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Partial mapping of methylated sequences in Xenopus laevis ribosomal RNA by preparative hybridization to cloned fragments of ribosomal DNA

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ABSTRACT

Xenopus laevis rRNA was hybridised to either of two cloned fragments of ribosomal DNA. One fragment, designated Xlrl1, contains a short region of the 18 S rRNA gene and most of the 28 S rRNA gene. The other fragment, Xlrl4, contains a short region of the 28 S gene and most of the 18 S gene. After hybridization the non-complementary rRNA was removed by digestion with T_1 RNase and the hybridized RNA was then eluted and examined by fingerprinting analysis. The 3' terminal sequence and the dimethyl-A-containing sequence of 18 S rRNA both hybridized to Xlrl1 rDNA, in agreement with the known direction of transcription of rDNA. The distribution of other methylated oligonucleotides between the various fingerprints permitted assignment of nearly all of the methylated sequences in 18 S and 28 S rRNA to either the short 3' region or the long 5' region of the respective molecules.

INTRODUCTION

Eukaryotic rRNA possesses numerous methyl groups[1-4]. Information is available on the nucleotide sequences immediately surrounding the methyl groups[1,5] and on the timing of methylation during ribosome maturation[1,6]. However, the locations of the methyl groups along the primary structures of 18 S and 28 S rRNA are unknown, except that a sequence with two adjacent dimethyl-A (m^6_2A) residues occurs close to the 3' end of 18 S rRNA[7-9]. Difficulties in the sequence analysis of these large RNA molecules have hindered direct approaches to this problem. Here we report on an indirect approach involving preparative hybridization of rRNA to cloned fragments of rDNA.

rDNA in Xenopus laevis occurs in a repeating array of the unit structure shown in Figure 1. Each unit contains two cleavage sites for the restriction endonuclease, EcoR1[10]. These sites have been utilized to clone the two fragments of rDNA designated Xlrl1 and Xlrl4 in the Figure[11-13]. In the present experiments 18 S or 28 S rRNA was

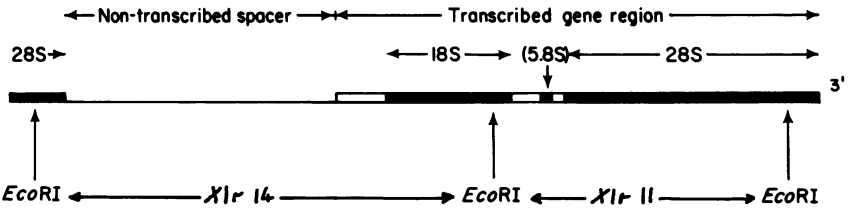


Figure 1. A unit of the repeating structure of rDNA in Xenopus laevis, showing the EcoRl cleavage sites and the Xlrll and Xlrl4 fragments.

hybridized to Xlrll or Xlrl4 rDNA. The non-complementary regions of rRNA were removed by digestion with T_1 RNase. The hybridized RNA was then eluted and examined by fingerprinting analysis. We shall refer to the rRNA regions that hybridize to Xlrll or Xlrl4 rDNA as the Xlrll and Xlrl4 regions of rRNA respectively. From the distribution of methylated oligonucleotides between the various fingerprints it was possible to assign nearly all of the methylated sequences in 18 S or 28 S rRNA to either the Xlrll or the Xlrl4 regions of the molecules.

Several lines of evidence indicate that rDNA in X. laevis is transcribed in the direction, 5'→18 S→28 S→3', that is, from left to right in Figure 1[14-17]. Additional evidence that this is the direction of transcription derives from results to be described in this paper. Thus, methylated sequences in the Xlrll region of 18 S rRNA are located towards the 3' end of the molecule, whereas in 28 S rRNA methylated sequences in the Xlrl4 region are located towards the 3' end.

