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# Nucleotide sequence of a crustacean 18S ribosomal RNA gene and secondary structure of eukaryotic small subunit ribosomal RNAs

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ABSTRACT

The primary structure of the gene for 18 S rRNA of the crustacean Artemia salina was determined. The sequence has been aligned with 13 other small ribosomal subunit RNA sequences of eukaryotic, archaebacterial, eubacterial, chloroplastic and plant mitochondrial origin. Secondary structure models for these RNAs were derived on the basis of previously proposed models and additional comparative evidence found in the alignment. Although there is a general similarity in the secondary structure models for eukaryotes and prokaryotes, the evidence seems to indicate a different topology in a central area of the structures.

## INTRODUCTION

The references to twenty-six papers reporting the primary structure of a small ribosomal subunit RNA (srRNA) are listed in Table 1. A number of secondary structure models have been proposed for this ribosome constituent, as summarized in the same table. The construction of these models, while sometimes involving experimental approaches (27,28,33-36) rests for a large part on the assumption that srRNAs in different species and organelles not only show primary structure homology, but also display similar base pairing patterns. The further refinement or confirmation of these models demands the examination of srRNA sequences in additional species. The reconstruction of evolutionary pathways from these sequences (37,38) will also benefit from such research.

Table 1 shows that in the animal kingdom, cytoplasmic srRNA sequences are known for four species, three of which are mammals, and all of which are vertebrates (phylum Chordata). Here we report the nucleotide sequence of the 18 S rRNA gene of the crustacean Artemia salina (phylum Arthropoda). We also propose an alignment of most of the srRNA sequences mentioned in Table 1, excluding those of animal, fungal and protist mitochondria. Suggested base-pairing schemes are superimposed on this alignment.

Table 1. Published small subunit rRNA sequences

An asterisk after a species name means that the sequence appears in the alignment of Fig. 4. Two slightly different sequences have been published in the case of rat cytoplasmic srRNA and yeast mitochondrial srRNA. Multiple references for the same sequence apply to the original report and to corrections published later. Among the papers on secondary structure models (last column) only the most recent and those judged most significant are cited.

| Origin | | | Chain length | Reference | |
|---|---|---|---|---|---|
| | | | | Sequence | Secondary structure |
| Eukaryotes | Animals | Rat 1 | 1869 | 1 | |
| | | 2* | 1874 | 2 | 2 |
| | | Mouse | 1869 | 3 | |
| | | Rabbit | 1858 | 4 | |
| | | Xenopus laevis* | 1826 | 5,27 | 27 |
| | | Artemia salina* | 1810 | this paper | |
| | Fungi | Saccharomyces cerevisiae* | 1799 | 6,28,33 | 28-30,33 |
| | Plants | Zea mays* | 1809 | 7 | |
| | | Oryza sativa | 1812 | 8 | |
| | Protist | Dictyostelium discoideum* | 1872 | 9 | 9 |
| Archaebacteria | | Halobacterium volcanii* | 1472 | 10 | 10,31 |
| Eubacteria | | Escherichia coli* | 1542 | 11,31 | 29-32 |
| | | Proteus vulgaris* | 1544 | 12 | 12 |
| | | Anacystis nidulans* | 1487 | 13 | |
| Chloroplasts | | Zea mays* | 1491 | 14 | 30 |
| | | Nicotiana tabacum | 1486 | 15 | |
| | | Chlamydomonas reinhardtii* | 1475 | 16 | |
| | | Euglena gracilis* | 1491 | 17 | 17 |
| Mitochondria | Animal | Human | 954 | 18 | 29,30 |
| | | Bovine | 955 | 19 | |
| | | Rat | 953 | 20 | |
| | | Mouse | 956 | 21 | |
| | Fungal | Aspergillus nidulans | 1437 | 22 | 22 |
| | | Saccharomyces cerevisiae 1 | 1659 | 23 | 30 |
| | | 2 | 1686 | 24 | |
| | Plant | Triticum aestivum* | 1955 | 25 | 25 |
| | Protist | Paramecium primaurelia | 1680 | 26 | |
| | | Paramecium tetraurelia | 1680 | 26 | |

## MATERIALS AND METHODS

### Isolation of small subunit ribosomal RNA

18 S rRNA was isolated by phenol extraction after preparation of the small ribosomal subunits (39) from Artemia cysts. For hybridization purposes, 18 S rRNA was labelled with $^{125}I$ (40) and unreacted iodine was removed by passage over a 2 ml Biogel P2 column. Treatment of 40 $\mu$g RNA with 0.5 mCi carrier-free $^{125}I$ yielded a specific activity of 6 $\mu$Ci/$\mu$g RNA.

### Preparation of DNA and cloning of the 18 S rRNA gene

Nuclear DNA was isolated from newborn larvae of Artemia salina according to the procedure of Cruces et al. (41). These authors demonstrated that the complete 18 S rRNA gene is present in a single Hind III fragment. Digested DNA was cloned in the Hind III site of pBR327 (42). Screening of the recombinants was batchwise, starting with 576 transformed cells, by extracting plasmid DNA by boiling-lysis (43) followed by fractionation on a 1% agarose gel, transfer to nitrocellulose (44) and hybridization with an $^{125}I$-labeled 18 S rRNA probe. The final positive clone, designated pRAs, was prepared in large quantity essentially according to Thompson et al. (45), the column purification step being replaced by a CsCl centrifugation according to Davis et al. (46). Large scale preparations of the inserted Hind III fragment were made by fractionation of the digested plasmid on low melting agarose and extraction (47).

### Sequencing of the 18 S rDNA

The entire 18 S rRNA gene is contained in a single Hind III fragment of about 3200 basepairs. Fig. 1 shows the detailed restriction map, as well as the position of the fragments subcloned in phage M13mp8 (48), and sequenced according to Sanger et al. (49) using a 27-mer primer isolated from an EcoR I digest of plasmid pSP16 (50). Sequencing gels were prepared according to Garoff and Ansorge (51) and run either at room temperature or at 55°C. The sequenced area extended from 210 nucleotides upstream of the 18 S rRNA 5'-terminus to the 3'-terminus. Overlaps were found for all but two 4-basepair and one 6-basepair restriction sites.

### Localization of the gene boundaries

The 3'-terminus of the 18 S rRNA was identified directly by sequencing of the [5'-$^{32}$P]pCp-labeled RNA according to Peattie (52) over a distance of 80 nucleotides adjoining the 3'-end. This sequence overlaps with that of a Sau3A I fragment extending to 5 nucleotides before the 3'-terminus (Fig. 1).

