide sequences in the rRNA molecules of the following vertebrate cultured cells were compared: human (HeLa); hamster (BHK/C13); mouse (L); chick-embryo fibroblast; *Xenopus laevis* kidney. In each species the combined 18S, 28S and 5.8S molecules possess approx. 110–115 methyl groups, and the methylated oligonucleotides released after complete digestion of the rRNA by T_1 ribonuclease encompass several hundred nucleotides. 'Fingerprints' of the three mammalian methyl-labelled 18S rRNA species were qualitatively indistinguishable. 'Fingerprints' of digests of 28S rRNA of hamster and mouse L-cells were extremely similar to those of HeLa cells, differing in one and three methylated oligonucleotides respectively. 'Fingerprints' of methyl-labelled rRNA from chick and *Xenopus* strongly resembled those of mammals in most respects, but differed in several oligonucleotides in both 18S and 28S rRNA. At least some of the differences between 'fingerprints' appear to be due to single base changes or to the presence or absence of methyl groups at particular points in the primary sequence. The findings strongly suggest that the methylated-nucleotide sequences are at least 95% homologous between the rRNA molecules of the two most distantly related vertebrates compared, man and *Xenopus laevis*

The metabolic processing of rRNA has been widely studied in vertebrates [for reviews see Hadjiolov & Nikolaev (1976) and Maden (1976)]. One important step in rRNA processing is methylation (Vaughan *et al.*, 1967). In HeLa cells the methylated nucleotide sequences of rRNA and precursor rRNA have been characterized in detail, and this information has played a crucial role in defining the maturation pathway for rRNA in the nucleoli of these cells (Maden & Salim, 1974). Limited data on rRNA methylation are also available for chick, *Xenopus* and mouse cells (Judes & Jacob, 1972; Griswold *et al.*, 1974; Hashimoto *et al.*, 1975; Khan & Maden, 1976*a*), but in these cases the sequence information is much less extensive than for HeLa cells.

Detailed information on rRNA methylation in other vertebrates should be of value for several reasons, including the following. (i) Because methyl labelling *in vivo* is frequently used as a means of specifically labelling rRNA, it would be desirable to know to what extent the sequences that are methylated resemble each other between species. (ii) Information on the methylated sequences should be useful in connection with more extensive studies on the primary structures of vertebrate rRNA, and especially in conjunction with studies that might be undertaken in the near future by 'indirect' methods, such as DNA sequencing. (iii) It might be possible from comparative studies on rRNA methylation to gain new insight into the degree of homology or divergence between nucleotide sequences within the high-molecular-weight rRNA of vertebrates.

We present here the results of a comparative analysis of the methylated nucleotide sequences within the rRNA of five vertebrate species. In addition to HeLa cells, the cell lines chosen were from two other mammalian sources, hamster (BHK/C13) cells and mouse L-cells, and two non-mammalian vertebrates, chick-embryo fibroblasts and *Xenopus laevis* cultured kidney cells. These cells and their species of origin have been widely used in studies on ribosome structure, formation and genetics.

Extensive homologies were found between the methylation patterns of the rRNA of all five species. The methylation patterns encompass more than 100 methyl groups and several hundred nucleotides in each species. The degree of homology between the methylated sequences in the rRNA of HeLa cells and *Xenopus laevis*, the two most distantly related species compared, is estimated to be about 95%. This high degree of homology implies extensive conservation, not only of the sequences that are methylated, but also of any other structural or

* Present address: Department of Molecular Biology, University of Islamabad, Islamabad, Pakistan.

metabolic factors which may be involved in their recognition by the methylating enzymes.

Methods

Preparation of rRNA

Methyl-labelled rRNA was prepared from HeLa, chick and *Xenopus* cells as previously described (Maden & Salim, 1974; Khan & Maden, 1976*a*), and from baby-hamster kidney cells (BHK-21/C13) and mouse L-929-cells by similar methods. When necessary 5.8S RNA was separated from 28S RNA by brief thermal treatment followed by re-centrifugation on a second sucrose gradient (Maden & Robertson, 1974); 18S RNA was also usually subjected to a second round of sucrose gradient centrifugation to achieve further purification.

'Fingerprinting' systems

T_1-ribonuclease digests. Such 'fingerprints' were prepared essentially as described previously (Maden & Salim, 1974). The standard conditions for achieving the separations in Plates 1 and 2 were: cellulose acetate, pH3.5 with 7M-urea, 3h at 4.5kV; DEAE-paper, 7% (v/v) formic acid, 16h at 1.3kV. In addition, long separations were carried out on T_1-ribonuclease digests of hamster and mouse rRNA, because the quality of 'fingerprints' of T_1-ribonuclease plus phosphatase digests can be poor (see below). In these long separations the first dimension was run for 5h at 4.5kV; the region ahead of the blue marker dye was transferred on to DEAE-paper and the second dimension was run for 40h at 1.3kV. This resulted in some improvement in the resolution of products above the broken lines in the keys (Plates 1*f* and 2*f*) as described under 'Quantification', below.

T_1-ribonuclease-plus-alkaline phosphatase digests. These 'fingerprints' were also prepared as described previously (Maden & Salim, 1974), except that the alkaline phosphatase/substrate ratio was lowered to approx. 1:20 (w/w). The separations were for 5h at 4.5kV in the first dimension and 40h at 1.3kV in the second dimension, the region ahead of the blue marker dye being transferred. The purpose of these 'fingerprints' was to maximize the degree of separation of products numbered from 44 upwards. This was successful for HeLa, chick, *Xenopus* and, in the 'fingerprints' shown, hamster. In our work with hamster and L-cells the recovery of the longest products was poor and several 'ghost' spots were apparent, suggesting secondary splitting. This problem is discussed under 'Quantification'.

Combined T_1-plus-pancreatic-ribonuclease digests. 'Fingerprints' of these were prepared as described by Khan & Maden (1976*a*). The information so obtained was helpful in determining partial sequences and molar frequencies of the longer T_1-ribonuclease products, as described below.

Sequence analysis of products

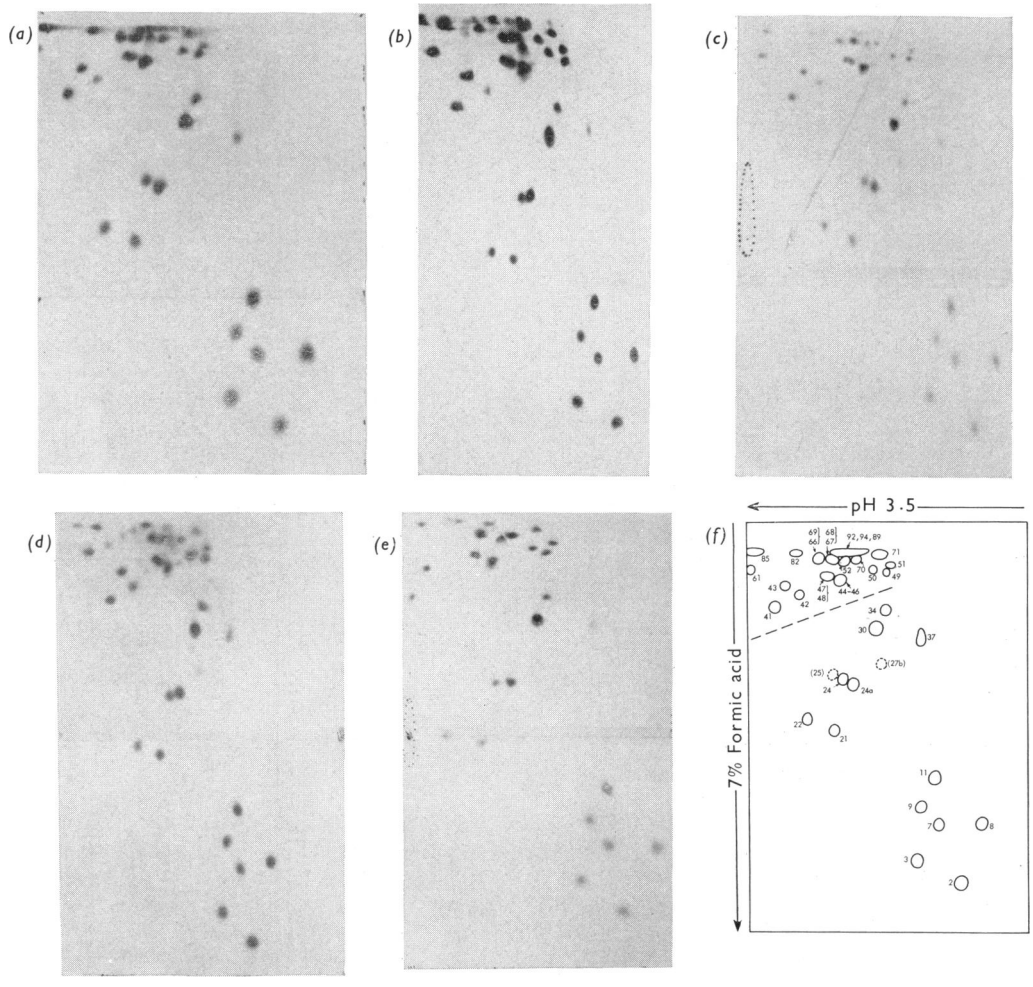
Most sequence information on the HeLa T_1-ribonuclease products was obtained previously (Maden & Salim, 1974). Minor additions and amendments have been made as a result of detailed correlation with information from 'fingerprints' of combined T_1-plus-pancreatic ribonuclease digests (Maden & Khan, 1977).