METHODS

Recombinant plasmids

The two rDNA fragments were cloned in the vehicle ColEl. The recombinant plasmid pXlrl4, containing the Xlrl4 insert, is one of a series of plasmids differing in the lengths of the non-transcribed spacer region of their inserts. The construction and restriction maps of these plasmids have been described[12,13]. pXlrll, containing the Xlrll insert, was derived during the same series of cloning experiments as pXlrl4 (unpublished work of R.H. Reeder and I.B. Dawid). The Xlrll insert is homologous to that of plasmid pX1212 of Boseley et al.[17]. Plasmid DNA was prepared as described[12,13]. Work with recombinant DNA followed the 1976 N.I.H. Guidelines.

rRNA

Radioactively labelled rRNA was prepared from X. laevis cultured kidney cells after growth in the presence of $^{32}PO_4$ or ^{14}C methyl-methionine. The procedures were as in Ref. 18, except that for labelling with $^{32}PO_4$ approximately 10^7 cells were labelled for 48-72 hr in the presence of 10mCi $^{32}PO_4$ in Eagle's medium containing one tenth of the normal phosphate concentration. Specific activities of 10^6 c.p.m. per microgram were thereby achieved.

Hybridization

Closed-circular plasmid DNA was opened with EcoR1 and the enzyme was then removed by phenol extraction. The DNA was precipitated with ethanol, denatured in 0.1N NaOH, and aliquots of 25µg were neutralized in 4 x SET (SET = NaCl 0.15M, EDTA 0.005M, Tris HCl 0.05M, pH 8). Immediately after neutralization each aliquot was filtered slowly onto a 2.5cm millipore disc[19]. Filters were dried in air and then for at least 2 hr at 80°. DNA-RNA hybridization was carried out at 40-50° overnight in 0.5 - 1ml of 4 x SET with 50% formamide with approximately equimolar quantities of rRNA and rDNA. After hybridization the filters were washed with several changes of 2 x SET at 25° and were then treated with T_1 RNase, 50-100 units/ml for 20min at 30 - 37° with stirring. (Pancreatic RNase was not included because of the possibility of introducing nicks at pyrimidines in the hybridized RNA regions, and thereby complicating the analysis of subsequent T_1 RNase fingerprints.) The filters were washed in several further changes of 2 x SET and rinsed briefly in 0.1 x SET. To release the hybridized RNA the filters were boiled for 15 min in 3ml of 0.1 x SET. More than 90% of the RNase-resistant radioactivity was released. The eluted RNA was adjusted to 0.15M NaCl and 0.01M $MgCl_2$. 20µg of carrier RNA and 10µg of DNase (Worthington, electrophoretically pure) were added. The solution was incubated for 10 min at 25° to degrade any eluted DNA (which otherwise caused streaking in the first dimension of the fingerprints). Phenol extraction was carried out, RNA was precipitated from the aqueous phase with ethanol, redissolved in 0.1-0.2ml of water and lyophilized for fingerprinting.

rRNA fingerprinting and assignment of methylated sequences to Xlrl1 or Xlrl4 regions

The yields of hybridized rRNA were adequate but not ample for the present analysis, and were usually lower in experiments with Xlrl4 than

with Xlrll rDNA. With ^{32}P-labelled rRNA a single filter yielded sufficient material for fingerprinting with an autoradiographic exposure of 2-3 days. With ^{14}C methyl-labelled rRNA six to eight filters yielded material for one fingerprint with an exposure of 8-12 weeks. A possible reason for low and rather variable yields in experiments with Xlrl4 rDNA is that the "simple sequence" regions in the non-transcribed spacer region of this rDNA[12,13] may have interfered with hybridization by causing renaturation of some DNA molecules before immobilization on the filter.