The 5'-terminus was identified by an indirect method. Comparison of the sequence of a subclone spanning the 5'-end (Fig. 1) with other eukaryotic

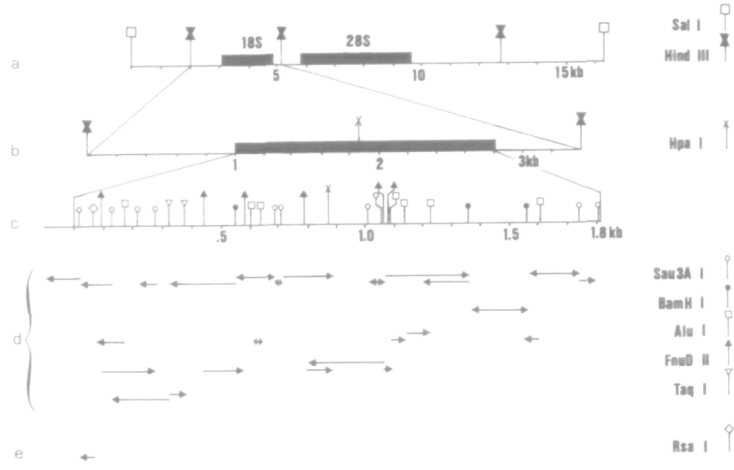



Fig. 1. Restriction mapping of Artemia rDNA and sequencing of 18 S gene.
a) Physical map of the A. salina rDNA repeat unit according to Cruces et al. (41).
b) Hind III restriction fragment cloned in pBR327, and containing the 18 S rRNA region. This fragment was usually recovered from the recombinant plasmid as two Hind III - Hpa I restriction products, in order to discriminate it from the 3.2 kb long vector.
c) Restriction map of the 18 S rRNA region; only those restriction sites used for sequencing in M13mp8 are shown.
d) Direction and sequencing distance of the sequenced subclones. The arrows point away from the dideoxy-sequencing primer. The M13mp8 R.F. DNA was opened with BamH I for BamH I or Sau3A I fragment insertion, with Sma I for Alu I and FnuD II fragment insertion, and with Acc I for Taq I fragment insertion.
e) The Sau3A I - Rsa I restriction fragment used for primer extension in the 5'-end determination.

18 S RNA sequences indicated a presumable terminus. This was confirmed by primer extension of a 50 nucleotide Sau3A I - Rsa I fragment, hybridized (53) to 18 S RNA and transcribed by AMV reverse transcriptase in the presence of deoxy- and dideoxytriphosphates and [$\gamma$-$^{32}$P]dATP. This experiment is illustrated in Fig. 2.

## RESULTS

### Primary structure of the Artemia 18 S rRNA gene

The sequenced area extends from nucleotide 210 upstream of the 18 S RNA 5'-terminus to the 3'-terminus. The 210-nucleotide sequence preceding the 5'-terminus is shown in Fig. 3. The 18 S RNA itself proved to be 1810 nucleotides long, which is slightly shorter than the other animal srRNAs

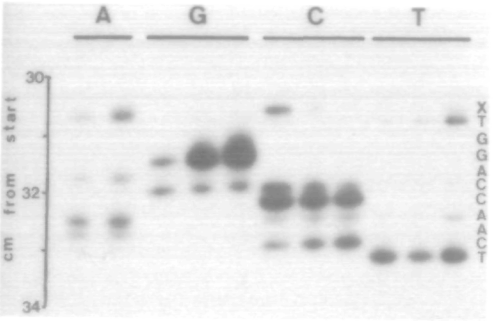



Fig. 2. Mapping of the 18 S RNA 5'-terminus.
18 S RNA was hybridized to the Sau3A I - Rsa I DNA fragment shown in Fig. 1 under the conditions described by Qu et al. (53). This template-primer system was reversely transcribed in four different reaction mixtures, each containing four dNTPs and one ddNTP, the latter in variable amounts. As an example, the C-specific reaction contained 50 $\mu$M dTTP, 50 $\mu$M dGTP, 2.5 $\mu$M dCTP, and 2.5 $\mu$Ci [$\gamma$-$^{32}$P]dATP at 3000 Ci/mmole. The ddCTP concentration was 2.5 $\mu$M (left lane), 1.2 $\mu$M (middle lane), or 0.6 $\mu$M (right lane). The mixture was incubated with 5 units AMV reverse transcriptase at 37°C. After 15 min, unlabeled dATP was added to 250 $\mu$M, 5 more enzyme units were added, and the mixture incubated a further 15 min before separation on a 6% polyacrylamide gel. In the T-, A- and G-specific reactions, ddNTP concentrations were about 10 times higher. The autoradiogram allows to read the sequence complementary to the 18 S RNA 5'-end from residue 2 to 11.

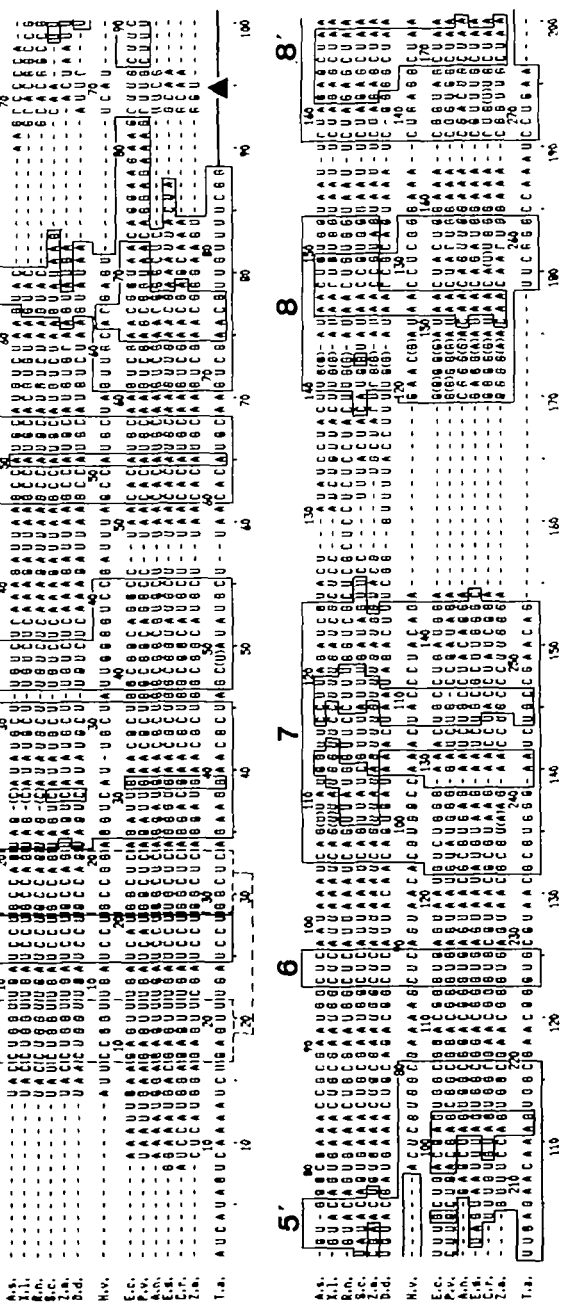
hitherto investigated (Table 1). Its sequence is shown in the alignment of Fig. 4 and in the secondary structure model of Fig. 5.