For the other cell lines one or more of the following procedures was carried out on nearly all products, using [^{14}C]methylated material.

Alkaline hydrolysis. Products were subjected to alkaline hydrolysis (in 10μl of 0.2M-NaOH, 37°C, 18h) followed by electrophoresis on Whatman 52 paper at pH3.5 (Brownlee, 1972). This revealed one or more alkali-stable (2'-*O*-methylated) compounds, a methylated base, or the alkali-conversion products of an unstable methylated base (Maden & Khan, 1977). The alkaline-hydrolysis products generally display characteristic mobilities at pH3.5, but alkali-stable isomers [Um-A, Am-U etc. (m, 2'-*O*-methyl)] are not distinguished (Maden & Salim, 1974). Nevertheless, for T_1-ribonuclease products up to trinucleotides it was safe to conclude that if the mobility of the original T_1-ribonuclease product and that of its alkaline-hydrolysis product were both the same as for the corresponding spot for HeLa cells the sequence was the same.

Snake-venom phosphodiesterase. For *Xenopus* the identity of many alkaline-hydrolysis products was confirmed by successive digestion with bacterial alkaline phosphatase and snake-venom phosphodiesterase, with re-electrophoresis at pH3.5 after each digestion (Maden & Salim, 1974). The final product was a [^{14}C]methylated nucleoside (Um, Am etc.). The results permitted distinction between possible alkali-stable isomers. (Often the dinucleoside monophosphate was incompletely digested and showed as an extra band of unchanged mobility.) Some of the original T_1-ribonuclease products from *Xenopus* RNA were also digested successively with alkaline phosphatase and snake-venom phosphodiesterase (Salim, 1972). This facilitated distinction between an internally located methylated compound (released as nucleotide) and one located at the 5'-end of the original product (released as nucleoside or dinucleoside monophosphate). The results were useful in connection with *Xenopus* products T8 and T40.

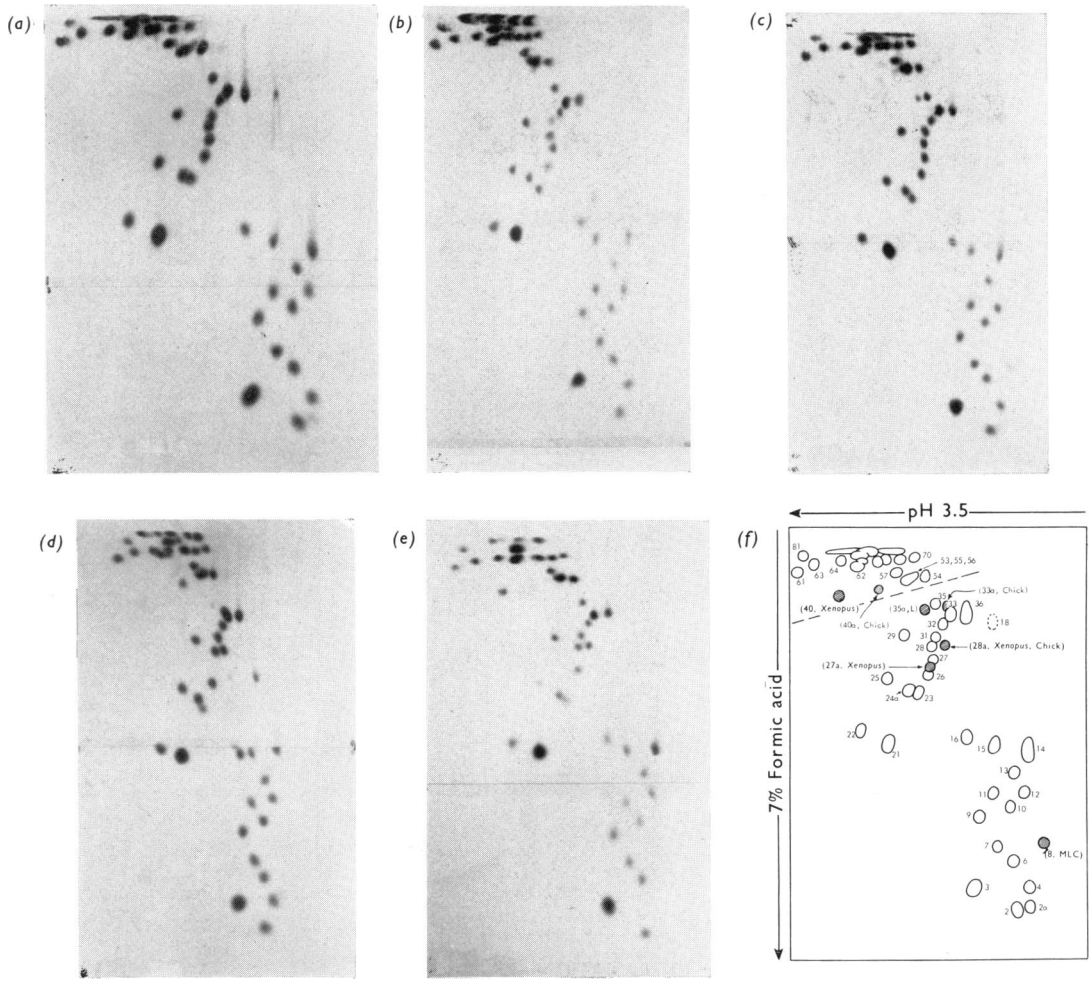
Pancreatic ribonuclease. For all species, all except the shortest T_1-ribonuclease products were digested with pancreatic ribonuclease (Brownlee, 1972). The resulting products were subjected to electrophoresis on DEAE-paper in 7% (v/v) formic acid (16h, 1kV), and the mobilities of the [^{14}C]methylated products



EXPLANATION OF PLATE 1

'Fingerprints' of T_1-ribonuclease digests of methyl-labelled 18S rRNA from (a) HeLa, (b) hamster, (c) mouse, (d) chick and (e) Xenopus laevis

