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Nucleotide sequence encoding the 5' end of Xenopus laevis 18S rRNA

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ABSTRACT

We have sequenced a region of cloned Xenopus laevis ribosomal DNA encompassing the last 24 nucleotides of the external transcribed spacer and the first 275 nucleotides of the 18S gene. The start of the 18S gene was identified by correlating the results obtained from RNA hybridization and fingerprinting with the DNA sequence. This 5' region of 18S rRNA contains five 2'-O-methyl groups and at least six pseudouridine residues. Several of these modified nucleotides are clustered into a relatively short region from nucleotides 99-124. Nucleotides 227-250 constitute a distinctive sequence of 24 consecutive G and C residues. Comparison with the first 160 nucleotides of a yeast 18S gene (25) reveals three blocks of high sequence homology separated by two short tracts where homology is low or absent. The external transcribed spacer sequences diverge widely from within a few nucleotides of the start of the 18S gene.

INTRODUCTION

Identification of the start of the 18S gene in ribosomal DNA (rDNA) is a necessary step in studying the structure and biosynthesis of vertebrate ribosomes. Boseley and co-workers (1,2) carried out extensive sequence analysis on the non-transcribed spacer and most of the external transcribed spacer of a cloned unit of X. laevis rDNA. They also sequenced a short, non-contiguous part of the 18S coding region, but the actual start of the 18S gene was not identified. In the present work we have combined the approaches of DNA sequence analysis and RNA hybridization and fingerprinting to identify the start of the 18S coding region and to characterize the 5' region of 18S rRNA.

METHODS

Which clone to sequence?

X. laevis rDNA consists of several hundred tandemly linked repeating elements, each element consisting of a transcription unit and a non-

transcribed spacer.

It may become useful to obtain extensive sequence information from a single transcription unit, rather than to reconstruct a composite sequence from different transcription units. For this purpose it is necessary to use a cloned rDNA sequence that is bounded by restriction sites located outside the transcription unit. Hind III cuts once per rDNA repeat at the site of termination of transcription (3). Thus in Hind III clones each transcription unit remains just intact. R. Reeder (personal communication) has constructed several such clones using the vector pMB9. One of these clones, pXlrl01, is in use in several laboratories (see, for example, ref.4). Therefore in this laboratory we have decided to approach sequencing objectives using pXlrl01. To facilitate this work we have subcloned various regions of the parent clone. The subclone relevant to the present analysis is called pXlrl01A. It contains the rDNA region from the last Bam HI site in the non-transcribed spacer to the Eco RI site in the 18S gene, ligated between the Bam HI and Eco RI sites of pBR322 (figure 1a). The general organization of this region of rDNA is shown in figure 3 of ref. 1. Further details relevant to sequencing are shown in figure 2, below.

DNA sequence analysis

This was carried out by the method of Maxam and Gilbert (5) with

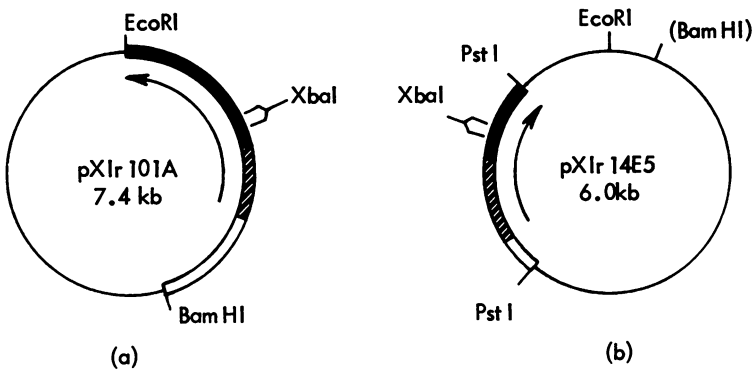


Figure 1. Diagrams of plasmids pXlrl01A and pXlrl4E5. The vector (single line) is pRR322(b) or the large Bam HI/Eco RI fragment of pBR322(a). The double line is the rDNA insert:- open region, part of non-transcribed spacer; shaded region, external transcribed spacer; black region, part of 18S coding region. The arrow indicates the 5' to 3' direction of the "s" strand of rDNA, i.e. the strand whose sequence in the coding region is synonymous to RNA. Only those restriction sites that are relevant to construction and primary digestions of the plasmids are shown. Further restriction sites are shown in figure 2.

recent modifications (6) using thin gels for electrophoresis (7). Sequencing gels were readable for 120-180 nucleotides.