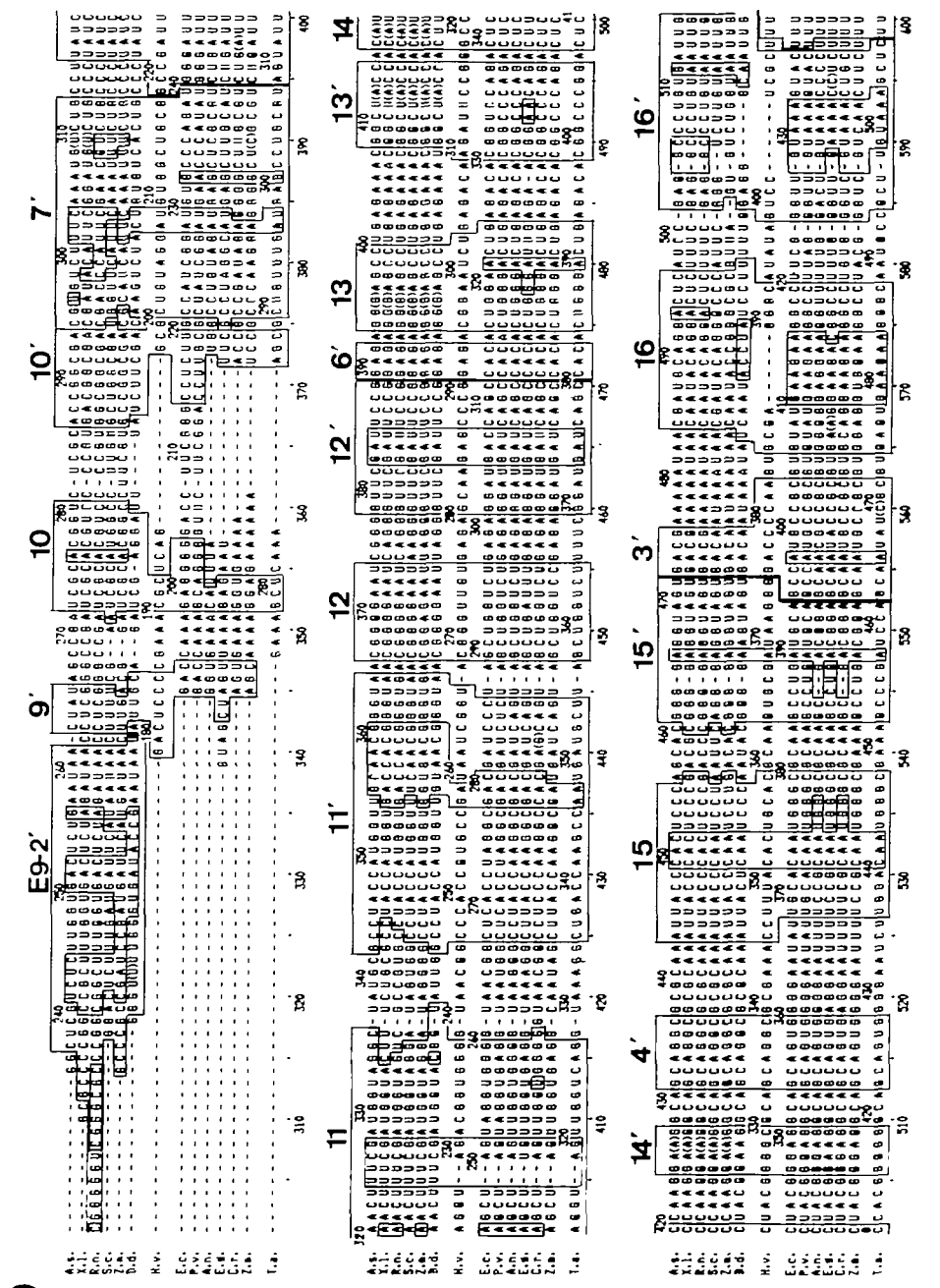
## Secondary structure of Artemia 18 S rRNA and srRNAs in general

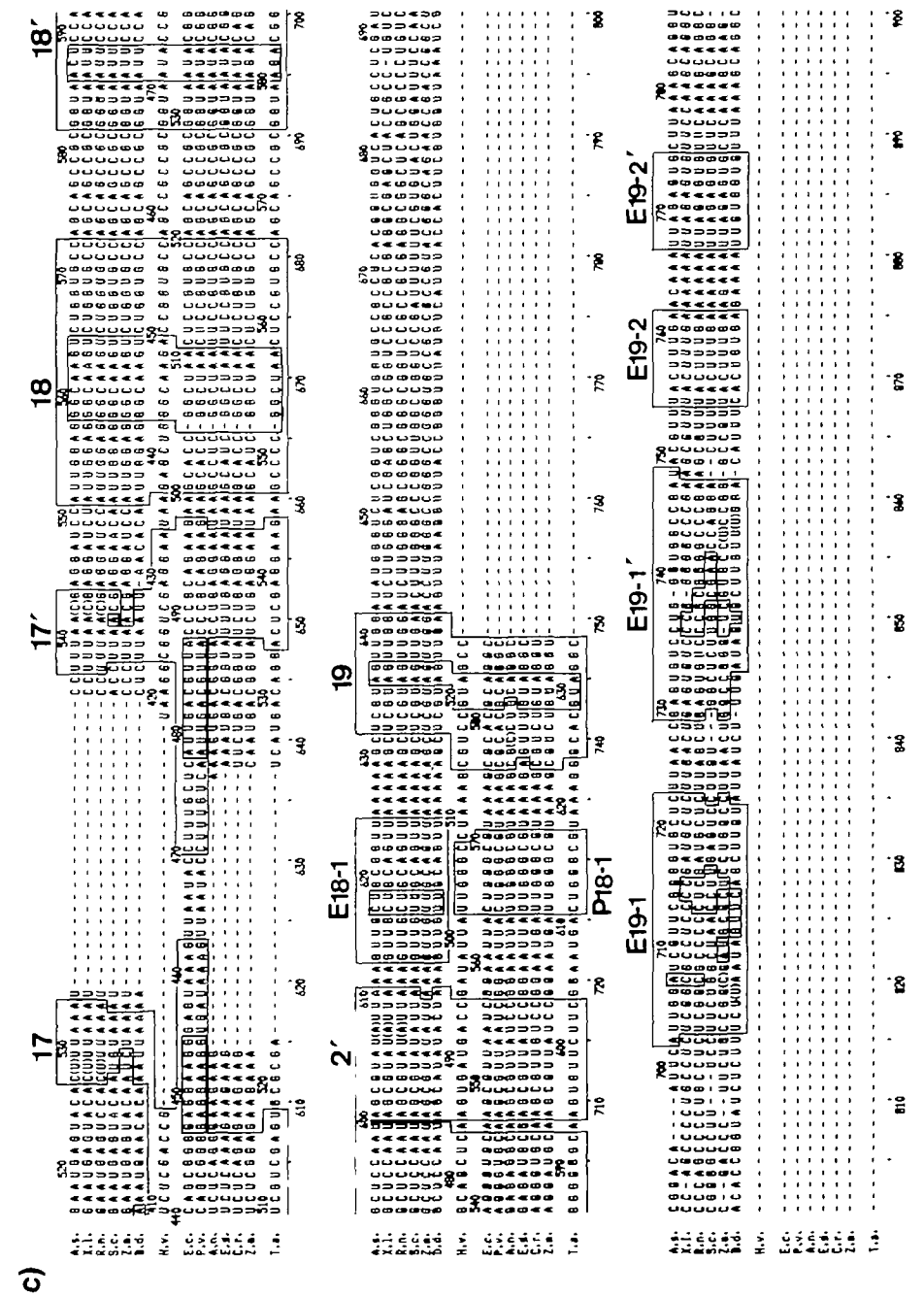
Fig. 4 shows an alignment of the Artemia 18 S RNA with corresponding srRNA sequences from 5 eukaryotes, 1 archaebacterium, 3 eubacteria, 3 chloroplasts and 1 plant mitochondrion. A single representative was chosen among the mammals, the higher plants, and the higher plant chloroplasts. In addition all the non-plant mitochondrial sequences were omitted because their

