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The sequence of the 5.8 S ribosomal RNA of the crustacean *Artemia salina*. With a proposal for a general secondary structure model for 5.8 S ribosomal RNA

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ABSTRACT

     We report the primary structure of 5.8 S rRNA from the crustacean Artemia salina. The preparation shows length heterogeneity at the 5'-terminus, but consists of uninterrupted RNA chains, in contrast to some insect 5.8 S rRNAs, which consist of two chains of unequal length separated in the gene by a short spacer. The sequence was aligned with those of 11 other 5.8 S rRNAs and a general secondary structure model derived. It has four helical regions in common with the model of Nazar et al. (J. Biol. Chem. 250, 8591-8597 (1975)), but for a fifth helix a different base pairing scheme was found preferable, and the terminal sequences are presumed to bind to 28 S rRNA instead of binding to each other. In the case of yeast, where both the 5.8 S and 26 S rRNA sequences are known, the existence of five helices in 5.8 S rRNA is shown to be compatible with a 5.8 S - 26 S rRNA interaction model.

INTRODUCTION

     5.8 S RNA, a constituent of the large subunit of the eukaryotic ribosome, has a chain length of approximately 160 nucleotides in most species. Small ribosomal RNAs such as 5 S and 5.8 S are attractive objects for studies in molecular evolution, not only because of their universal occurrence, but also because gel sequencing methods for small RNAs[1,2] allow a relatively rapid examination of their primary structures in a variety of organisms. Some 17 sequences of 5.8 S rRNAs, among which 6 from vertebrates, are known at present[3]. As the collection grows, it should be possible to deduce a generally valid secondary structure model with some confidence. Most of the proposed base pairing schemes rely on the model[4] originally put forward by Nazar and collaborators for mammalian and yeast 5.8 S rRNAs. However, this model assumes base pairing between the 5'- and the 3'-terminal sequences of the molecule, and does not account for the binding of 5.8 S rRNA to 28 S rRNA[5], which is thought to involve precisely these terminal sequences[6,7]. The recent elucidation of two partial [8,9] and one complete[10] 28 S rRNA sequences has come with some detailed proposals for the interaction with 5.8 S rRNA, which

are discussed below.

The eubacterial ribosome does not contain any 5.8 S rRNA, but its coun-
terpart is found in a sequence of about 160 nucleotides at the 5'-terminus of
23 S rRNA[11,12], the rest of this sequence being related to the 28 S rRNA. In
eukaryotes, 5.8 S rRNA also precedes 28 S rRNA in the ribosomal RNA genes,
but is separated from it by a spacer, which has a length of 234 nucleotides
in yeast[13]. In the insects Drosophila melanogaster[7] and Sciara coprophila[14],
the 5.8 S rRNA is further split into two pieces kept together by base pairing.
The mean chain lengths of the 5'-terminal part, spacer, and 3'-terminal part
are 123, 25, and 30 nucleotides. Therefore it was of some interest to exam-
ine if the same situation prevails in other representatives of the phylum
arthropoda, but belonging to a different class, the crustacea.

MATERIALS AND METHODS
Artemia 5.8 S rRNA

Ribosomes were prepared from dry cysts of Artemia salina, dissociated
into subunits and fractionated by zonal ultracentrifugation under the condi-
tions described by Zasloff and Ochoa[15]. Large subunit RNA was obtained by
phenol extraction and ethanol precipitation. 150 OD_{260} units of RNA were
dissolved in 600 μl of 8 M urea containing 0.05 M Tris-boric acid, pH 8.3,
and 0.001 M EDTA. The solution was heated for 10 minutes at 80° to promote
dissociation of the 5.8 S rRNA from the 28 S rRNA, cooled in ice, and loaded
on an 0.4 x 20 x 40 cm slab gel consisting of an upper 5 cm layer of 3% poly-
acrylamide cast on top of a 10% polyacrylamide gel, both layers containing
the same buffer as used for the dissociation treatment. After electro-
phoresis overnight at 350 Volt approximately 3 OD_{260} units could be extracted
from the 5.8 S rRNA band.