RNA hybridization and fingerprinting

A second rDNA subclone, pXlrl4E5 (figure 1b) was used for most of the RNA hybridization experiments. The parent clone, pXlrl4, is described in refs. 8 and 3. The subclone is one of a series that was constructed to facilitate preparative hybridization of rRNA to defined regions of rDNA (B.E.H.M., unpublished work). It contains a ∿1600 bp fragment extending from the last Pst I site in the non-transcribed spacer to the Pst I site in the 18S gene (ref. 1, figure 3), ligated into the Pst site of pBR322 (figure 1b).

General procedures for preparative RNA hybridization, which were previously described (9), have been slightly modified in this work. The modified procedure yielded RNA regions of high purity and in adequate yield for analytical purposes. The procedure was as follows. A purified restriction fragment from 20-50 µg of subclone was filtered onto a single nitrocellulose filter (10). (For experiments with ^{14}C methyl labelled rRNA, to be reported elsewhere, restriction fragments from up to 100 µg of subclone were filtered and were adequately retained on single filters, as judged by recovery of hybridized RNA). Each filter was then processed throughout in a separate scintillation vial. Hybridization was for 6-12 hr at 65° using a slight molar excess of ^{32}P labelled 18S rRNA (10^6 c.p.m. per microgram) to DNA, in approximately 0.5 ml of 5 x SET without formamide (1 X SET = 0.15 M NaCl, 1 mM EDTA, 0.05 M Tris HCl, pH 8). Unbound RNA was then removed and the filter was washed by vortexing with several changes of 2 x SET. "Trimming" of hybridized RNA was carried out using 6 µg T1 ribonuclease (approx. 25 units) in 0.5 ml 2 x SET. Vials were vortexed to ensure complete mixing, and were incubated at 37° for 15 min. T1 ribonuclease was then removed and the filters were washed with four further changes of 2 x SET. After a brief rinse in 0.1 x SET the hybridized RNA was eluted in 2 ml of 0.1 x SET by placing the vials in a boiling waterbath for 15 min. The eluate was removed, the filter and vial were rinsed with 1.5 ml 0.1 x SET and the rinse was added to the eluate. Usually 80-90% of the T1 resistant hybrid was eluted, as checked by monitoring filters for radioactivity before and after elution. To the eluate the following additions were made:- NaCl to 0.3 M, $MgCl_2$ to 0.01 M, 20-40 µg of carrier RNA (purified from HeLa cells) and 10 µg of DNase (worthington, electrophoretically pure). After 15 min at 20° the eluate was

extracted with phenol and the aqueous phase precipitated with ethanol. The DNase step was to degrade any eluted DNA, which otherwise interfered with fingerprinting. To maximize the recovery of RNA, ethanol precipitates were stored at least overnight at -20° and then for 20 min in dry ice and and ethanol before centrifugation. RNA fingerprinting and subsequent operations were carried out as described (11-13). Data in the results section (figure 4, table 1) show that RNA prepared in this way is bounded by the T1 cleavage site(s) next to the corresponding restriction site(s) on rDNA.

RESULTS

Before this work was commenced it had been estimated that 18S rRNA is roughly 2000 nucleotides long (14,15), that a unique Eco RI site in the 18S gene is some 300 nucleotides from the 3' end (16) and that the first of two Xba I sites (the promotor proximal or "left" Xba site) is about 1500 nucleotides from the Eco RI site (ref. 1 and P. Boseley, personal communication). This suggested that the 18S gene starts a short distance in the 5' direction from the left Xba site (see figure 2). We shall show that this is true by correlating the results of DNA sequencing and RNA fingerprinting.

DNA sequencing

Figure 2 shows the rDNA region that was sequenced, together with the sequencing strategy (upper part of figure). The part of the sequence discussed in this paper extends from just to the left of the Sma I/Ava I site to the second Ava I site 285 base pairs to the right. Figure 3a shows part of a sequencing gel reading leftwards through the start of the 18S gene (identified below) into the external transcribed spacer. At the points indicated a single C residue was read on one strand whereas two G residues were read on the other strand. This implied the presence of an Eco RII site with a methylated C residue on each strand (17). This was confirmed by cleavage with Bst NI, which cuts at methylated Eco RII sites. Figure 3b shows a sequencing gel reading rightwards from the left Xba I site. A secondary structure effect, discussed later, interfered with reading at the point indicated, but the sequence was clearly read at this point from the other strand. Figure 4 shows the determined DNA sequence with the inferred RNA sequence above.