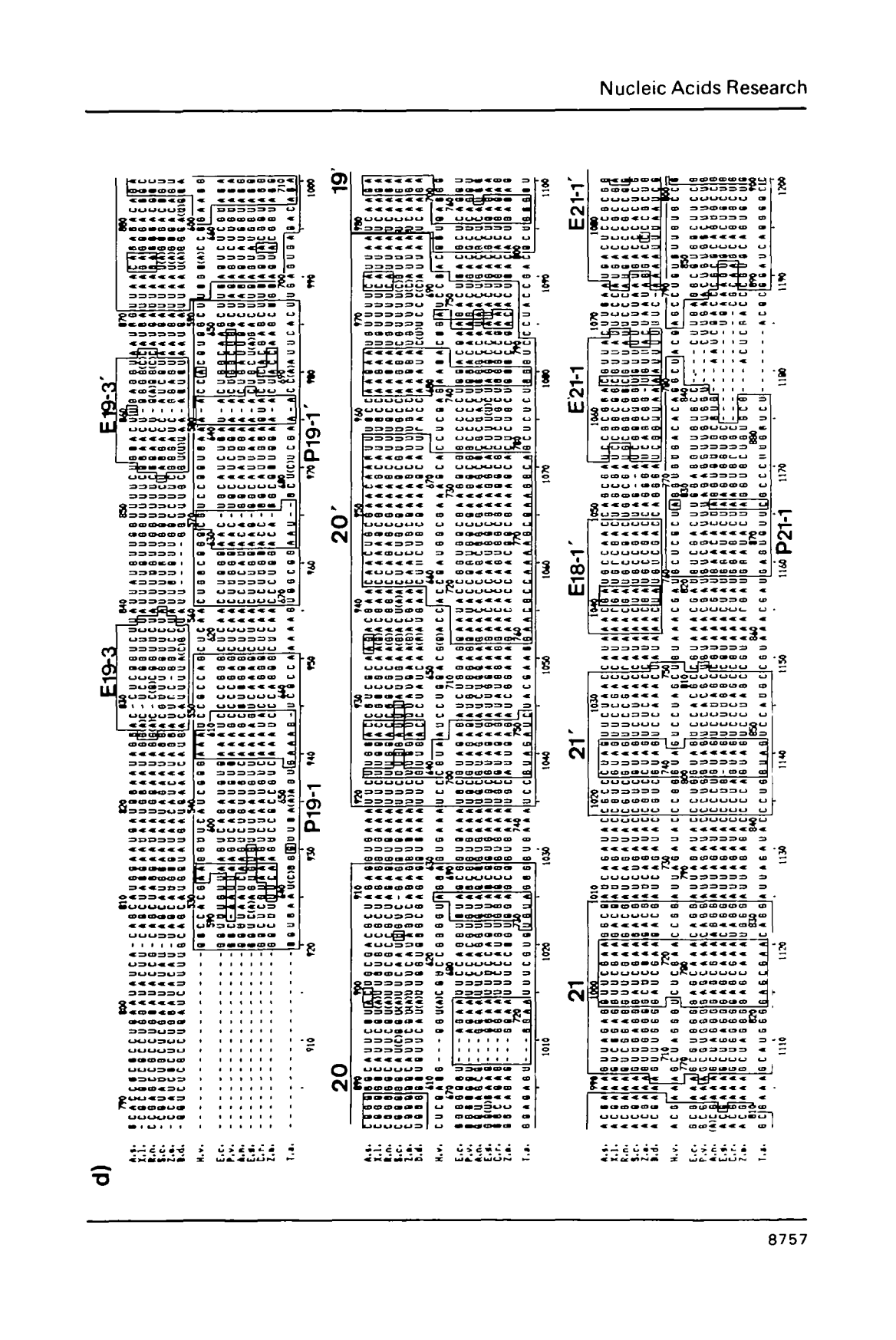
```
-210                                                              -141
 GATCCAAACATCTGGTGAGCTGAGACTGAATACGTTGGTTTAGTAGTGGAATCCTTGGATTACGGTAATG
-140                                                               -71
 ACTTTGGTATCATTGGAGCCTTTGACTAATTGGGATTTAAGGGTCTTCAAAGGACATGGCAAGGTGGCCT
 -70                                                                -1
 ACTCCTAGAGGGAAATTCTCAGGATTTCAATGCGTTTTCTAATGTGACCTGATGGTTAACGTGAATGTGT
```