Fingerprinting was carried out after digestion of RNA with T_1 RNase [1,4] or with combined T_1 plus pancreatic RNase[18,20]. Methylated sequences were assigned to the Xlrll or Xlrl4 regions of rRNA on the basis of the following lines of evidence. 1). T_1 RNase fingerprints were obtained of methyl-labelled material from the Xlrll regions of 18 S and 28 S rRNA, and are shown below (Figures 3b and 4b, results section). ii). Faint but well resolved T_1 RNase fingerprints were obtained of methyl-labelled material from the Xlrl4 regions of 18 S and 28 S rRNA. iii). Combined T_1 plus pancreatic RNase fingerprints were obtained of the Xlrll and Xlrl4 regions of 18 S and 28 S rRNA, using ^{32}P-labelled rRNA and ^{14}C methyl-labelled rRNA in separate experiments. Products were identified by their mobilities in the fingerprinting systems[4,18] and, where necessary, by hydrolysis with alkali or T_2 RNase; alkali-stable or other characteristic products were identified by their electrophoretic mobilities on Whatman 52 or 3MM paper at pH 3.5. Use of the two fingerprinting systems permitted cross-checking of assignments[20]. Also, certain methylated products that are not well resolved in T_1 RNase fingerprints are represented in combined T_1 plus pancreatic RNase fingerprints by shorter, well resolved products whose relationship to the T_1 products is known. (The relationship between the two fingerprinting systems for X.laevis rRNA is similar in most respects to that described for HeLa cell rRNA[20]; unpublished observations of B.E.H.M.).

A full catalogue of more than one hundred methylated sequences in X. laevis rRNA is given in Ref. 4. Here, for simplicity, we list only these methylated sequences that hybridize to the short, Xlrll region of 18 s rRNA and the short, Xlrl4 region of 28 S rRNA. The many other methylated sequences listed in Ref. 4 were identified in the long regions of 18 S or 28 S rRNA by at least one of the criteria listed above.

RESULTS

^{32}P-labelled 18 S rRNA

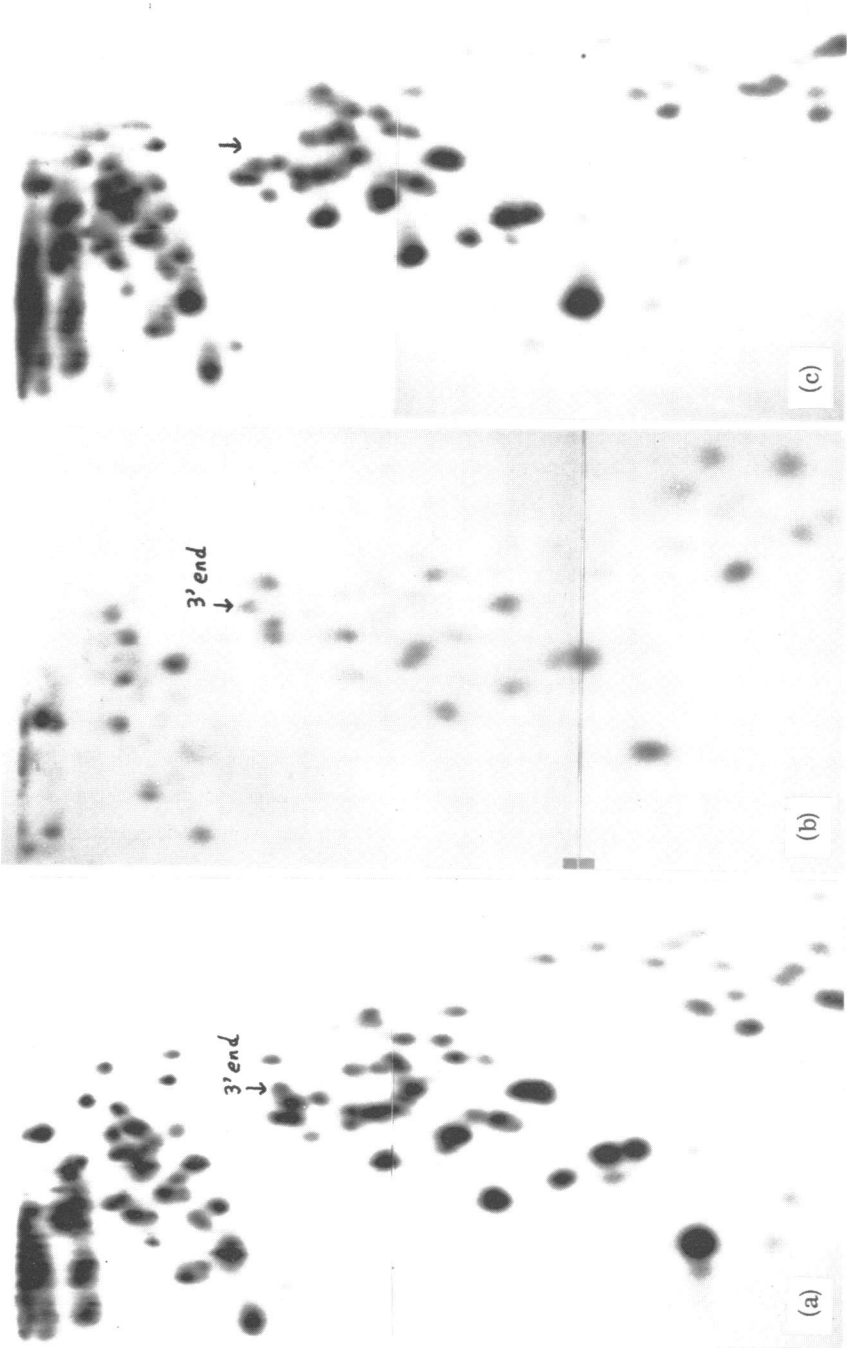
Figure 2 shows fingerprints of ^{32}P-labelled 18 S rRNA and of the Xlrll and Xlrl4 regions of 18 S rRNA. The Xlrll fingerprint (Figure 2b) is much simpler than that of whole 18 S rRNA (Figure 2a), especially in the upper region to which most large, unique oligonucleotides migrate. This indicates that a specific part of 18 S rRNA has been purified by hybridization.