Fingerprint of 5' end of 18S rRNA

Figure 5a shows a T1 ribonuclease fingerprint of the region of 18S

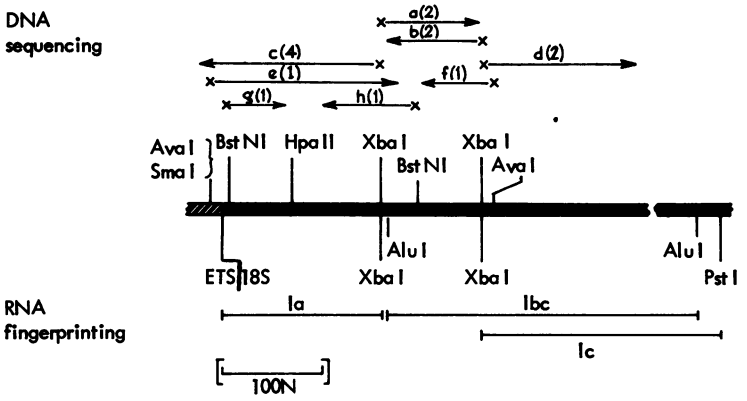


Figure 2. Region of X. laevis rDNA from the 3' end of the external transcribed spacer (ETS, shaded) to the Pst I site in the 18S gene. The Pst site is approximately 600 nucleotides from the start of the 18S gene. The upper arrows indicate the sequencing strategy. Stars indicate kinase-labelled ends, and the numbers in parenthesis indicate the number of sequencing runs on each fragment. Fragments a-d were from an Eco RI plus Xba I digest of pXlrl01A (see figure 1a). Fragments a and b were sequenced after strand separation, fragments c and d after secondary restriction. Fragment e was from an Sma I digest. (The indicated site together with another Sma I site beyond the Pst site gave rise to a 750 bp fragment, which was labelled and then subjected to secondary restriction with Ava I). Fragment f was from an Ava I digest, using Bst NI for secondary restriction. Fragments g and h were from the fragment bounded by the indicated Bst NI sites, using Hpa II for secondary restriction. The lower lines indicate the regions of 18S rRNA that were hybridized to restriction fragments. Regions Ia and Ic were purified after hybridization to the respective DNA fragments obtained when pXlrl4E5 (figure 1b) was digested with Eco RI plus Xba I. Region Ibc was purified by hybridization to the indicated Alu I fragment.

rRNA that hybridizes to rDNA to the left of the Xba sites (region Ia in the lower part of figure 2). This fingerprint was reproducibly obtained whether pXlr 14E5 or pXlrl01A was used for hybridization. Data on the oligonucleotides are summarized in table 1. The 5' terminal oligonucleotide is readily identified as the only product yielding pUp on alkaline hydrolysis:- spot 21. Additional data on this product gave the partial sequence, pU(AC,C,U)Gp. This is consistent with the sequence reported for the 5' end of mouse L cell 18SrRNA, pUACCUGp (18) and also with partial analytical data reported elsewhere for the 5' ends of several eukaryotic 18S rRNAs including Xenopus (19).