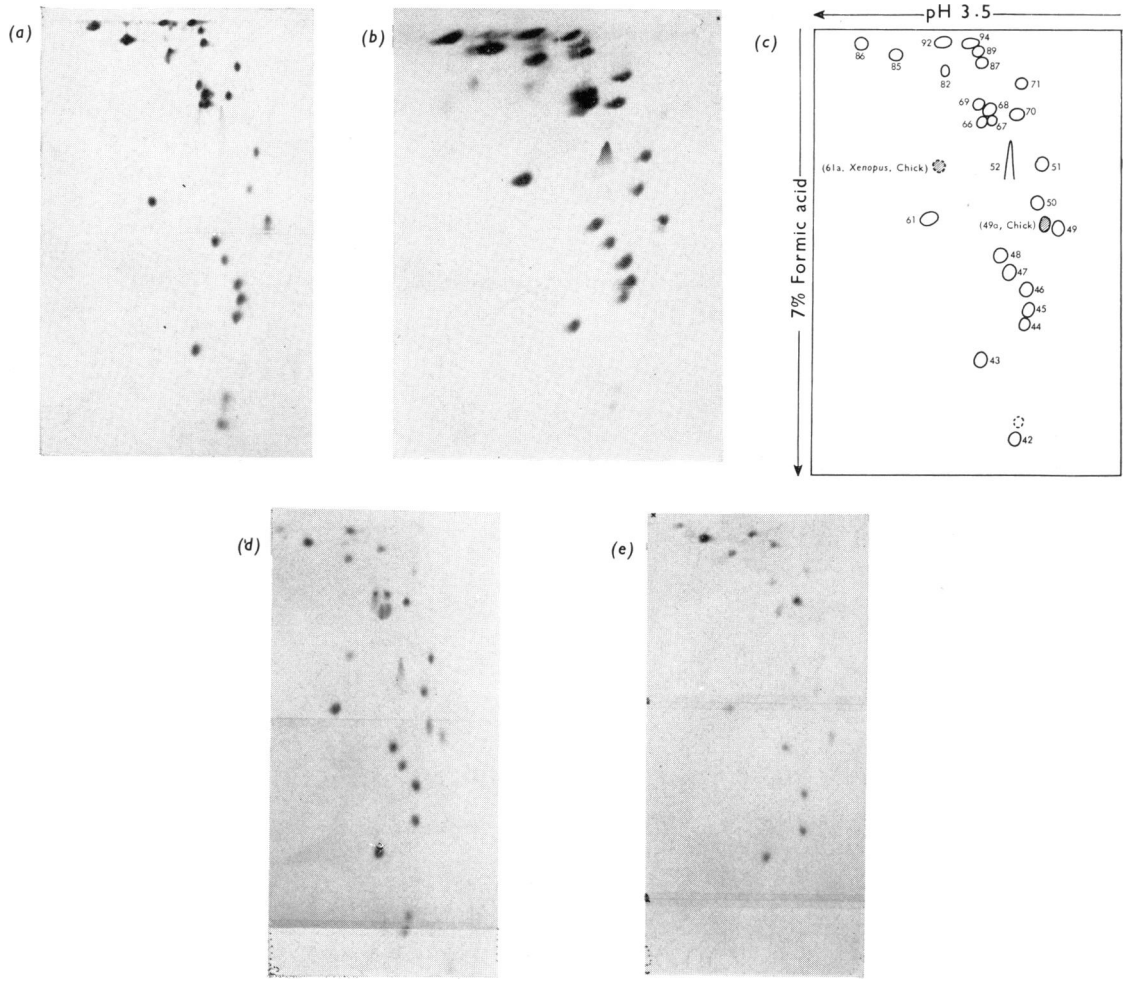
Products above the broken lines in the key (*f*) are better resolved in Plate 3. Broken circles in the key represent weakly labelled and variable products. Dotted ellipses near the edges of some of the 'fingerprints' represent the position of the blue marker dye. The anode is to the left in the first dimension (pH3.5) and at the bottom in the second dimension (7% formic acid). See the text and Table 1 for further details.



EXPLANATION OF PLATE 2

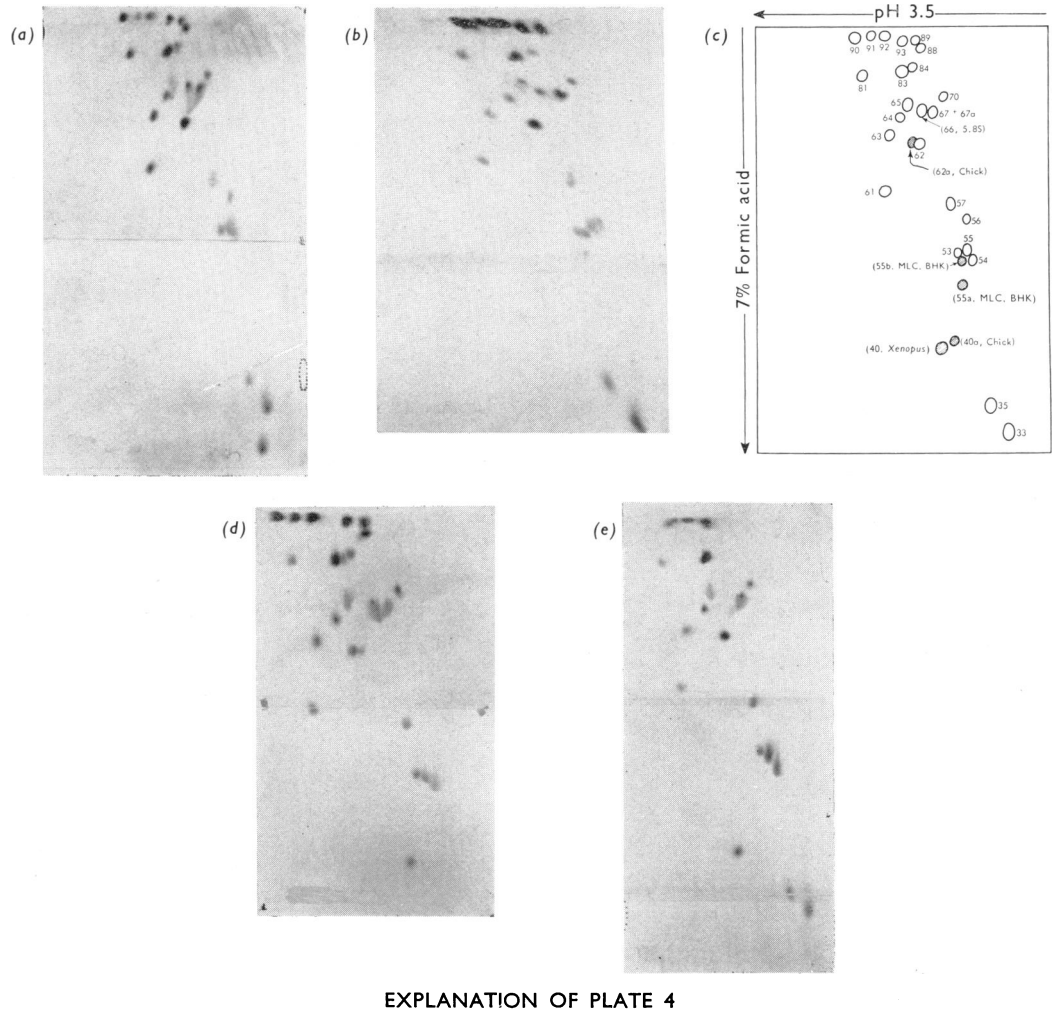
'Fingerprints' of T_1-ribonuclease digests of methyl-labelled 28S rRNA from (a) HeLa, (b) hamster, (c) mouse, (d) chick and (e) Xenopus laevis

Shaded circles in the key represent products that are not present in the 'fingerprint' of HeLa-cell rRNA. Other conventions are as in Plate 1. See the text and Table 2 for further details. (*f*) Key (MLC, mouse L-cells).



EXPLANATION OF PLATE 3

'*Fingerprints*' *of combined* T_1*-ribonuclease-plus-alkaline phosphatase digests of methyl-labelled* 18*S rRNA from* (*a*) *HeLa*, (*b*) *hamster*, (*d*) *chick and* (*e*) *Xenopus laevis*
Conventions are as in Plates 1 and 2. (*c*) Key.



EXPLANATION OF PLATE 4

'Fingerprints' of combined T_1-ribonuclease-plus-alkaline phosphatase digests of methyl-labelled 28S rRNA from (a) HeLa (b) hamster, (d) chick and (e) Xenopus laevis

The *Xenopus* 'fingerprint' is of 5.8S-RNA-free 28S RNA and therefore lacks spot 66. (*c*) Key (MLC, mouse L-cells).

were compared with those of products in 'fingerprints' of combined T_1-plus-pancreatic-ribonuclease digests. The rationale of this procedure and the findings obtained have been described in detail for HeLa-cell rRNA (Maden & Khan, 1977). In general the results obtained with other rRNA species supported the sequence assignments tentatively derived from the mobilities of the oligonucleotides and their alkaline-hydrolysis products, compared with the respective HeLa products. In these cases it was assumed that the sequence was the same as that of the corresponding HeLa product. In a few cases, however (particularly with *Xenopus*), the results of pancreatic-ribonuclease digestion revealed the presence of material different from or additional to that found in the correspondingly numbered HeLa product, for example 18S products T69, T70 and T82.