Primary structure determination

The primary structure was derived mainly by gel sequencing methods.
Peattie's[1] chemical degradation method was applied to RNA labeled at the 3'-
terminus with [5'-^{32}P] pCp in the presence of RNA ligase. The sequence in
the neighbourhood of the 5'-terminus was confirmed and completed by a two di-
mensional combination of gel- and thin layer electrophoresis as described by
Tanaka et al.[2].

Length heterogeneity at the 5'-terminus was investigated by labeling a
5.8 S rRNA preparation with [γ-^{32}P] pppA in the presence of polynucleotide
kinase after dephosphorylation. The precise conditions are mentioned in the
legend of Fig. 1, which shows the gel separation of the 5'-terminally labeled

RNA chains. Each component was degraded partially with U_2 RNAse, yielding as the main labeled hydrolysis product an oligonucleotide extending from the 5'-terminus to the A residue at position 14. Each oligonucleotide was degraded partially with pancreatic-, T_1-, and U_2 RNAse, and completely with alkali, and the digests were separated by electrophoresis on DEAE-cellulose paper at pH 1.9. The sequences could be read from the 5'-terminus on the basis of M-values[16] over a length sufficient to establish an overlap with the sequence derived from the gel methods. The sequence at the 3'-terminus of the 5.8 S rRNA was completed by moving spot analysis[17] of [^{32}P]pCp-labeled RNA. The end group was identified by alkaline hydrolysis.

Modified nucleotides could be identified and localized on the Peattie gels. Residues containing a 2'-O-methylribose moiety manifest themselves by a gap in the ladder of formamide degradation bands, whereas the base-specific reactions are unimpeded. A pseudouridine on the contrary, gives a band upon formamide degradation but no band under any of the four base-specific reaction conditions. The localization of the Ψ residue was confirmed by extraction of the corresponding band from a Tanaka-gel, complete hydrolysis with P_1 nuclease, and identification of the labeled pΨ by TLC[2].

Secondary structure estimation rules

Tinoco et al.[18] have provided sets of ΔG values that allow to calculate the free energy change associated with the formation of a postulated secondary structure, and thus to make a choice among different possible models. Unfortunately, their values allow only free energy predictions for hairpin structures, including interior- and bulge loops, but not for complete molecules with features such as multibranched loops and unpaired termini. As a consequence, different investigators have completed these rules by different assumptions, as discussed by Salser[19]. Moreover, the ΔG values are not sufficiently precise to allow the systematic prediction, in conjunction with a computer program[20], of the cloverleaf as the most stable secondary structure for tRNAs. For these reasons, we preferred to resort to a set of ΔG values taken from Ninio[21], who thorougly investigated the problem of tRNA secondary structure prediction. He tested different binding models, which are sets of ΔG values for stacking interactions between base pairs. One set is derived from thermodynamical experiments, another from statistical observations, a third from empirical manipulation of the values. Each binding model can be combined with a topological model, which is a set of energetic penalties for loop structures. The test for different combinations of binding- and topological models consisted in the obtained predictability of

the tRNA cloverleaf.

To derive the 5.8 S rRNA model described below and to calculate the free energies we followed Ninio's[21] thermodynamical binding model and the topological model with the following options : a helix segment undisturbed by bulges or interior loops should have a minimum length of two base pairs. It may contain terminal G·U pairs and, under certain conditions, odd base pairs, i.e. interior loops consisting of only two bases. The same set of rules was previously employed and described more explicitly in the derivation of a uniform 5 S rRNA secondary structure model[22].