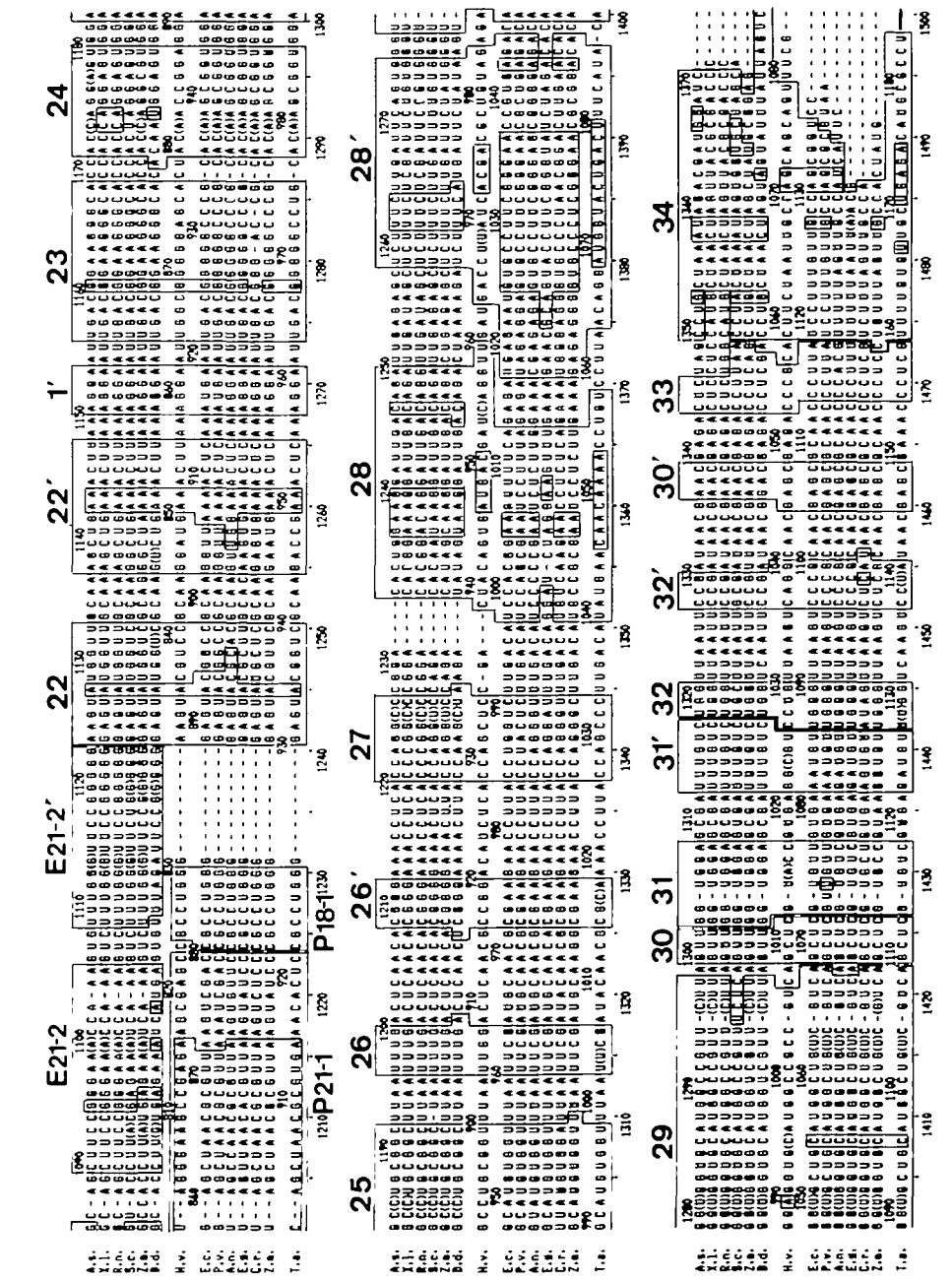
Fig. 3. Sequence of 210 nucleotides preceding the 18 S RNA 5'-terminus. The sequence was derived by analysis of a Sau 3A I fragment spanning the 5'-terminus (Fig. 1d).

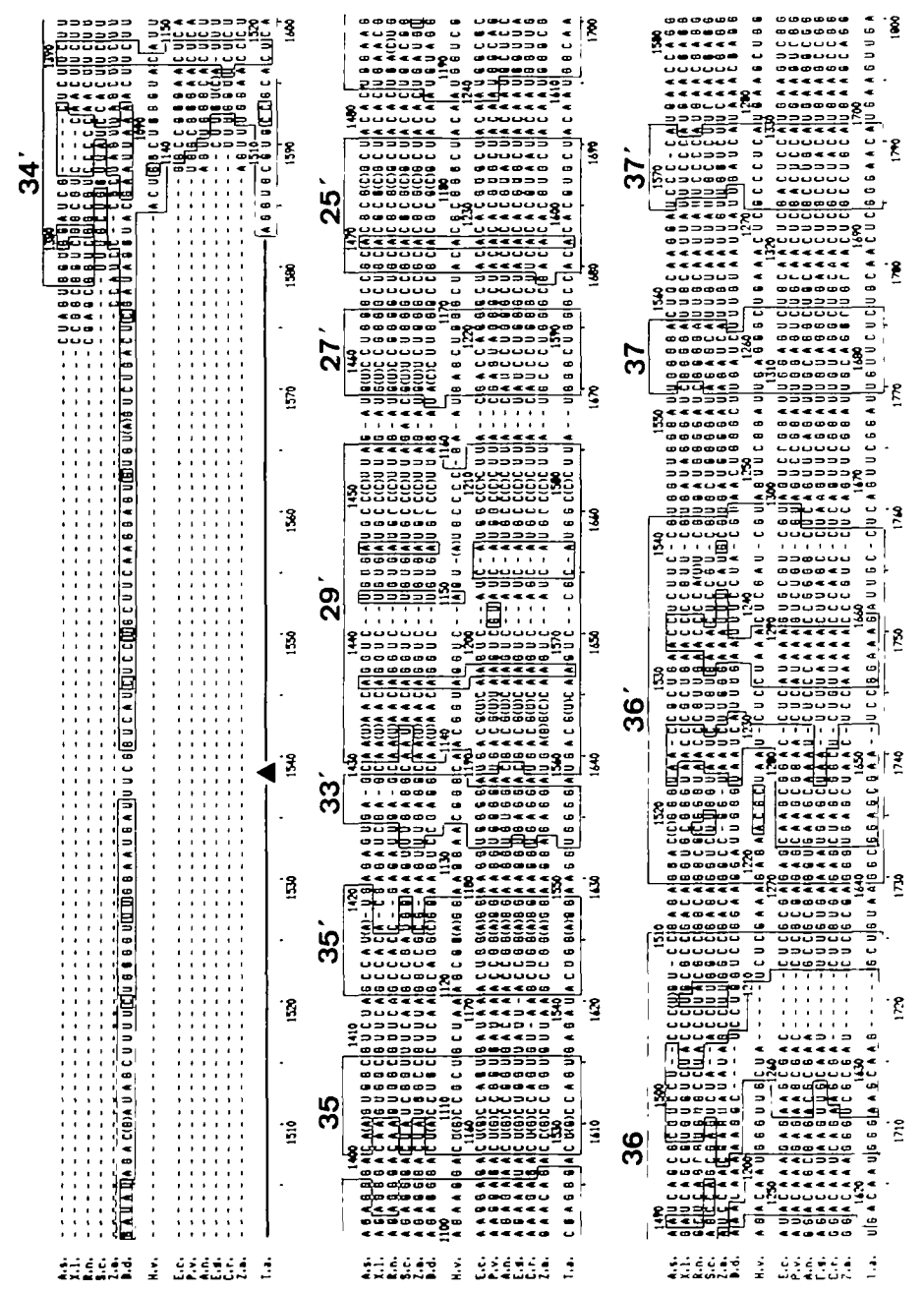

a)
1
2
3
4
5
6
7
8
8′
9
E9-1
E9-1′
E9-2
5′


b)
E9-2'
9'
10
10'
7'
11
11'
12
12'
6'
13
13'
14
14'
4'
15
15'
3'
16
16'
A.s.
X.l.
R.n.
S.c.
Z.m.
B.d.
K.v.
E.c.
P.v.
A.n.
E.s.
C.r.
Z.a.
T.a.