The 3' terminal T_1 oligonucleotide of X. laevis 18 S rRNA is A-U-C-A-U-U-A_{OH}[21], as in many other eukaryotes[8]. This product is resolved from others in the 18 S fingerprint[21]; its position is shown in Figure 2a. The product is present in the Xlrll fingerprint (Figure 2b), in which its identity was confirmed by base composition analysis. It is absent from the Xlrl4 fingerprint (Figure 2c). Specific hybridization of the 3' end of 18 S rRNA to Xlrll rDNA is consistent with the extensive evidence, already cited[14-17], that the direction of transcription of rDNA in X. laevis is 5'→18 S→28 S. It will be shown below that the m_2^6A-containing sequence of 18 S rRNA, which is located near the 3' end[7-9], also hybridizes to Xlrll rDNA.

The Xlrl4 fingerprint differs from that of whole 18 S rRNA in the absence of the 3' terminal oligonucleotide, already mentioned, and the absence of another unique product which migrates just to the right of the 3' spot in Figure 2a, and in many quantitative respects. The fact that most of the differences between the two fingerprints are quantitative rather than qualitative (using the present separation conditions) is due to the sequence complexity of the ^{32}P 18 S fingerprint; many "spots" consist of high yield products or mixtures of products, components of which appear in either the Xlrll or the Xlrl4 fingerprint. (Several specific examples of this were noted during partial quantitative analysis of these fingerprints: unpublished observations of B.E.H.M.).

18 S rRNA:methylated sequences

Figure 3a shows a T_1 RNase fingerprint of methyl-labelled 18 S rRNA from X. laevis. The molecule contains some 40 methyl groups distributed between 34 chemically different T_1 RNase digestion products[4]. Figure 3b shows a T_1 RNase fingerprint of the part of methyl-labelled 18 S rRNA that hybridizes to Xlrll rDNA. Six methylated oligonucleotides are present. Data on these methylated sequences were obtained from several experiments and are summarized in Table 1.