RESULTS AND DISCUSSION
Primary structure and length heterogeneity at the 5'-terminus

Table 1 gives a summary of the experiments that allowed us to establish the Artemia 5.8 S rRNA sequence, which is shown in Fig. 2 aligned with 11 other 5.8 S rRNAs, and in Fig. 3c in the form of a secondary structure model. Contrary to the situation in insects, the RNA chain is uninterrupted. However, the 5.8 S rRNA from Artemia consists of a mixture of components with slightly different chain length. The sequences are identical except for having a different starting point at the 5'-terminus. This phenomenon does not interfere with and is not revealed by the sequencing methods of Peattie[1] and Tanaka et al.[2], because in both methods labeled fragments extending from an internal position to the 3'-terminus are analyzed. The presence of three

Table 1. Experiments that established the primary structure

Method used, reference	Positions identified (a)
M-value analysis[16] of 5'-terminal RNAse U_2 oligonucleotides obtained from 5'-labeled RNA	1 - 5 2 - 6 (b) 4 - 6
Gel sequencing according to Tanaka et al.[2]	1 - 38
Gel sequencing according to Peattie[1]	10 - 160
Moving spot analysis[17] of 3'-labeled RNA	149 - 161
Alkaline hydrolysis of 3'-labeled RNA	162

(a) Numbering system as in Fig. 3c; the numbering in the 3'-half of the
    sequence alignment (Fig. 2) is different because gaps were introduced
    to optimize homology.
(b) A separate experiment was performed on oligonucleotides obtained from
    each of the 3 main components, starting at position 1, 2, and 4.

components is demonstrated in Fig. 1, which shows a gel separation of 5.8 S
rRNA labeled at the 5'-terminus. The main components have chain lengths of
159, 161, and 162 nucleotides. The approximate concentrations are 45%, 25%
and 25% respectively. The 5'-terminal base of the 162 nucleotide component
was chosen as base number 1 in Figs. 2 and 3c, but the mixture actually con-
tains a few percent of chains up to 164 nucleotides long. The sequence pre-
ceding residue 1 is probably G-U. Heterogeneity at the 5'-terminus of 5.8 S
rRNAs has been reported in other organisms, as indicated in Fig. 2, and seems
to be due to an inaccuracy in the processing event generating this terminus.

Artemia 5.8 S rRNA contains two 2'-O-methylribose residues and one pseu-
douridine. An HPLC analysis of pseudouridine content[23] led to a calculated
amount of 1.8 residues per molecule. Hence it is possible that one or more
extra U residues are partially modified into Ψ. This would not be detected
by Peattie[1] sequencing since the unmodified molecules would yield a band.

Secondary structure of 5.8 S rRNA

We did not attempt to investigate the Artemia 5.8 S rRNA secondary
structure experimentally, but certain inferences can be drawn from the com-
parison of 17 sequences now available[3]. The search for common sequence com-
plementarities has been a rather fruitful approach of RNA secondary structure
investigation. It has led to the cloverleaf model for tRNA, and to the Fox-
Woese model[24] and some more refined models (reviewed in ref. 22) for 5 S rRNA.
In both cases the predictions have, to different extents, been verified ex-
perimentally. The same approach is now being exploited in the case of larger

Fig. 1. Separation of Artemia 5.8 S
rRNA components.

10 μg Artemia 5.8 S rRNA was incubated
for 2 h at 37° with 1 μg of alkaline
phosphatase from calf intestinal mucosa
in 10 μl 0.1 M Tris chloride, 0.1% SDS
(pH 8). The RNA was purified by phenol
extraction and ethanol precipitation,
redissolved in 0.01 M Tris chloride,
0.006 M dithiotreitol, 0.01 M MgCl$_2$
(pH 8), containing 10^8 dpm [γ-^{32}P] pppA
and 2 units polynucleotide kinase, and
incubated for 1 h at 37°. The mixture
was then separated on an 8% gel as used
for sequencing. The position number of
the 5'-terminal residue (see Fig. 2) is
marked alongside the bands. The bands
migrated some 35 cm, but only the rel-
evant gel area is shown.