Start of 18S coding sequence

The above findings provide three criteria for identifying the start

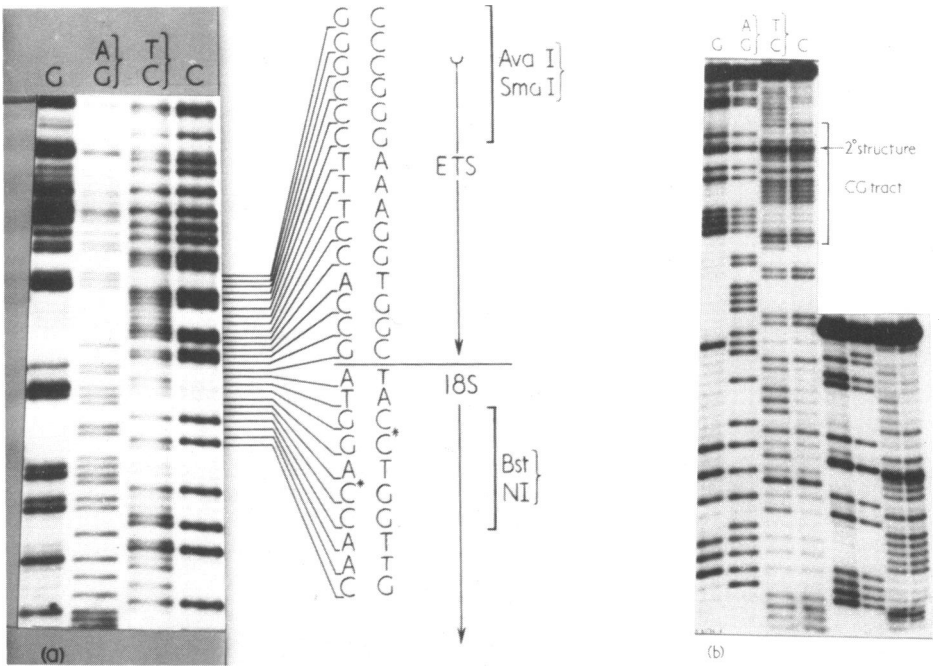


Figure 3. (a) Detail of sequencing gel reading leftwards from left Xba I site of figure 2, on "complementary" DNA strand. Gel shows three features of interest:- i) Methylated C residues (asterisks) near to start of 18S gene. These showed as spacing anomolies; G was read on opposite strand and sequence was confirmed by cleavage with Bst NI and sequencing from cleavage site. ii) Start of 18S gene. iii) Change to GC-rich sequence in ETS. The G and C tracks are dense in this gel, but four gels gave the same sequence. The "synonymous" strand was read from the SmaI/AvaI site - sequence is shown in right hand column. (b) Detail of sequencing gel reading rightwards from left Xba I site. CG tract is evident and the sequence is easily read up to region of secondary structure (see text).

of the 18S coding sequence in rDNA. i) The coding sequence must start with T(AC,C,T)G, with the nucleotides in parentheses probably in the order shown. ii) The DNA sequence to the right of the putative 18S start sequence should be consistent with the oligonucleotides in table 1. iii) The DNA sequence to the left of the 18S start sequence should not correspond to any distinctive oligonucleotides in table 1.

There is only one site to the left of the Xba sites which possesses the sequence, T(AC,C,T)G (figure 4). The entire DNA sequence from this putative 18S start to the first Xba site is compatible with the oligonucleo-