3' end
3' end
(a)
(b)
(c)

The heavily labelled product, T30, is the m_2^6A-containing sequence, with four methyl groups. This sequence is known to be located some 20 nucleotides from the 3' end of 18 S rRNA in several eukaryotes[7-9]. Its presence in the Xlrll fingerprint is consistent with the other evidence, already noted, on the direction of transcription of the ribosomal genes.

The methylation pattern of the Xlrl4 region was examined by the various techniques outlined in the methods section. All of the 18 S methylated sequences[4] that are not listed in Table 1 were positively identified in fingerprints of the Xlrl4 region, using either the T_1 or the combined T_1 plus pancreatic RNase fingerprinting systems, except that two of the longest T_1 products (T87, T94) were not individually identified because of poor resolution at the origin of the second dimension of the fingerprints. Of the sequences that hybridized to Xlrll rDNA, four were absent from Xlrl4 fingerprints, or present only in trace amounts (products T8, T30, T34 and T48). Absence of product T69 was not satisfactorily established because this product would comigrate, if present, with product T67.

Product T49, containing m^7G, hybridized efficiently to Xlrl4 as well as to Xlrll rDNA (Table 1). The reason for this is unknown; one possible explanation is that the sequence, ...m^7G-A-A-U..., might be encoded at the EcoRl site of the 18 S gene. (The EcoRl sequence reads G-A-A-U-U-C when transcribed into RNA). In this event the unmodified G at one or other end of the oligonucleotide would be protected from trimming by T_1 RNase after hybridization to Xlrl4 or Xlrll rDNA respectively. Further RNA and DNA sequence data should enable this possibility to be examined.

Figure 2 (opposite). T_1 ribonuclease fingerprints of ^{32}P-labelled 18 S rRNA from Xenopus laevis: (a) whole 18 S rRNA, (b) Xlrll region, (c) Xlrl4 region. Electrophoresis is from right to left in the first dimension (cellulose acetate, pH 3.5) and downwards in the second dimension (DEAE paper, 7% formic acid). Separation conditions were chosen to maximize resolution in the "one-uridylate" graticule. Spots that migrate rapidly in the second dimension (G, AG, etc.,) have migrated off the end; UG is the prominent spot towards the lower left corner of the fingerprints. The 3' terminal oligonucleotide, A-U-C-A-U-U-A_{OH}, is indicated and is absent in (c) (arrow). (The mobility of this spot is anomalous with respect to uridine content because of the absence of G and the free hydroxyl end, both of which cause enhanced mobility in the second dimension.)