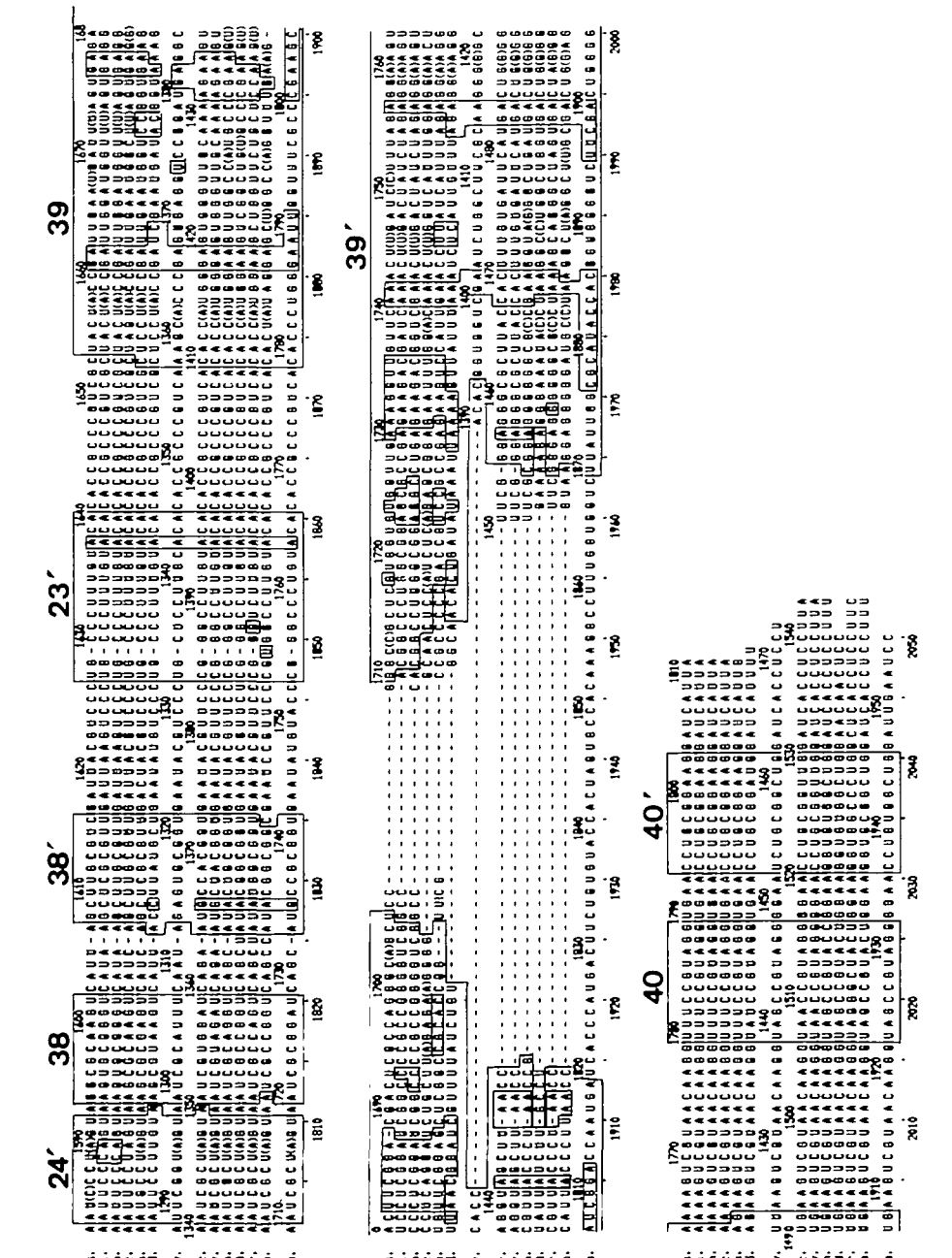

c)
17
17′
18
18′
2′
E18-1
19
P18-1
E19-1
E19-1′
E19-2
E19-2′


d)
E19-3
E19-3′
P19-1
P19-1′
20
20′
19′
21
21′
E18-1′
E21-1
E21-1′
P21-1


e)
E21-2
E21-2'
22
22'
1'
23
24
P21-1
P18-1
25
26
26'
27
28
28'
29
30
31
31'
32
32'
30'
33
34



f)


g)
24′
38
38′
23′
39
39′
40
40′

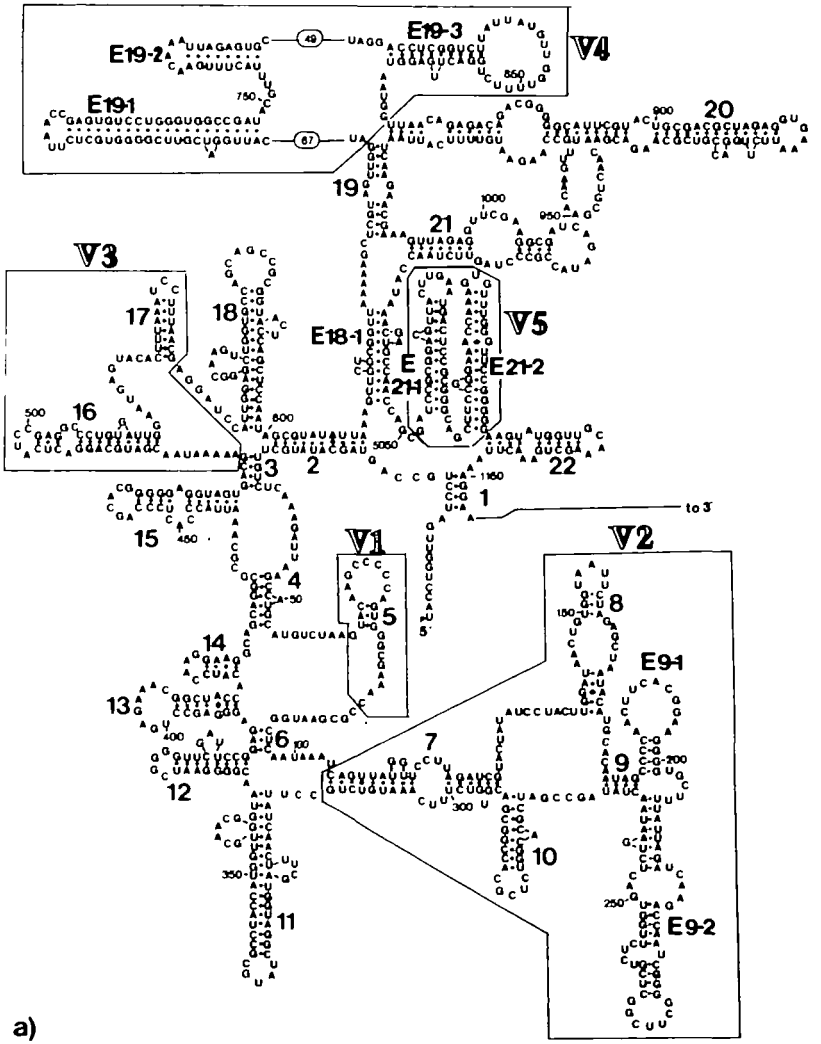
alignment is problematic in some cases and certain areas. Moreover, when added to the alignment, the extra information did not facilitate the derivation of a generalized eukaryotic srRNA secondary structure model and its comparison with the prokaryotic models (29-32), which was our main purpose.