Quantification

In HeLa, chick and *Xenopus* rRNA the molar yields of several methylated products were determined with reference to the total ^{32}P content of the rRNA molecules, by using 'fingerprints' of combined T_1-plus-pancreatic-ribonuclease digests (Khan & Maden, 1976*a*). From the inter-relationship between several T_1-plus-pancreatic-ribonuclease products and the corresponding T_1-ribonuclease products it was evident that most of the latter occurred approximately once per RNA molecule. The same was assumed to be true for hamster and mouse rRNA. Therefore, to quantify the individual T_1-ribonuclease products, several products whose yields appeared to be approx. 1 mol/mol of rRNA were selected in each 'fingerprint' (about ten 18S products and 20 28S products). The mean of their molar yields was defined as unity, and all individual yields were expressed relative to this mean. The products above the broken lines (in Plates 1*f* and 2*f*) were quantified by using 'fingerprints' of T_1-ribonuclease digests with long separations, or 'fingerprints' of T_1-ribonuclease-plus alkaline phosphatase digests. In these cases quantification was relative to the mean of several reference products, whose combined molar yields had already been determined from standard 'fingerprints' of T_1-ribonuclease digests (for example spots 43–48 and 61 in 18S RNA). The great majority of the observed values in Tables 1 and 2 are means from three or four 'fingerprints'. For a few individual products, which were not well resolved in all 'fingerprints', the values are based on one or two 'fingerprints'.

Some of the longest oligonucleotides produced by T_1 ribonuclease gave persistently low yields, especially in 'fingerprints' of T_1-ribonuclease-plus-phosphatase digests, but also to some extent in standard 'fingerprints' of T_1-ribonuclease digests. This may have been partly due to incomplete transfer from the first dimension to the second dimension of the 'fingerprints'. However, secondary splitting, possibly by an impurity in the enzyme or enzymes, seems also to have been partly responsible, as some extra weakly labelled products were variably present (such as 25a and 27a in Plate 1*f*). The T_1-ribonuclease products whose observed yields were most open to question were, in 18S RNA, T37, T50, T51, T68–T71 and T87 upwards and in 28S RNA, T70 and T88 upwards. These products are each 10–20 nucleotides long, but fortunately several of them correspond to short, 1 mol/mol products in 'fingerprints' of combined T_1-plus-pancreatic ribonuclease digests. Table 3 shows the molar yields of the T_1-plus-pancreatic-ribonuclease products that correspond to some of the T_1-ribonuclease products in question.

The suggested molar frequencies in Tables 1 and 2 are based on all the available data from the various 'fingerprinting' systems (including Table 3), and with the additional assumptions that if a short oligonucleotide is recovered in low yield this is likely to reflect a true submolar frequency of this oligonucleotide, whereas if a long oligonucleotide is recovered in low yield the reason may be that it is an artifact and the suggested frequency is given tentatively as 1.

Results

Plates 1 and 2 show 'fingerprints' of T_1-ribonuclease digests of methyl-labelled rRNA from the five species. Plates 3 and 4 show 'fingerprints' of combined T_1-ribonuclease-plus-alkaline phosphatase digests of HeLa, hamster, chick and *Xenopus* methyl-labelled rRNA. The latter procedure resolves several large uridylate-rich oligonucleotides, which are not well resolved in standard 'fingerprints' of T_1-ribonuclease digests. Clearly the methylation patterns are very similar to each other, with only a relatively small number of oligonucleotides differing between species. From the procedures described in the Methods section the sequence information and quantitative data in Tables 1 and 2 were obtained. Several points arising from the Tables are summarized below.

HeLa-cell rRNA and general points

The HeLa-cell data are basically those of Maden & Salim (1974) with a few additions and minor amendments. The observed molar yields have been adjusted slightly by the inclusion of further data from more recent 'fingerprints'. The molar yields are expressed as numbers of methyl groups per 'spot'. Thus product T30 from 18S RNA occurs once per molecule, but contains four methyl groups. Conversely, product T21 from 28S RNA is singly methylated but occurs several times per molecule.

Further additions to the HeLa-cell data have resulted from carrying out a systematic correlation

Table 1. *Sequence data and molar yields of methylated oligonucleotides in T_1-ribonuclease digests of vertebrate 18S rRNA*

2′-*O*-Ribose-methylated nucleotides are denoted Cm, etc.; base-methylated nucleotides as mA etc., and hyphens denote internal phosphate residues. The molar yields are expressed as numbers of methyl groups. For example, product T30 occurs once per molecule, but contains four methyl groups. A plus sign indicates that an oligonucleotide is present, but the observed molar-yield data were unreliable for experimental reasons. In some cases additional supporting data for the suggested molar yields were obtained from 'fingerprints' of combined T_1-plus-pancreatic-ribonuclease digests (see Table 3 and the Methods section). A minus sign indicates absence of a methylated oligonucleotide from the 18S rRNA of that species. Spots that are present only in 18S rRNA species other than HeLa are listed at the bottom of the Table. Notes: (*a*) see Table 3 for additional quantitative data on these sequences; (*b*) this product may also contain a small amount of an extra unidentified component; (*c*) this oligonucleotide is present in fractional yield in HeLa, but the sum of Um-C plus Gm-A is approximately one methyl group. The presence of Gm-A was not confirmed in other species owing to poor recovery of the spot, but the results from combined T_1-plus-pancreatic-ribonuclease 'fingerprints' (Table 3) indicate the presence of A-Gm-A-G in all species except *Xenopus*. See also Fuke & Busch (1977), discussed in the text. The parenthetic G residue is included as it contributes to the specificity of cleavage by the T_1 ribonuclease (see the Discussion section for further details).

		Yield (mol of methyl groups/mol of oligonucleotide)										
		Observed					Suggested					
Spot no.	Sequence	HeLa	Hamster	Mouse	Chick	*X. laevis*	HeLa	Hamster	Mouse	Chick	*X. laevis*	Notes
T2	(G)-Cm-G	1.06	1.03	0.95	1.77	1.06	1	1	1	2	1	
T3	(G)-Am-G	1.07	1.15	1.05	1.01	1.06	1	1	1	1	1	
T7	(G)-A-Cm-G	0.95	1.17	0.94	1.03	1.03	1	1	1	1	1	
T8	(G)-Cm-C-C-G plus C-Cm-C-G	1.55	1.52	1.19	1.62	0.96	1.5	1.5	>1	1.5	1	(Cm-C-C-G only in *Xenopus*)
T9	(G)-Am-A-G	0.91	1.09	0.98	1.00	0.97	1	1	1	1	1	
T11	(G)-A-Am-C-G	1.91	1.94	1.87	1.84	1.74	2	2	2	2	2	
T21	(G)-Gm-G	1.05	1.05	1.10	1.08	1.08	1	1	1	1	1	
T22	(G)-Um-G	0.99	1.03	1.08	1.00	1.01	1	1	1	1	1	
T24	(G)-U-Am-G	0.93	0.95	1.04	0.89	0.96	1	1	1	1	1	
T24a	(G)-A-Gm-G	1.71	1.81	1.49	2.14	1.87	2	2	2?	2	2	
T30	(G)-m_2^6A-m_2^6A-C-(C,U)-G	3.75	3.56	3.88	4.16	3.78	4	4	4	4	4	
T34	...(m^6A,A)-C...	0.82	0.78	0.84	0.77	0.86	1	1	1	1	1	
T37	...amΨ-C...	0.50	0.53	0.45	+	0.43	1	1	1	1	1	(*a*)
T41	(G)-Gm-U-G	1.14	1.03	1.03	0.87	1.05	1	1	1	1	1	
T42	(G)-C-U-Um-G	0.51	0.59	0.55	0.67	0.11	0.5	0.5	0.5	0.7	0.1	
T43	(G)-A-Um-U-G	1.01	1.08	1.07	1.03	1.00	1	1	1	1	1	
T44	(G)-A-U-Um-C-C-G	0.99	1.01	0.86	0.83	0.98	1	1	1	1	1	
T45	(G)-(A,C,U)-Cm-U-G	1.04	1.06	1.13	—	—	1	1	1	—	—	
T46	...Am-U-G	1.03	0.77	0.96	0.98	0.98	1	1	1	1	1	
T47	(G)-A-A-C-U-Um-G	0.84	0.86	0.88	0.77	—	1	1	1	1	—	
T48	(G)-A-U-U-Am-A-G	0.96	0.84	0.84	0.97	0.98	1	1	1	1	1	
T49	...m^7G-A-A-U...	1.01	0.81	0.77	0.79	0.84	1	1	1	1	1	
T50	...A-Gm-G	0.76	0.68	0.60	0.65	0.70	1	1	1	1	1	
T51	...Cm-C...	0.59	0.57	0.56	0.58	0.72	≃1	≃1	≃1	≃1	≃1	
T52	...(A_3,Am-A)-G	1.00	1.12	1.08	0.71	0.94	1	1	1	1	1	(*b*)