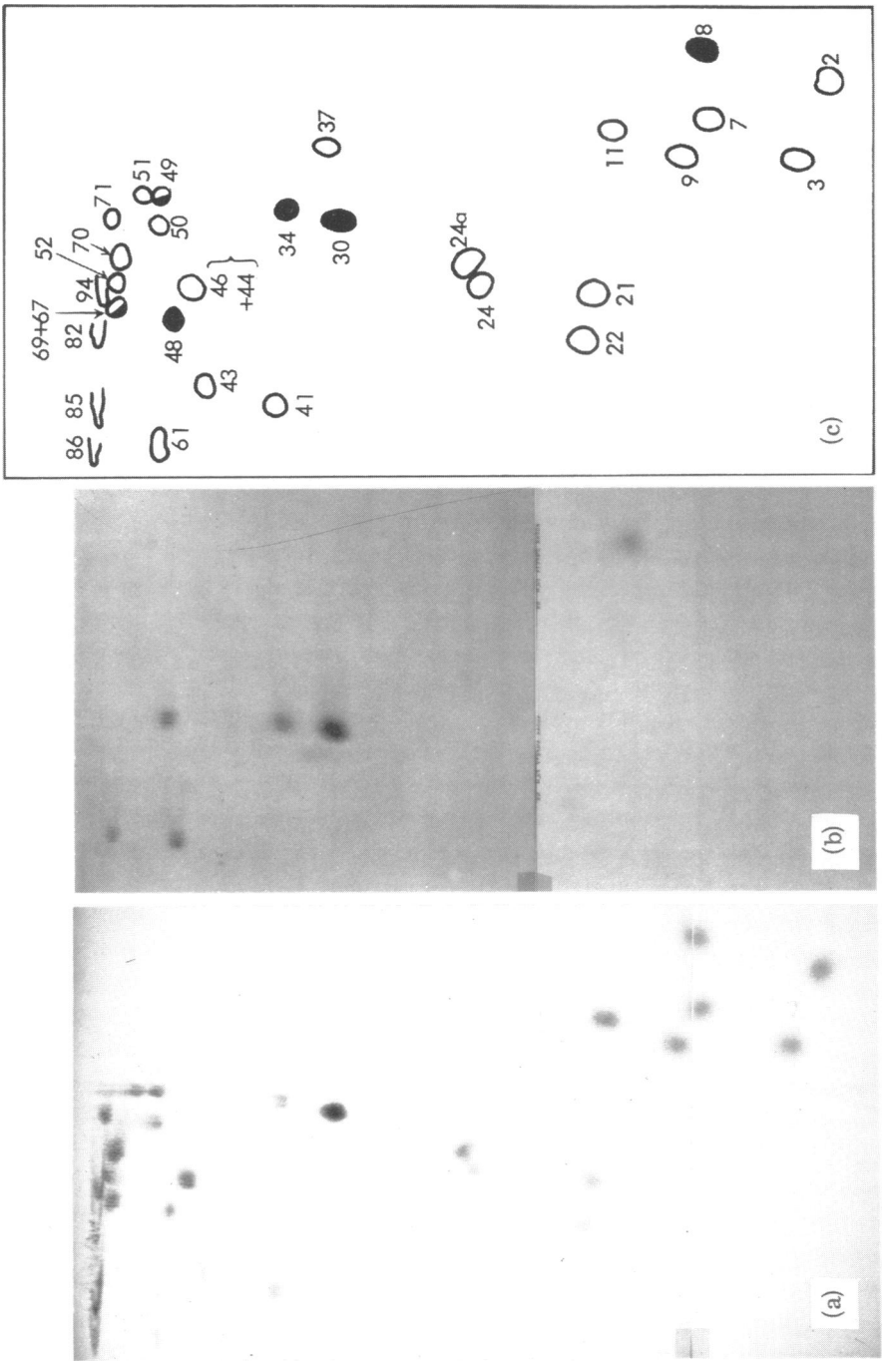

(a)
(b)
(c)
69+67
52
82
94
70
71
86
85
51
49
50
48
46
+44
43
34
37
30
41
61
24a
24
22
21
11
9
8
7
3
2

Table 1
Methylated sequences in 18 S rRNA that hybridize to Xlrll rDNA

Product	Sequence	Relative molar yield (methyl groups)(a). Xlrll	Xlrl4	18 S	Notes
T8	Cm-C-C-G	1.22	-	1.10 (0.96)	(b)
T30	m_2^6A-m_2^6A-C-C-U-G	3.91	-	3.95 (3.78)	(c)
T34	...(m^6A,A)C...	1.08	-	1.06 (0.86)	
T48	A-U-U-Am-A-G	0.96	-	0.78 (0.98)	(b)
T49	...m^7G-A-A-U...	0.77	1.06	0.89 (0.84)	(d)
T69	...Um-G	0.83	?	<1 (<1)	(b)(e)

Notes:- (a) Xlrll molar yields are the means of theee determinations, two from T_1 fingerprints and one from a combined T_1 plus pancreatic fingerprint in which the corresponding products[20] were present. Molar yields for whole 18 S RNA are the means of two determinations in this work, followed by published values[4] in parentheses. (b) These products were eluted from a ^{32}P fingerprint of Xlrll rRNA and were digested with T_2 RNase. The expected alkali-stable dinuclotides were released. (c) The molar yield of T30 is expressed as numbers of methyl groups; there is one mole of this oligonucleotide per mole of 18 S rRNA. (d) The molar yield of product T49 in Xlrl4 rRNA is the mean from a T_1 fingerprint of methyl labelled RNA, a T_1 plus pancreatic fingerprint of methyl labelled RNA (spot TP65, m^7G-A-A-U) and a T_1 plus pancreatic fingerprint of ^{32}P-labelled RNA. (e) Molar yield of T69 in whole 18 S is not accurately known due to co-migration with another spot (T67).