Presumed double-stranded areas are indicated on the alignment by pairs of boxes numbered 1 and 1', 2 and 2', etc. Corresponding numbers 1, 2, etc. identify the helices in the secondary structure model for Artemia 18 S RNA in Fig. 5. The helix numbering system is as follows. A separate number is given to each helical area, which may be an uninterrupted helix, or a set of helical segments connected by bulges or internal loops. In order to bear a different number, helices or helical areas must be separated from each other by a multibranched loop, or by a single-stranded area that does not form a loop. This numbering system makes abstraction of the fact that some helix segments may or may not be stacked one upon another (have a common axis), a fact which is difficult to assess without knowledge of the tertiary structure. Some proposed helices are specific for eukaryotic srRNAs, either because they occur in a eukaryote-specific insertion, or because a different pairing scheme is assumed for eukaryotes and prokaryotes in the area. Such helices are labeled Ex-y, where x is the number of the preceding universal helix and y a serial number. As an example, helices E9-1 and E9-2 are eukaryote-specific helices encountered after universal helix 9 in the alignment. Prokaryote-specific helices are similarly labeled Px-y. All the proposed helices are supported to some extent by the existence of compensating substitutions, except E18-1 and

Fig. 4a to g. Alignment of srRNA sequences.
The following sequences are aligned and indicated by the initials of genus and species name (from top to bottom) : Artemia salina, Xenopus laevis, Rattus norvegicus, Saccharomyces cerevisiae, Zea mays, Dictyostelium discoideum, Halobacterium volcanii, Escherichia coli, Proteus vulgaris, Anacystis nidulans, Euglena gracilis chloroplast, Chlamydomonas reinhardtii chloroplast, Zea mays chloroplast, Triticum aestivum mitochondrion (references in Table 1). The latter sequence is incompletely listed, with two deleted stretches indicated by a line segment and a black triangle. Boxes labeled 1-1', 2-2' etc. correspond with helices present in all sequences here aligned. If the helix is eukaryote-specific its number is preceded by E, if it is prokaryote-specific (archaebacterial, eubacterial, chloroplast and plant mitochondrial sequences) it is preceded by P. Helices separated only by internal loops or bulges are given the same number. The interior loops and bulges are defined by nested boxes. Residues forming non standard base pairs (pairs other than G·C, A·U, and G·U, or interior loops of 2 bases) are put in parentheses. The broken-line boxes in the beginning of the alignment indicate an interaction that according to some models may coexist with helix 1. Nucleotide numbering specific for A. salina, H. volcanii, E. coli and T. aestivum mitochondrion srRNA is placed above those sequences; a general numbering system for the alignment is found at the bottom of the alignment.

P18-1. This is because the complementary sequences that may give rise to the latter two helices are strongly conserved among the eukaryotic, respectively the prokaryotic, srRNAs.

In establishing the alignment and defining the concomitant base-pairing schemes, we were aided to a great extent by previously proposed models (references in Table 1) for prokaryotic and eukaryotic srRNAs. The prokaryotic base-pairing scheme is identical to that published by Woese et al. (31) except that we propose the presence of an extra helix labeled 9, and that certain



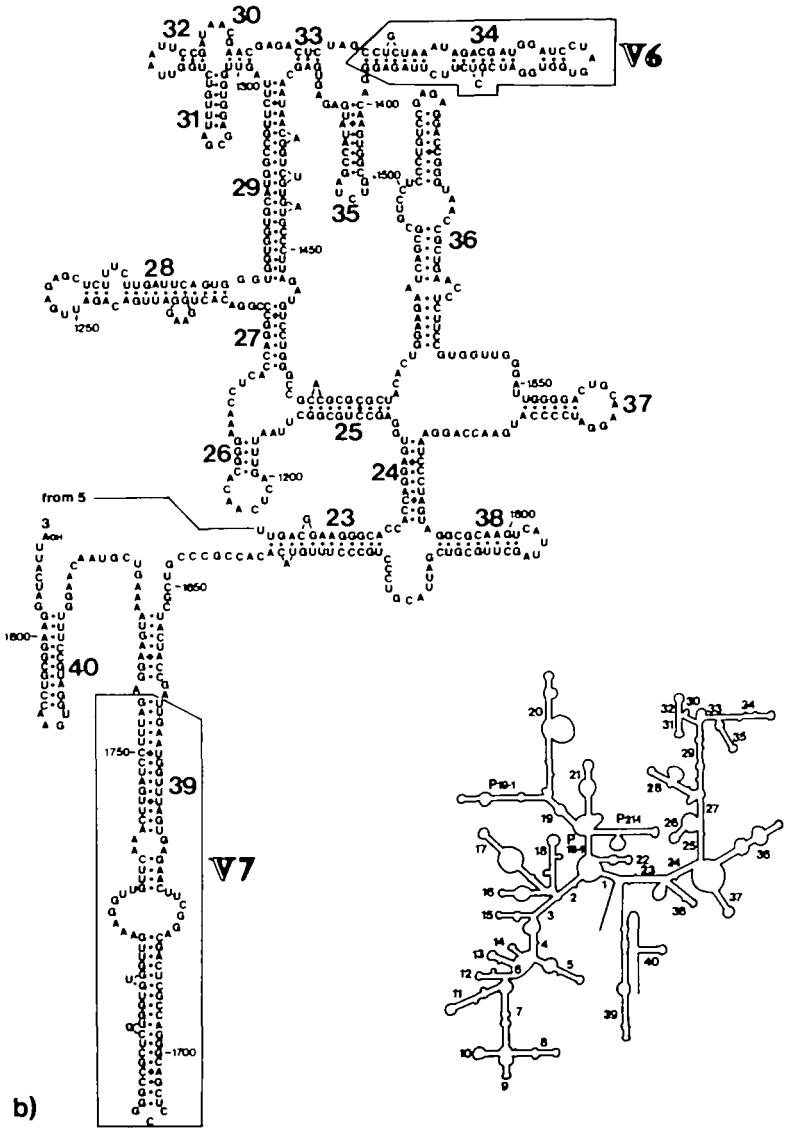



Fig. 5a and b. Secondary structure model for Artemia srRNA compared with the bacterial srRNA model (31).
The helix numbering system is explained in the text and in the legend to Fig. 4. Boxes labeled V1 to V7 enclose areas showing variable primary and secondary structure when different srRNAs are compared. In area V4, the secondary structure of a stretch of 67 nucleotides between helices 19 and E19-1, and a stretch of 49 nucleotides between E19-2 and E19-3, remains undefined for the time being. Non-standard base pairs are symbolized by a losenge instead of a dot. The insert in Fig. 5b shows the application of our helix numbering system to bacterial srRNA secondary structures, drawn in the shape adopted by Woese et al. (31).

helical areas are extended at the expense of nearby single-stranded areas on the basis of comparative evidence, even if there is no proof from compensating substitutions. For eukaryotic srRNAs, a number of slightly different models have been proposed (2, 9, 27-30, 33). None of these models is completely analogous to the prokaryotic model (31), even if one disregards the presence of eukaryote-specific insertions. The model shown in Fig. 5 for Artemia 18 S RNA comes closest to the model (9) recently proposed for Dictyostelium 18 S RNA. The insert in Fig. 5 allows a comparison between the models defined here (Fig. 4) for eukaryotic and prokaryotic srRNAs.