T61	(G)-Ψ-U-Gm-G	1.12	1.03	1.03	1.05	0.79	1	1	1	1	1	
T66	...Am-U...	0.92	0.82	0.77	0.90	—	1	1	1	1	—	
T67	...Um-A-A-U...	0.93	0.72	0.97	0.97	0.90	1	1	1	1	1	
T68	...A-(A,Um-A)-Cm-A-U...	1.82	1.40	1.50	—	—	2	≃2	≃2	—	—	(*a*)
T69	...Gm-G	0.86	0.56	0.50	0.3	—	1	<1?	<1?	0.5?	—	
T70	...A-Gm-C...	0.76	0.76	0.77	0.75	1.66	1	1	1	1	2	(Plus Cm-C in *Xenopus*)
T71	...Am-A-A-U...	0.72	0.52	0.52	+	0.79	1	1	1	1	1	(*a*)
T82	...A-Am-U...	0.95	0.78	0.86	0.75	—	1	1	1	1	—	
T85	...Um-C...Um-G	2.02	1.99	1.78	2.14	1.82	2	2	2	2	2	
T86	(G)-Um-U-U-A-C-U-U-U-G	1.11	0.72	1.05	0.66	0.88	1	1	1	1	1	
T87	...Am-U...	0.80	+	+	0.61	0.86	1	1	1	1	1	
T89	...Um-C...	0.47	+	+	—	—	0.5	0.5	0.5	—	—	
T92	...A-A-Am-U	0.66	+	+	?	?	0.6	1	1	?	1	(*a*)
T94	...Gm-A,Um-C...	1.07	+	+	0.70	0.87	1	1	1	1	1?	(*a*), (*c*), (Um-C only in *Xenopus*)
T49a	...A-Cm-U...	—	—	—	0.73	—	—	—	—	1	—	
T61a	...Um-U...	—	—	—	0.36	0.26	—	—	—	0.4	0.3	
T68 chick	...A,(A,Um-A)-C...	—	—	—	0.71	—	—	—	—	1	—	(*a*)
T69 *Xenopus*	...Um-G	—	—	—	—	0.55	—	—	—	—	<1?	
T82 *Xenopus*	...Am-U...	—	—	—	—	0.87	—	—	—	—	1	
Total methyl groups, 18S RNA							45	≃45	≃45	≃43	≃40	

Table 2. *Sequence data and molar yields of methylated oligonucleotides in T_1-ribonuclease digests of vertebrate 28 S rRNA*

All general conventions are as in Table 1. Note that several spots from digests of *Xenopus rRNA* contain different or additional methylated components to the correspondingly numbered HeLa spots. Notes: (*a*) see Table 3 and the Methods section for additional molar-yield data on these products; (*b*) m^1A was not satisfactorily demonstrated in product T14 in chick, but its presence was inferred from the corresponding T_1-plus-pancreatic-ribonuclease digest spot TP56 (Khan and Maden, 1976*a*; Maden & Khan, 1977). Spot T14 from a digest of *Xenopus* rRNA may possibly contain an extra methylated component (see Table 3). (*c*) In chick, spot T35 is largely replaced by a related product, T33a, which is barely resolved from T33 (Plate 2, see also the text). (*d*) It is possible that spot T90 is an unresolved mixture of products, and that Um-C in product T91 represents a partly resolved component. The parenthetic G residue is included as it contributes to the specificity of cleavage by the T_1 ribonuclease (see the Discussion section for further details).

Spot no.	Sequence	Yield (mol of methyl group/mol of oligonucleotide) Observed HeLa	Hamster	Mouse	Chick	*X. laevis*	Suggested HeLa	Hamster	Mouse	Chick	*X. laevis*	Notes
T2	(G)-Cm-G	1.08	1.08	1.14	1.13	1.05	1	1	1	1	1	
T2a	(G)-mC-G	0.20	—	±	—	—	0.2	—	±	—	—	
T3	(G)-Am-G	4.42	4.28	4.18	3.48	4.29	4.5	≏4	≏4	3.5	≏4	
T4	(G)-C-Cm-G	0.93	1.05	0.96	1.00	—	1	1	1	1	—	
T6	(G)-C-Am-G	1.15	1.00	1.08	1.11	1.18	1	1	1	1	1	
T7	(G)-A-Cm-G	1.14	1.11	1.19	0.90	1.11	1	1	1	1	1	
T9	(G)-A-Am-G	1.15	1.08	1.02	1.01	1.12	1	1	1	1	1	
T10	(G)-A-Cm-C-G	1.02	0.98	0.95	0.97	0.92	1	1	1	1	1	
T11	(G)-A-Am-C-G	1.05	1.05	0.92	0.81	0.97	1	1	1	1	1	
T12	(G)-C-C-Cm-A-G	1.03	0.91	0.90	1.05	0.98	1	1	1	1	1	
T13	(G)-Cm-C-A-A-G	0.93	0.82	0.81	0.91	0.84	1	1	1	1	1	
T14	...(m^1A,A)-C,Am-C...	1.85	1.32	1.26	1.12	1.96	2	2	2	2?	2?	(*a*), (*b*)
T15	(G)-A-A-C-Am-G	0.85	0.90	0.80	0.64	0.77	1	1	1	1	1	
T16	(G)-C-C-Gm-G	0.79	0.87	0.87	1.07	0.79	1	1	1	1	1	
T18	...Am-G?	0.42	0.17	0.44	0.40	—	0.5	0.2	0.5	0.5	—	
T21	(G)-Gm-G	6.97	6.50	6.29	6.09	5.11	7	≏7	≏7	≏7	5	(*a*)
T22	(G)-Um-G	1.05	1.13	1.16	≏1	1.20	1	1	1	1	1	
T23	(G)-Cm-U-G	0.85	1.08	0.96	0.33	0.80	1	1	1	0.3	1	
T24a	(G)-A-Gm-G	1.09	1.05	0.98	1.01	0.80	1	1	1	1	1	
T25	(G)-Am-U-G	1.03	1.06	1.08	0.95	0.42	1	1	1	1	0.5	
T26	(G)-Um-A-C-G	0.99	0.88	0.98	0.96	—	1	1	1	1	—	
T27	(G)-Cm-A-U-G	1.01	1.16	1.11	—	—	1	1	1	—	—	
T28	...(m^6A,A)-C...	0.98	0.93	1.07	0.86	1.22	1	1	1	1	1	
T29	(G)-A-A-Ψm-G	0.90	1.06	1.02	0.88	1.07	1	1	1	1	1	
T31	(G)-A-(A,Gm-A)-G	0.93	0.79	0.77	1.01	1.19	1	1	1	1	1	
T32	(G)-(C,U)-A-Am-A-G	1.03	0.97	1.04	0.99	1.04	1	1	1	1	1	
T33	...mC,A-A-Am-U...	2.21	1.77	2.05	≏2	2.14	2	2	2	2	2	(*c*)
T35	(G)-A-A-C-A-A-Um-G	0.93	0.82	0.87	0.16	0.97	1	1	1	0.2	1	(*c*)
T36	(G)-A-C_{3-5}-Gm-A-Am-A-G	1.80	1.47	1.51	1.74	1.56	2	2	2	2	2	(*a*)
T53	...Gm-U...	0.89	0.83	0.78	0.88	0.99	1	1	1	1	1	
T54	...Cm-C...	0.90	0.81	0.98	0.90	1.96	1	1	1	1	2	(Plus Um-C in *Xenopus*)
T55	...Um-C...	0.95	0.97	0.83	0.90	2.07	1	1	1	1	2	(Plus Cm-U in *Xenopus*)
T56	...Cm-U...	0.78	—	—	—	—	1	—	—	—	—	