28 S rRNA: methylated sequences

Figure 4a shows a T_1 fingerprint of methyl-labelled 28 S rRNA. The major 28 S sequence (exclusive of 5.8 S RNA) contains approximately 68 methyl groups in some 45 chemically different T_1 products[4]. Figure 4b shows a T_1 fingerprint of the part of methyl labelled 28 S RNA that hybridizes to Xlrll rDNA. A faint T_1 fingerprint of material that hybridized to Xlrl4 rDNA was obtained, and combined T_1 plus pancreatic

Figure 3 (opposite). T_1 RNase fingerprints of ^{14}C methyl-labelled 18 S rRNA: (a) whole molecule, (b) Xlrll region, (c) Key. Numbering system according to Ref. 4. In the Key filled spots denote oligonucleotides that hybridized to Xlrll rDNA, open spots denote those that hybridized to Xlrl4 rDNA. Spot 49 (half filled) hybridized to both rDNA fragments. Products 67 and 69 are unresolved from each other. 67 hybridized to Xlrl4, 69 to Xlrll. Spot 94 comigrates with 87 and 92; all of this material was inferred to hybridize to Xlrl4 rDNA. Spots 2-11 migrated somewhat more slowly in the second dimension than in ref. 4, relative to other spots. This appears to have been due to use of a batch of DEAE paper with somewhat different ion exchange properties to that used previously.

(a)
(b)
(c)
2
3
6
7
9
10
11
12
13
14
15
16
18
21
22
23
24a
25
27
28
28a
29
31
32
33
35
36
40
54
55
+53
57
62
65
63
64
66+67
67a
70
81
83
90
92a
93

RNAse fingerprints were obtained of both regions. The Xlrll region contains most of the 28 S sequence (Figure 1) and, as can be seen from Figure 4b, most of the methyl groups. However, a number of methylated products are absent from the Xlrll fingerprint (see Figure 4c, Key), and some others are present in lower yield than in whole 28 S RNA. These products were identified qualitatively in the various fingerprints of the Xlrl4 region and are summarized in Table 2. In addition to the numbered products in the Table, three others are probably present in the Xlrl4 region but were not individually identified. These were in the following regions of the T_1 fingerprint:- one component from spots 53-54, one component from spot 67 or 67a and one component (not Um-Gm-U, see below) from spot 83.

Product T92a, containing Um-Gm-ψ, is of particular interest as it occurs in all eukaryotic 28 S RNAs that have been examined (Refs. 18 and 22 and references therein). Since the product remains at the origin of the second dimension in a T_1 fingerprint it was not identifiable with confidence using this fingerprinting system alone. It was assigned to the Xlrl4 region on the basis of the following evidence. The combined T_1 plus pancreatic RNase fingerprint of vertebrate 28 S RNA yields a spot, TP1, that contains one mole each of Um-Gm-U and Um-Gm-ψ per mole of RNA[18,20]. Combined T_1 plus pancreatic RNase fingerprints of the Xlrll and Xlrl4 regions each yielded approximately one mole of this spot, indicating that one component is present in each region of the molecule. The two components are distinguishable by successive treatment of the spot with alkaline phosphatase and snake venom phosphodiesterase, followed by chromatographic analysis for pU or pψ (see Figure 2 of Ref. 18). The ^{32}P-labelled Xlrll component was found by this procedure to be Um-Gm-U only, with none of the ψ-containing component. It was therefore inferred that the Xlrl4 component is Um-Gm-ψ, although the amount of Xlrl4 material was

Figure 4 (opposite). T_1 RNase fingerprints of ^{14}C methyl-labelled 28 S rRNA: (a) whole molecule, (b) Xlrll region, (c) Key. Numbering system according to Ref. 4. In the Key, filled spots denote products that hybridized to Xlrl4 rDNA, open spots denote those that hybridized to Xlrll rDNA. Half filled spots contain more than one T_1 product, one of which hybridized to Xlrl4 rDNA. Identification is tentative for spot 54. No hybridization was detected of the weakly labelled products T18 or T25. The conventions for filled circles in Figures 3 and 4 are such that products in the short 3' regions of the molecules are marked black in each case.