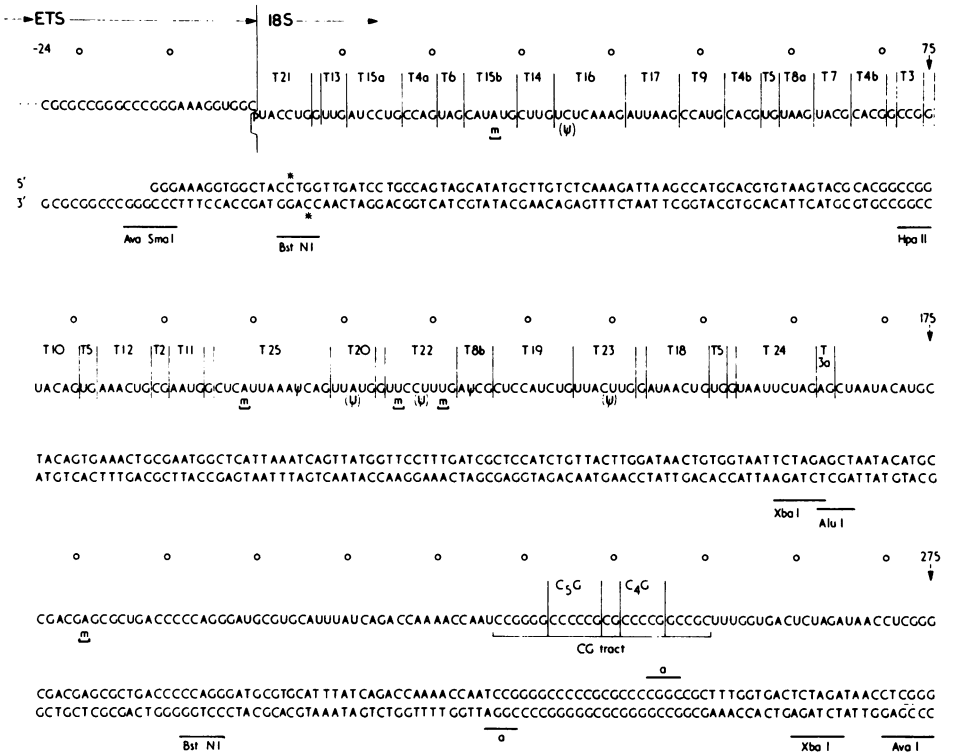


Figure 4. Nucleotide sequence in region of start of 18S gene, with inferred RNA sequence above. Restriction sites which were used in the analysis are indicated. Asterisks denote methylated C residues. "a" denotes secondary structure seen on sequencing gel of indicated strand. Nucleotides are numbered from start of 18S gene, negative numbers being used for external transcribed spacer (ETS, raised in RNA sequence). Small circle denotes every tenth nucleotide. T21, etc. refer to T1 ribonuclease products (figure 5 and table 1). Subscript m denotes 2'-O-methyl group in RNA. Ψ denotes pseudouridine. (Ψ) beneath an oligonucleotide indicates a pseudouridine-containing olionucleotide in which the pseudouridine has not yet been placed.

tide data in table 1. (Among the RNA products, only G and CG were recovered in appreciably higher yields than predicted by the DNA sequence, presumably because of minor, non-specific impurities in the recovered hybrid). A few nucleotides to the left of the putative 18S start, the DNA sequence GAAAG is not represented by AAAG in the fingerprint of the 5' region of 18S RNA, in agreement with criterion iii). Therefore by all three criteria the start of the 18S gene is located at the position shown in figure 4.