T57	(G)-A-Am-C-Ψ-A-U-G	0.98	1.04	1.00	0.97	1.10	1	1	1	1	1	
T61	(G)-U-U-Gm-G	1.19	1.10	1.15	1.05	0.78	1	1	1	1	1	
T62	(G)-Cm-A-Gm-U-U-G	2.21	2.10	2.21	0.24	2.13	2	2	2	0.2	2	
T63	(G)-m^{3}U-U-Ψ-A-G	1.18	1.01	1.04	1.03	1.05	1	1	1	1	1	
T64	(G)-A-U-Cm-U-U-G	1.08	1.11	1.15	1.07	1.21	1	1	1	1	1	
T65	...A-Am-C...	0.98	1.15	1.26	1.03	1.22	1	1	1	1	1	
T67	...mC,Am-U...	2.51	1.17	1.15	≐2	2.47	1.5	1.5	1.5	2	1.5	(mC migrates with product 66 in chick)
T67a	...A-Am-U...		0.90	1.15			1	1	1		1	
T70	...Am-U...	0.65	0.66	1.00	0.94	0.92	≐1	≐1	1	1	1	
T81	(G)-U-A-U-Gm-U-G	1.06	1.03	0.94	0.73	0.78	1	1	1	1	1	
T83	...Um-Gm-U...	1.93	2.26	2.31	1.99	3.98	2	2	2	2	4	(*a*), (plus Gm-G, A-Gm-C in *Xenopus*)
T84	...Gm-U...	0.76	1.08	0.90	0.57	—	1	1	1	1?	—	
T88	...A-Cm-U...	0.80	+	+	1.47	—	1	1	1	1	—	(Co-migrates with product 93 in *Xenopus*)
T89	...Cm-C...	0.81	+	+	1.10	—	1	1	1	1	—	
T90	...Gm-U,Um-C,(Gm-A,A)-C...	2.53	+	+	2.52	2.71	3	3	3	3	≐3	(May lack Gm-U in *Xenopus*)
T91	...Um-C...	0.92	?	?	1.23		?	?	?	1	?	(*d*)
T92a	...Um-Gm-Ψ...	1.83	+	+	2.96	3.15	2	2	2	2	2	(*a*)
T92b	...Gm-U...	0.52	?	?	?	(+)	0.5	?	?	?	1	
T93	...Am-Gm-Cm-A-A-A-U...	1.63	+	+	—	4.57	≐3	≐3	≐3	—	4	(*a*), (plus A-Cm-U in *Xenopus*)
T8	...Cm-C...	—	0.10	0.30	—	—	—	0.1	0.3	—	—	
T27a	(G)-U-Cm-A-G	—	—	—	—	0.96	—	—	—	—	1	
T28a	...Cm-U...	—	—	—	0.99	0.99	—	—	—	1	1	
T33a	(G)-(A,C_2)-A-A-Um-G	—	—	—	+	—	—	—	—	1	—	(*c*)
T35a	(G)-A_5-Um-G	—	—	0.37	—	—	—	—	0.4	—	—	
T40	(G)-Um-A-U-G	—	—	—	—	1.19	—	—	—	—	1	
T40a	...Cm-U...	—	—	—	0.51	—	—	—	—	0.5	—	
T55a	?	—	0.40	0.17	—	—	—	0.4	0.2	—	—	
T55b	...Cm-U	—	0.40	0.81	—	—	—	0.4	0.8	—	—	
T62a	...A-Gm-U...	—	—	—	0.86	—	—	—	—	1	—	
T84a	?	—	—	—	0.48	—	—	—	—	0.5	—	
T93a	...Am-Gm-C...	—	—	—	1.75	—	—	—	—	2	—	
5.8 SRNA												
T22	(G)-Um-G	0.21	+	+	0.7	0.4	0.2	≐0.3	≐0.3	0.7	0.4	
T66	(G)-A-A-U-U-Gm-C-A-G	0.95	+	0.84	1.11	0.85	1	1	1	1	1	
Total methyl groups, 28S+5.8S RNA							≐70	≐70	≐70	≐67	≐70	

between information obtained from 'fingerprints' of T_1-ribonuclease digests and of combined T_1-plus-pancreatic-ribonuclease digests (Maden & Khan, 1977). A similar correlation between the two 'fingerprinting' systems was carried out for the other rRNA species, as outlined in the Methods section. The results are not described in detail, but certain points are summarized below and in Table 3.

Spot T37 contains a hypermodified nucleoside which, during its biosynthesis, incorporates label from carboxyl-labelled methionine as well as from methyl-labelled methionine. This compound, 3-amino-3-carboxypropyl-1-methylpseudouridine, was first characterized in hamster 18S rRNA (Saponara & Enger, 1974) and has since been identified in several eukaryotic 18S rRNA species (Maden *et al.*, 1975). Its biosynthetic pathway has been described (Brand *et al.*, 1978). The low yield of the product in 'fingerprints' of T_1-ribonuclease digests is an artifact (probably due to poor transfer from the first dimension to the second dimension, and/or to streaking). A smaller derivative product in 'fingerprints' of combined T_1-plus-pancreatic-ribonuclease digests occurs at a yield of approx. 1 mol/mol (Table 3).

Sequence information on several other oligonucleotides has been extended slightly by correlating T_1-ribonuclease (T) products with combined T_1-plus-pancreatic-ribonuclease (TP) products. Examples are 18S rRNA product T68, now known to be a single doubly methylated oligonucleotide in mammals, and 28S rRNA product T14, now known to contain an alkali-labile base, 1-methyladenine (Table 3) (Maden & Khan, 1977). On re-analysis, 18S spot T69 was found to contain Gm-G, not Um-A, and spot T86 is Um-U-U... (Khan & Maden, 1977), not U-Um-U as was previously reported by Maden & Salim (1974). (For abbreviations, see legend to Table 1.) We did not, however, complete the sequence analysis of many long oligonucleotides from T_1-ribonuclease digests. Several of these are poorly resolved in 'fingerprints' of ^{32}P-labelled rRNA with the present 'fingerprinting' systems (Salim, 1972); however, see the reference to Fuke *et al.* (1976) in the next section.

18S rRNA: hamster and mouse

There were no significant qualitative differences between the methylation patterns of the three mammalian 18S rRNA species. Difficulty was experienced with low recovery of the longest oligonucleotides from T_1-ribonuclease digests, and the presence of variable weakly labelled products (in approx. 0.15 molar yield) such as T25a and T27a (Plate 1*f*). Both of these effects were almost certainly due to impurities in the enzyme preparations, as discussed in the Methods section. The only reproducible difference between the mammalian 18S patterns was the somewhat lower yield of A-A-Am-U (Table 3) in HeLa-cell 18S rRNA than in the other two 18S rRNA species.

Fuke *et al.* (1976) have resolved several long ^{32}P-labelled oligonucleotides from various mammalian 18S RNA species in 'fingerprints' involving homochromatography on DEAE-cellulose thin layers in the second dimension. A number of their oligonucleotides contain alkali-stable products. From the mobilities of their oligonucleotides in the first dimension (cellulose acetate), and from the alkali-stable dinucleotide contents, we suggest the following relationships between their products and ours: their product 1 = our T92; 3 = T94; 4 = T71; 6 = T89; 9 = T50; 12 = T87 (probably); 14 = T37; 18 = T86. Fuke & Busch (1977) have determined the sequence of their product 3 (corresponding to our T94). It contains 18 nucleotides, and includes the sequence -Um-C-, and ends with the sequence -A-Gm-A-G. The non-terminal G residue was incompletely methylated, as we also found, but the extent of methylation was higher than in our Table 3. When this G residue is unmethylated it is cleaved by T_1 ribonuclease, producing their product 6 (our T89) instead.