Table 2
Methylated sequences in 28 S rRNA that hybridize to Xlrl4 rDNA

Product	Sequence	Xlrl4	Molar yield (methyl groups)(a) Xlrll	28 S	Notes
T3	Am-G	+	3.00	4.95 (4.29)	
T10	A-Cm-C-G	+	-	1.11 (0.92)	(b)
T21	Gm-G	+	3.98	5.77 (5.11)	
T28a	...Cm-U...	+	-	0.93 (0.99)	
T61	U-U-Gm-G	+	-	0.54 (0.78)	
T63	m^3U-U-Ψ-A-G	+	-	1.04 (1.05)	(b)
T81	U-A-U-Gm-U-G	+	-	0.58 (0.78)	
T92a	...Um-Gm-Ψ	+	-	(2)	(b)(c)
Plus ∿3 unidentified products					

Notes:- (a) Xlrl4 sequences were scored qualitatively. Molar yields for whole 28 S RNA are based on two determinations in this work, and are followed by published values[4] in parentheses. (b) These methylated sequences give rise to unique T_1 plus pancreatic RNase products, which were identified in T_1 plus pancreatic fingerprints of Xlrl4 28 S RNA. (c) Molar yield expressed as numbers of methyl groups; there is one mole of this oligonucleotide per mole of 28 S RNA (see text for identification of this product).

insufficient for final confirmation of pψ by chromatography.

DISCUSSION

The experiments described in this paper provide partial data on the distribution of methylation sites along the 18 S and 28 S rRNA of *X. laevis*. In particular they enable an approximate estimate to be made of the frequency of methylation sites in the short 3' regions as compared with the long 5' regions of the molecules. The EcoRl site in the 18 S gene was estimated by electron microscopy to be located some 20% of the gene length from one end (the 3' end) of the 18 S gene[10]. Further restriction mapping data suggest a distance of 15% from the 3' end (unpublished data of R.H. Reeder). We shall assume a distance of 15-20% of the gene length for this calculation. The 3' region of 18 S rRNA, defined by the present experiments, contains eight or nine of the forty methyl groups in the molecule, or five to six of the methylation sites if the m^6_2A-containing sequence is counted as a single site. (The

uncertainty between five or six sites is due to the uncertain location of the m^7G site, discussed under Results). Thus the 3' terminal 15-20% of 18 S rRNA contains 20-23% of the methyl groups, or 14-17% of the separate methylation sites. A comparable calculation may be made for 28 S rRNA. The EcoR1 site in the 28 S gene was estimated to be 10% of the distance from the (3') end of the gene[10]. The corresponding 3' part of 28 S rRNA contains some 12 of the 68 methyl groups in the molecule, or ~18% of the total methyl groups. Although these estimates involve some uncertainties, they suffice to indicate that there is no gross clustering of methylation sites in the short 3' regions of the molecules; the majority of methyl groups evidently occur in the long 5' regions. The present findings for 18 S rRNA are in marked contrast to those for E. coli 16 S rRNA, in which seven out of a total of eleven methyl groups are clustered within the 3' 15% of the molecule (four of these being in the m_2^6A-containing sequence)[23].

It should be of considerable interest to extend these mapping experiments so as to provide details on the distribution of methylation sites within the long 5' regions of 18 S and 28 S rRNA. Detailed restriction maps that are now available of the Xlrl1 rDNA fragment[17] and the transcribed region of the Xlrl4 fragment (unpublished data of R.H. Reeder and B. Sollner-Webb) should facilitate such further analysis.

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