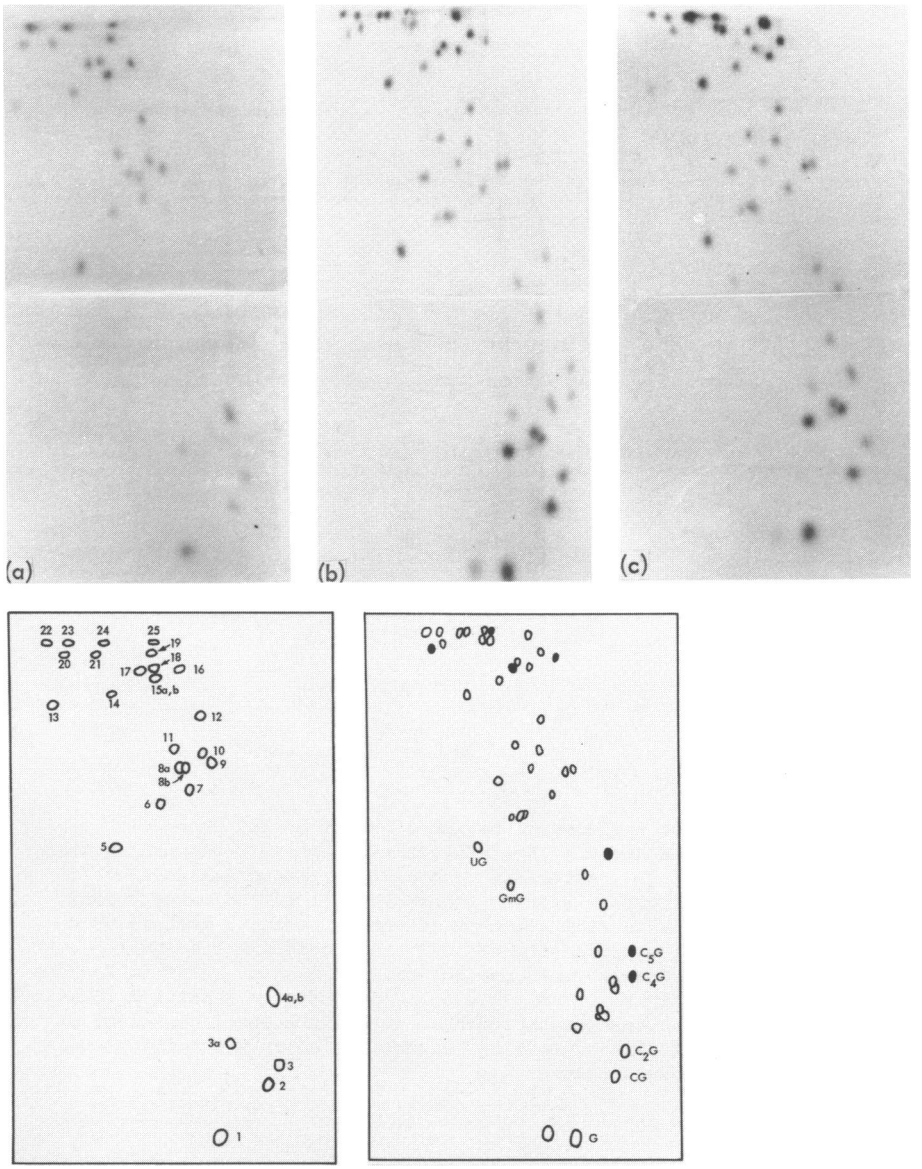


Figure 5. (a) Fingerprint of 18S rRNA region from 5' end to first Xba I site (figure 2). Key (below) gives numbering system. Spot 22 ran off end of fingerprint shown, but was retained in other fingerprints. (b) Fingerprint of 18S rRNA that hybridizes to Alu I fragment of figure 2. (c) Fingerprint of 18S rRNA that hybridizes from second Xba I site to Pst I site (figure 2). Spots marked black in key are present in (b) but not (c). For further explanation see text.

Table 1

Tl oligonucleotides in figure 5a

Spot	Composition[a]	Panc[b]	Inferred Sequence[c]	Molar Yield Observed	Molar Yield Predicted[d]	Notes
1	G		G	9.98	7	
2	C,G		CG	1.92	1	
3	2C,G		CCG	1.25	1	
3a	AG			0.79	0-1	(e)
4a }	2C,A,G	AC,AG	CCAG	2.93 }	1	
4b }			CACG	}	2	(f)
5	U,G		UG	3.16	3	
6	A,U,G	AG	UAG	0.91	1	
7	C,A,U,G	AC	UACG	0.87	1	
8a	2A,U,G	AAG	UAAG	0.86	1	
8b	C,A,Ψ,G	AΨ	AΨCG	0.92	1	(g)
9	2C,A,U,G	AU	CCAUG	0.99	1	
10	C,2A,U,G	AC,AG	UACAG	1.00	1	
11	2A,U,G	AAU	AAUG	1.04	1	
12	C,3A,U,G	AAAC	AAACUG	0.93	1	
13	2U,G		UUG	0.82	1	
14	C,2U,G		CUUG	1.35	1	
15a }	C,A,AmU,U,G	AU,AmU	AUCCUG	1.59 }	1	
15b }			CAUAmUG	}	1	(h)
16	2C,3A,U,Ψ,G	AAAG	UCUCAAAG (1Ψ)	0.94	1	(j)
17	3A,2U,G	AU,AAG	AUUAAG	1.03	1	
18	C,3A,2U,G	AU,AAC	AUAACUG	0.77	1	
19	C++,A,3U,G	AU	CUCAUCUG	0.96	1	
20	A,2U,Ψ,G	AU	UUAUG (1Ψ)	1.08	1	(j)
21	pUp,C,2A,U,G	AC	pUACCUG	0.73	1	
22	C,UmC,2U,Ψ,UmG		UUmCCUUUmG (1Ψ)	1.07	1	(j)
23	C,A,3U,Ψ,G	AC	UUACUUG (1Ψ)	1.31	1	(j)
24	C,A++,4U,G	AAU,(AG)	UAAUUCUAG	1.08	1	
25	C++,A++,AmU,U, Ψ,G	AAAΨ AmU,(AG)	CUCAmUAAAΨ CAG	0.94	1	(k)