18S rRNA: chick and Xenopus

The methylation pattern of chick 18S rRNA differs from that of mammals in certain respects. G-Cm-G (product T2) occurs twice per molecule in chick, the hexanucleotide T45 is missing and products T49a and T61a are weakly labelled extra spots. Interestingly, product T68 displays the same mobilty as that from HeLa cells, but is only singly methylated (at Um, but not at C). The chick product is therefore listed separately at the foot of Table 1, though its primary sequence must be similar to or identical with product T68 in HeLa cells. Product T92 is absent from chick. Product T94 is present, product T89 is absent, and the combined T_1-plus-pancreatic-ribonuclease product A-Gm-A-G, corresponding to the fractionally methylated G in product T94 (discussed above), was present in higher yield in chick than in mammals (Table 3).

A few very weakly labelled products in the chick 18S-rRNA 'fingerprint' in Plate 1 are probably results of secondary splits of the longest oligonucleotides.

The chick 18S-rRNA pattern obtained in the present work with freshly cultured fibroblasts corresponds exactly to the pattern published by Judes & Jacob (1972) for chick-embryo brain tissue, except in one minor respect: we find no obvious equivalent to their streaking spot 24. We have retained our numbering system for correlating the data with those from mammalian rRNA.

The *Xenopus* 18S-rRNA methylation pattern differs from that of mammals in several respects. The following products are absent: one of the two components of product T8; T42 (present in fractional yield in other 18S rRNA species); T45 (also absent

Table 3. *Interrelationships between some T_1-ribonuclease products and the corresponding T_1-plus-pancreatic-ribonuclease products*

For details, see the text. Notes: (*a*) the value given for product TP23 is for the unresolved mixture of Am-A-A-U and A-A-Am-U. On alkaline hydrolysis Am-A-A-U (equivalent to spot T71) accounted for 1 mol in all species, whereas A-A-Am-U had a yield of approx. 1 mol/mol in hamster, mouse and *Xenopus*, 0.6 mol/mol in HeLa cells and was absent from chick. (*b*) Um-C is present in other T_1-ribonuclease products as well. (*c*) Another T_1-ribonuclease product in chick 28S RNA may contain Am-C, but this has not been identified. Product TP56 in *Xenopus* was not satisfactorily characterized, but may contain an additional methylated component to m^1A.

	T_1 ribonuclease	T_1+pancreatic ribonuclease	Sequence	Yield from T_1-plus-pancreatic ribonuclease digestion (mol of methyl groups/mol of oligonucleotide) HeLa	Hamster	Mouse	Chick	*X. laevis*	Suggested
18S	T37	TP50	amΨ-C	0.94	0.87	0.99	0.75	0.90	1
	T68	TP9′	A-(A,Um-A)-Cm-A-U	1.72	1.54	1.67	—	—	~2 (Mammals)
	T68 chick	TP66	A-(A,Um-A)-C	—	—	—	0.95	—	1 (Chick)
	T71	TP23	Am-A-A-U	1.67	1.85	1.96	0.91	1.81	1 (*a*)
	T92	TP23	A-A-Am-U				—		⩽1 (None in chick)
	T94	TP8′ (+TP55)	A-Gm-A-G (Um-C)	0.51	0.47	0.62	0.75	—	~0.5 (None in *Xenopus*) (*b*), Um-C only in *Xenopus*
28S	T14	TP53	Am-C	1.07	0.98	1.01	(1.84)	1.12	1 [2 in chick (*c*)]
		TP56	(m^1A,A)-C	1.13	0.78	0.86	0.86	1.69	(*c*)
	T16	TP4	Gm-G	8.93	9.30	9.53	8.98	7.66	9 (8 in *Xenopus*)
	T21								
	T61								
	T83 *Xenopus*								
	T36	TP9	Gm-A-Am-A-G	1.64	1.64	1.70	1.72	1.66	2
	T83	TP1	Um-Gm-U	4.25	3.77	3.79	4.32	4.19	2 methyl groups each
	T92a		Um-Gm-Ψ						
	T93	TP11	Am-Gm-Cm-A-A-A-U	2.22	2.03	2.09	—	2.48	2–3 (Am-Gm-C in chick)

from chick), T47, T66, T68 (doubly methylated in mammals, but singly methylated in chick), T69, T82, T89 and A-Gm-A-G in T94. At least three differences would not have come to light without partial sequence analysis: product T69 in *Xenopus* contains Um-G instead of Gm-G, and is therefore chemically different from the correspondingly numbered HeLa-cell product. Product T70 in *Xenopus* contains Cm-C as well as A-Gm-C, these two components probably being within separate co-migrating oligonucleotides. Product T82 in *Xenopus* contains Am-U instead of A-Am-U. This product may be related to one of the HeLa products, T66 or T82, but it is clearly not identical with either of them.

28S RNA: hamster and mouse

Unlike 18S RNA, the mammalian 28S RNA species differ from each other in two or three respects. The low-yield product, T2a, was detectable only in HeLa RNA. Perhaps more interestingly, product T56, containing Cm-U, also occurs only in HeLa RNA, but product T55b, which is present in hamster and mouse, contains Cm-U and may possibly be related to product T56. (Hamster and mouse 28S RNA also contained a weakly labelled uncharacterized product, T55a; this may possibly have been due to a secondary split in a longer T_1-ribonuclease product.)

Mouse 28S-RNA 'fingerprints' reveal two further products in fractional yield: a small quantity of T8, which occurs only in 18S RNA in other species, and a relatively long oligonucleotide, T35a, which was not found in any other 28S RNA.

28S RNA: chick and Xenopus

Chick and HeLa 28S RNA differ in a number of respects. Chick 28S RNA lacks products T27 and T56. Spot T35 is also absent, but a neighbouring, extra spot, T33a (barely resolved from T33) contains the partial sequence A-A-Um-G. From the relative mobilities of products T35 and T33a we suggest that the latter is related to the former by an A → C change: (A,C_2)-C-A-A-Um-G.

The doubly methylated product T62 is almost absent from chick 28S rRNA, but is replaced by an apparently related product, which migrates marginally faster in the first dimension and contains the singly methylated partial sequence, A-Gm-U. Product T-93 is a long oligonucleotide, which remains near the origin of the second dimension. In mammals and *Xenopus* it contains the triply methylated sequence, Am-Gm-Cm-A. In chick it contains Am-Gm-C. Again it is likely that this represents the same sequence as in mammals and *Xenopus*, differing in absence of a 2′-*O*-methyl group from the C residue. All the above findings are consistent with data previously obtained from 'fingerprints' of combined T_1-plus-pancreatic-ribonuclease digests of chick 28S RNA (Khan & Maden, 1976*a*). Finally, chick 28S RNA contains the extra products T28a and T40a and an uncharacterized product in fractional yield, T84a.

Xenopus 28S RNA also differs from HeLa 28S RNA in several respects. Products T4, T26, T27, T56, T84 and T89 are absent from *Xenopus*. *Xenopus* contains the extra product, T40, Um-A-U-G. This could possibly be related to either product T26, Um-A-C-G, or T27, Cm-A-U-G, by a C → U change. [Circumstantial evidence suggests that the relationship is to T26. This is based on the high susceptibility of product T26 in HeLa and product T40 in *Xenopus* to digestion of 28S RNA by S_1 nuclease under experimental conditions previously described for 5.8S rRNA (Khan & Maden, 1976*b*; unpublished work of M. S. N. Khan & B. E. H. Maden).]

Product T27a in *Xenopus* possesses the sequence U-Cm-A-G. Despite the topographical relationship of this product to spots T26 and T27 in the HeLa 'fingerprints', there is no obvious sequence relationship to either.

Product T88 is absent in *Xenopus*, but is replaced by an oligonucleotide that co-migrates with product T93, and that contains A-Cm-U. This may therefore be related to product T88 in the other 28S RNA species. Additional *Xenopus* 28S components, not present in HeLa RNA, are product T28a (also present in chick), an extra Um-C which co-migrates with product T54, an extra Cm-U which co-migrates with product T55, and two extra components, A-Gm-C and Gm-G, which co-migrate with product T83.

5.8S RNA

Vertebrate 5.8S RNA contains two methylation sites (see bottom of Table 2). As discussed elsewhere (Nazar *et al.*, 1975; Khan & Maden, 1976*a*), one of the sites is fractionally methylated to an extent that differs between species, and possibly also between tissues of the same species.