## DISCUSSION

### Sequence alignment and proposed base pairing patterns

The alignment of srRNA sequences (Fig. 4) presented most problems in certain areas where the primary as well as the secondary structure seems to be especially variable. Seven such variable areas are indicated on the secondary structure model (Fig. 5) by the boxes $V_1$ to $V_7$. In two of these, $V_1$ and $V_3$, prokaryotic sequences tend to be longer than the eukaryotic counterparts, while in the other five areas the reverse situation prevails. In some of the areas, considerable variability exists even among the eukaryotes (or among the prokaryotes). Examples can be seen in alignment positions 749 to 986 (area V4 in Fig. 5), which contain a long eukaryotic insertion with poorly conserved sequence, and in alignment positions 1497 to 1573 (area V6), where Dictyostelium discoideum srRNA contains a much longer hairpin than other eukaryotes. In the variable areas the choice of the sequence alignment is often equivocal. This also thwarts the detection of base-pairing patterns because there is no stringent frame in which to search for compensating substitutions. As a consequence, the secondary structures proposed in such areas should be considered as minimal and subject to improvement.

Spencer et al. (25) distinguished five variable areas when comparing wheat mitochondrial and E. coli srRNAs. Their areas V1, V2, V4 and V5 correspond respectively with areas V1, V2, V6 and V7 in the model of Fig. 5.

### Secondary structure topology in the central area of srRNA

Six of the seven variable areas are situated at the periphery of the secondary structure topology, and coincide with the presence or absence of one or several hairpin structures. An exception is area V5, situated centrally and surrounded by universal helices 1, 2, 19, 21, and 22. If the secondary structure models shown in Fig. 5 and defined in the alignment are correct in

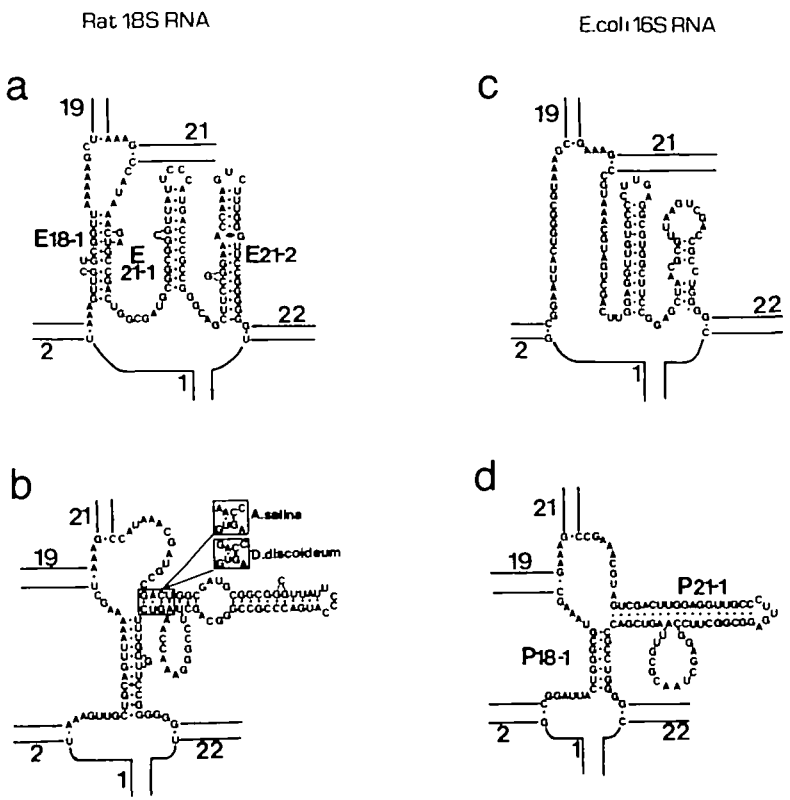



Fig. 6. Central area of srRNA secondary structure.
The area comprised between universal helices 1, 2, 19, 21, and 22 is drawn in two possible conformations for a eukaryotic srRNA (Rat) and a prokyarotic srRNA (E. coli). The conformations adopted in Fig. 4 and 5 are (a) for eukaryotes and (d) for prokaryotes. However, some authors (2,28,29,33) propose (b) or similar topologies for eukaryotes, or consider (27) conformation (c) for prokaryotes. Note however that the boxed structure in model (b) cannot be formed in some other eukaryotes, and that an equivalent of helix E18-1 is not generally possible in prokaryotes.

outline, then this area confers a fundamental difference in folding pattern to prokaryotic and eukaryotic srRNAs.

Authors of secondary structure models for yeast (28,33) and rat (2) 18 S RNA and of an early model (29) for Xenopus 18 S RNA tried to fit these sequences in a topology similar to that advocated for prokaryotes (31). In contrast, recent models for Xenopus (27) and Dictyostelium (9) 18 S RNAs assume the existence of a eukaryote-specific topology, which we have also adopted in

Fig. 5. The two possible topologies are illustrated for the central area of rat 18 S RNA in Fig. 6a and b. However, as indicated in Fig. 6b, a part of the prokaryotic-like bulged helix cannot be constructed in the case of Dictyostelium and Artemia.

Conversely, Atmadja et al. (27) postulate the existence of a switch in the central area of E. coli 16 S RNA, where one of the structures would resemble the eukaryotic topology. This is illustrated in Fig. 6c and d. In this case however, the equivalent of eukaryote-specific helix E18-1 cannot be formed in most prokaryotic srRNAs. It may be premature to decide whether such a switch actually exists, or whether the topology is kingdom-specific. In this paper we have proposed the eukaryotic topology of Fig. 6a because on the basis of presently available data it looks more stable and more conserved. The alternative structures illustrated in Fig. 6 may point to an evolutionary switch rather than a presently existing dynamic switch. At one point in time, mutation may have led to an alternative base pairing opportunity, adopted in one evolutionary line while the other conserved the original topology. Or the switch may still exist in bacterial srRNas, eukaryotic srRNAs being frozen in one of the conformations.

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Note added in proof

Two papers reporting the sequence of a srRNA came to our attention after this study was completed. The 16 S RNA of the halophilic archaebacterium Halococcus morrhuae (54) has a chain length of 1475 nucleotides and is 89% homologous with Halobacterium volcanii 16 S RNA. The 18 S RNA of maize mitochondria (55), with a chain length of 1968 nucleotides, is 97% homologous with wheat mitochondrial srRNA. The prokaryotic srRNA secondary structure model (31) followed in this paper is applicable to both sequences.