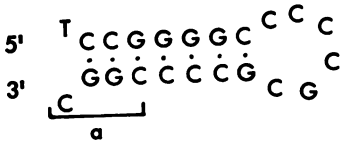
Footnotes to table 1:- (a) Composition was obtained from alkaline hydrolysis supplemented by positional information from fingerprint (13). Ψp was separated from Up by one-dimensional chromatography (23). (b) Pancreatic ribonuclease products larger than mononucleotides are shown. Tl products lacking A were not analysed. (c) The simplest sequences were derived from (a) and (b). All other sequences were derived by matching data from (a) and (b) to the DNA sequence. (d) Observed molar yields are means from three independent hybridization and fingerprinting experiments. Predicted yields are from the 18S rDNA sequence to the first Xba I site. (e) Product AG was recovered in variable yield. The corresponding DNA sequence, GAG, coincides with the 3' end of the first Xba I site. (f) These two products were not separated from each other. Observed molar yield is for the mixture. (g) Products 8a and 8b were separated in two of the three experiments. 8b is the Ψ-containing product. (h) These products did not separate; yield is for mixture. Two lines of evidence place AmU where shown:- "AU" in 15a sequence is preceded by G. Pancreatic ribonuclease fingerprint did not yield GAmU. Therefore AmU must be in product 15b. Partial venom digestion of corresponding spot in HeLa cell 18S rRNA (methylated spot 46, ref. 11) yielded sequence assignment ...AmUG. Since most methylated sequences in Xenopus 18S RNA are homologous to HeLa (12), this location for the methyl group is almost certainly correct also for Xenopus. (j) Each of these products possesses one Ψ, but exact location within the product has not yet been ascertained. (k) AAA Ψ migrates differently from AAAU in combined Tl plus pancreatic ribonuclease fingerprints (24). A Tl plus pancreatic fingerprint of this region of 18S RNA yielded AAAΨ but no AAAU (in agreement with the presence of only one AAAT in the DNA sequence to the first Xba site). This places Ψ unambiguously as shown within product T25.

Modified nucleotides

Analysis of the oligonucleotides in table 1 revealed four 2'-O-methyl groups and six pseudouridine residues. The 2'-O-methylated oligonucleotides had been encountered previously in the analysis of methylated sequences in X. laevis rRNA (12). Data on the methylated oligonucleotides (table 1) together with the DNA sequence data permitted the complete sequences of these methylated oligonucleotides to be inferred, and also their positions in the overall sequence. Some of the pseudouridine residues have not yet been precisely located within their respective oligonucleotides. The first methylated nucleotide in 18S rRNA is 2'-O-methyl A at position 27. A pseudouridine occurs a few nucleotides later. Several modified nucleotides are clustered between positions 99 and 124 (figure 4).

Region between Xba I sites

A striking feature of this region is an uninterrupted run of 24 C and G residues from positions 227 to 250. As mentioned above, this feature gave rise to secondary structure effects in DNA sequence analysis. The expected secondary structure in the "S" strand of DNA (synonymous to RNA) is as follows:-



Three of the bases marked "a" in figure 4 and above contribute the fifth, sixth and seventh consecutive CG pairs in a hairpin structure. Presumably the strength of this secondary structure exceeds the denaturing capacity of the sequencing gel system, so that over this short region the normal relationship between sequence length and mobility does not hold. The sequence was easily read from the other strand at this point, and vice versa for the other end of the CG tract.

The tract is reminiscent of the stable CG stem in vertebrate 5.8S rRNAs (20-22), except that in the present instance the loop as well as the stem consist of C and G residues. By means of hybridization and fingerprinting experiments we have obtained evidence that the tract is indeed present in 18S rRNA. Figures 5b and c show fingerprints of 18S rRNA that hybridize to two overlapping restriction fragments of rDNA designated Ibc and Ic in figure 2. The former possesses, and the latter lacks, the rDNA region between the Xba I sites, which we shall call region Ib. The CG tract should encode the T1 products C_4G and C_5G. These are present in figure 1b and absent from figure 1c, indicating that they occur in rRNA

encoded by region Ib. Figure 1b also shows several other distinctive oligonucleotides which are absent from figure 1c. Preliminary base composition data on these products are consistent with sequence assignments predicted by the DNA sequence of region Ib. Finally, data from ^{14}C methyl-labelled rRNA, to be described elsewhere (B.E.H.M., in preparation) indicate that region Ib contains a single methyl group within the sequence PyGAmG, which can therefore be placed at the position shown in figure 4.

DISCUSSION

In these experiments the start of the 18S gene has been identified in a cloned unit of X. laevis rDNA, and the first part of the 18S gene has been sequenced. Good correlation has been obtained with the fingerprint of the 5' region of 18S rRNA. Several modified nucleotides have been exactly or approximately located in this 5' region. The external transcribed spacer has been sequenced for a short distance to the left of the 18S gene.