Discussion

From the suggested molar yields in Tables 1 and 2 we can calculate the approximate numbers of methyl groups in these vertebrate rRNA species. These numbers are shown at the bottom of the Tables. There are only small differences between the numbers for *Xenopus* and chick rRNA as compared with mammalian rRNA.

It is also possible to estimate the degree of interspecies homology between the methylated sequences on the basis of certain assumptions. Since these estimates are of some interest, their derivation is described in outline.

First we estimated the numbers of nucleotides encompassed by the HeLa oligonucleotides T2 to T86. Products from T87 upwards were omitted from quantitative consideration, as they are somewhat

poorly characterized. The estimates were based on the oligonucleotide lengths (including in each case the preceding G residue, which contributes to determining the specificity of cleavage by T_1 ribonuclease) and their molar frequencies. Where the lengths were not known exactly they were derived approximately from partial sequence data (Salim, 1972), from the locations of the products in 'fingerprints' of ^{32}P-labelled RNA together with the general mobility rules of Brownlee & Sanger (1967), or by correlation with the data of Fuke *et al.* (1976) mentioned above. For multiply methylated products the frequency of the oligonucleotide was scored, not that of the number of methyl groups. (For example the 18S-rRNA product T30, though containing four methyl groups, occurs only once per molecule.) In this way it was calculated that the HeLa 18S-RNA products T2 to T86 encompass approx. 280 nucleotides and the HeLa 28S products T2 to T84 encompass approx. 300 nucleotides (thus these oligonucleotides represent some 14% of the 18S sequence and 6% of the 28S sequence respectively).

Most of the methylated oligonucleotides are common to either the 18S or the 28S RNA of all five species. It therefore seems likely that, in general, differences between the 'fingerprints' are caused by small differences in the respective nucleotide sequences, or in their state of methylation, and not by radical differences. As mentioned in the Results section, there are several probable examples of such small differences. It is likely that product T33a in chick is related by a single base change to product T35 in mammals and *Xenopus*, that product T40 in *Xenopus* is related to product T26 in mammals, and that A-Cm-U in product T93 in *Xenopus* is related to product T88 in mammals. Sequences which apparently differ in their state of methylation between species include: 18S-RNA spot T68, which is doubly methylated in mammals, singly methylated in chick, and possesses no obvious methylated equivalent in *Xenopus*; 28S-RNA spot T62 is doubly methylated in mammals, but the corresponding spot T62a is singly methylated in chick; the triply methylated Am-Gm-Cm-A, within the long oligonucleotide T93 in mammals and *Xenopus*, is replaced by Am-Gm-C (also T93) in chick. By analogy there might be sequences that are singly methylated in one species but are unmethylated, and therefore undetectable, in 'fingerprints' of [^{14}C]-methylated RNA, in another species.

One can place lower limits on the degree of interspecies homology between the methylated sequences by ignoring the above considerations and assuming that each oligonucleotide which is present in the 'fingerprint' of one species, but absent from that of another, represents a block change of all the bases in that part of the nucleotide sequence. However, it is more realistic to make an estimate incorporating the above considerations. Clearly uncertainties are involved, but one can average the effects of possible contributions from single base changes, differences in state of methylation and, perhaps, occasional more extensive sequence differences by assuming that each oligonucleotide that is present in the 'fingerprint' of one species, but absent from that of another, represents on average one, or alternatively two, base changes.

These considerations may be applied to the rRNA of HeLa cells and *Xenopus*, the two most distantly related species studied. Among the 280 nucleotides encompassed by spots T2–T86 of HeLa cell 18S RNA, there are eight oligonucleotides that are absent from, or chemically different in, the *Xenopus* 'fingerprints'. These are: a fractional component of spot T8, oligonucleotide T42 which occurs in fractional yield in HeLa cells, and products T45, T47, T66, T68, T69 and T82. If each of these oligonucleotides were replaced by a totally different sequence in *Xenopus* the degree of homology calculated from the remaining common oligonucleotides would be approx. 75%. If, however, the differences are the result of, on average, one base change per oligonucleotide between the two species, then the degree of homology is:

$$\simeq(280-8)/280 = 0.97, \text{ or } 97\%$$

If each difference results from, on average, two base changes, then the degree of homology is 94%. For 28S RNA the minimum estimate of homology is approximately 85%, if one assumes that, of the HeLa products T2 to T84, those that are absent from *Xenopus* are replaced by totally different sequences. If each HeLa product that is absent from the *Xenopus* 'fingerprint' represents, on average, one base change, the degree of homology is 97.5%; if on average two base changes are involved, the degree of homology is 95%. On the basis of similar considerations the mammalian methylated sequences are more than 99% homologous to each other.

It is interesting that the relatively few interspecies differences are all between sequences with methyl groups on the 2′-hydroxyl position of ribose moieties. These sequences are methylated rapidly during transcription of precursor rRNA (Greenberg & Penman, 1966). We have suggested that the specificity for this type of methylation may be determined by conformational features within precursor rRNA, rather than by primary structure *per se* (Maden & Salim, 1974). The few sequences with methyl groups on the heterocyclic bases seem to be even more highly conserved than those with methyl groups on the ribose moieties, considered as a class. The base-methylated sequences in 18S rRNA are represented by oligonucleotides T30, T34, T37 and T49, and in 28S rRNA by products T14, T28, T33, T63 and T67. The base-methylated sequences in 18S rRNA are methylated

later during ribosome maturation than the ribose-methylated sequences (Maden & Salim, 1974; Brand *et al.*, 1978). A plausible inference would be that these sequences themselves are critically important in determining both the specificity of methylation and functions that are performed by these parts of the rRNA molecules, either during the final stages of ribosome maturation or in protein synthesis.

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References

Brand, R. C., Klootwijk, J., Planta, R. J. & Maden, B. E. H. (1978) *Biochem. J.* **169**, 71—77

Brownlee, G. G. (1972) *Determination of Sequences in RNA*, North-Holland/Elsevier, Amsterdam

Brownlee, G. G. & Sanger, F. (1967) *J. Mol. Biol.* **23**, 337–353

Fuke, M. & Busch, H. (1977) *FEBS Lett.* **77**, 287–290

Fuke, M., Busch, H. & Rao, P. N. (1976) *Nucleic Acids Res.* **3**, 2939–2957

Greenberg, M. & Penman, S. (1966) *J. Mol. Biol.* **21**, 527–535

Griswold, M. D., Brown, R. D. & Tocchini-Valentini, G. P. (1974) *Biochem. Biophys. Res. Commun.* **58**, 1093–1103

Hadjiolov, A. A. & Nikolaev, N. (1976) *Prog. Biophys. Mol. Biol.* **31**, 95–144

Hashimoto, S., Sakai, M. & Muramatsu, M. (1975) *Biochemistry* **14**, 1956–1964

Judes, C. & Jacob, M. (1972) *FEBS Lett.* **27**, 289–292

Khan, M. S. N. & Maden, B. E. H. (1976*a*) *J. Mol. Biol.* **101**, 235–254

Khan, M. S. M. & Maden, B. E. H. (1976*b*) *FEBS Lett.* **72**, 105–110

Maden, B. E. H. (1976) *Trends Biochem. Sci.* **1**, 196–199

Maden, B. E. H. & Khan, M. S N. (1977) *Biochem. J.* **167**, 211–221

Maden, B. E. H. & Robertson, J. S. (1974) *J. Mol. Biol.* **87**, 227–235

Maden, B. E. H. & Salim, M. (1974) *J. Mol. Biol.* **88**, 133–164

Maden, B. E. H., Forbes, J., de Jonge, P. & Klootwijk, J. (1975) *FEBS Lett.* **59**, 60–63

Nazar, R. N., Sitz, T. O. & Busch, H. (1975) *FEBS Lett.* **59**, 83–87

Salim, M. (1972) Ph.D. Thesis, University of Glasgow

Saponara, A. G. & Enger, M. D. (1974) *Biochim. Biophys. Acta* **349**, 61–77

Vaughan, M. H., Soeiro, R., Warner, J. R. & Darnell, J. E. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1527–1534