The part of the 18S gene sequence that was determined by Boseley et al. (1), using a different rDNA clone, can now be located in the gene. It corresponds to nucleotides 76-158 in our sequence, and matches the latter at every nucleotide except for the first two, where TA in our sequence replaces T in theirs. T1 product T10 (U,AC)AG matches well with our sequence at this point, whereas none of the oligonucleotides match TCAG. We therefore believe that our sequence is representative of most RNA molecules at this point.

The same group (2) also compared available sequence data from the X. laevis 18S gene and external transcribed spacer with corresponding sequence data from yeast (Saccharomyces cerevisiae) rDNA (25). They noted strong homology within the gene region and lack of homology in the E.T.S. We can now complete this comparison (figure 6). Some interesting points emerge. There is strong homology between the first 64 nucleotides of the X. laevis sequence and the corresponding region of the yeast sequence, the latter differing from X. laevis by only three substitutions and an insertion. A tract of eleven nucleotides then follows with almost no homology, a GC rich region in X. laevis being replaced by an AT (or AU) rich region in yeast. There then follow fifty more nucleotides where homology is strong (starting with UACAG, mentioned above), another short region with only partial homology, and finally a block of 20 nucleotides with strong homology to the first Xba site, where the yeast data end.

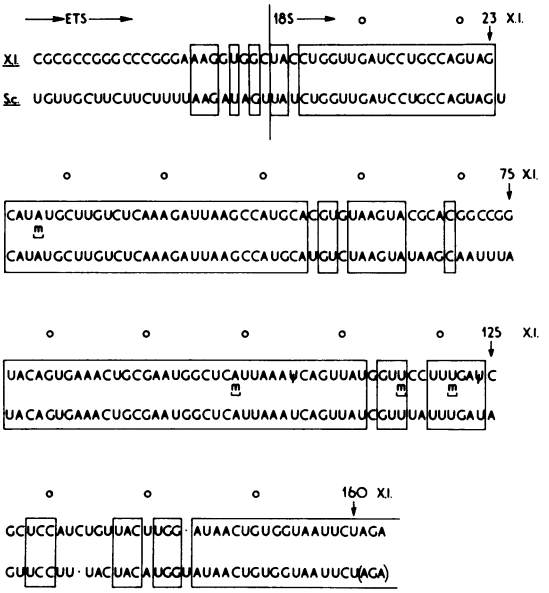


Figure 6. Alignment of last part of external transcribed spacer (ETS) and first part of 18S RNA of X. laevis (X.l.) and Saccharomyces cerevisiae (S.c.), inferred from rDNA sequences. S.c. is from ref. 25. The last three nucleotides, AGA, were not shown in ref. 25 but can be inferred from the presence of an Xba I site. Positions of modified nucleotides are not known in yeast. X. laevis pseudouridine residues whose locations are only approximately known (figure 4) are not included in this figure.

Thus, in the region of the gene for which comparative data are available, there are relatively long tracts of high homology interrupted by short regions where homology is low or absent. On a larger scale, a similar pattern of "extensive but interrupted" homology has been found for 28S rRNA from different taxonomic groups (26,27). It will be interesting to discover whether the pattern of high but interrupted homology seen in figure 6 is typical of 18S RNA as a whole, and, in particular, whether or not the CG tract from nucleotides 227-250 is conserved between different taxonomic groups.

The ETS sequences diverge widely a short distance from the 18S gene. In figure 6 partial homology is seen over nucleotides -1 to -8. Then the X. laevis ETS becomes GC rich and the yeast ETS AU rich, these patterns being maintained throughout most of the ETS (1,2,25).

The 5' end of E. coli 16S rRNA (28,29) shows no large tracts of high

homology with either the X. laevis or yeast sequences. When the E. coli and eukaryotic sequences are suitably aligned a few tracts of partial homology can be inferred. However, before the evolutionary relationship between prokaryotic and eukaryotic ribosomes can usefully be discussed, further eukaryotic sequence data will be needed.

Finally it may be noted that an important part of the characterization of any eukaryotic rRNA is the placing of the many modified nucleotides. This was acheived in this work by correlating the results of RNA hybridization and fingerprinting experiments with the DNA sequence. One of us (B.E.H.M.) has extended these hybridization and fingerprinting experiments to obtain an approximate map of the distribution of methyl groups along the whole of X. laevis 18S and 28S rRNA. A report of this work is in preparation.

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