Figure 2

1 XENOPUS LAEVIS
2 DROSOPHILA MELANOGASTER
3 SCIARA COPROPHILA
4 ARTEMIA SALINA
5 CRYPTHECODINIUM COHNII
6 ACANTHAMOEBA CASTELLANII
7 NEUROSPORA CRASSA
8 SACCHAROMYCES CEREVISIAE
9 THERMOMYCES LANUGINOSUS
10 TRITICUM AESTIVUM
11 VICIA FABA
12 CHLAMYDOMONAS REINHARDTII

Conserved bases

1 XENOPUS LAEVIS
2 DROSOPHILA MELANOGASTER
3 SCIARA COPROPHILA
4 ARTEMIA SALINA
5 CRYPTHECODINIUM COHNII
6 ACANTHAMOEBA CASTELLANII
7 NEUROSPORA CRASSA
8 SACCHAROMYCES CEREVISIAE
9 THERMOMYCES LANUGINOSUS
10 TRITICUM AESTIVUM
11 VICIA FABA
12 CHLAMYDOMONAS REINHARDTII

Conserved bases

rRNAs[10,25-28]. Anyway, experimental studies on isolated 5.8 S rRNA are not entirely relevant to the in vivo situation because of the interaction with 28 S rRNA.

For two reasons, the alignment in Fig. 2 contains only one vertebrate sequence. First, all vertebrate 5.8 S rRNAs are strongly homologous, so little information would be gained by including them all. Second, most of these sequences, which were among the first examined, were established by the older RNA sequencing methods involving fingerprinting of uniformly ^{32}P-labeled RNA and partial RNAse digestion. These methods entail the risk that an oligonucleotide may be misplaced in the sequence. Errors of this type have been committed in 5 S rRNA sequencing and have retarded the emergence of secondary structure models by blurring alignments until the sequences were revised[22]. In the case of Xenopus laevis 5.8 S rRNA a comparison of the RNA sequence[29] with the gene sequence[8] has revealed a few errors in the former, including a misplaced G-C. Due to the extreme conservation of verte-brate sequences, it seems quite possible that an original error has been perpetuated in subsequent results. Until this matter is clarified, it seems safe to rely only on the DNA-derived sequence of Xenopus laevis as a repre-sentative of the vertebrates.

The alignment in Fig. 2 is similar to the one published by Hinnebusch et al.[30], except that it is more complete, that it contains the corrected vertebrate sequence and that in the highly variable region numbered 120-148 we have concentrated all the gaps in positions 131-136. The search for common sequence complementarities yielded 6 potential double-stranded areas labeled A-A' to F-F'. In the insect sequences, the stable helix formed by pairing of areas F and F' is not closed by a hairpin loop, because this is where the spacer is cut out of the precursor. The model most frequently used to represent 5.8 S rRNA secondary structures is the one originally proposed

Fig. 2. Alignment of 5.8 S rRNA sequences.
The insect sequences (nrs. 2 and 3) consist of a long chain ending at position 130 and a short one starting at position 137. Heterogeneous 5'-termini are indicated by commas, and Artemia 5.8 S rRNA contains minor components extend-ing to the left of position 1. The boxes A-A', B-B' etc. delimit potential base-paired areas. Nested boxes indicate bulges or small interior loops within these areas. Odd base pairs are bracketed. Single stranded regions are labeled M (multibranched loop), I (internal loop) or H (hairpin loop). The sequences proposed to interact with 28 S rRNA in different models (see text and Fig. 4) are underlined in Xenopus laevis, Neurospora crassa, and Saccharomyces cerevisiae.

by Nazar and collaborators[4] for the rat tumour and yeast sequences and is
illustrated in Fig. 3a. Helices C, D, E, and F of our base pairing scheme
(Fig. 2 and 3b) are essentially as in Nazar's model. In some cases, such as
helix D in the plant sequences, we chose different pairing schemes for the
sake of uniformity. Helix A is identical with the Nazar model in the case
of Xenopus and Chlamydomonas, but had to be adapted extensively in other
sequences for reasons of consistency in secondary structure prediction rules.
In the case of Acanthamoeba castellanii, no satisfactory base pairing scheme
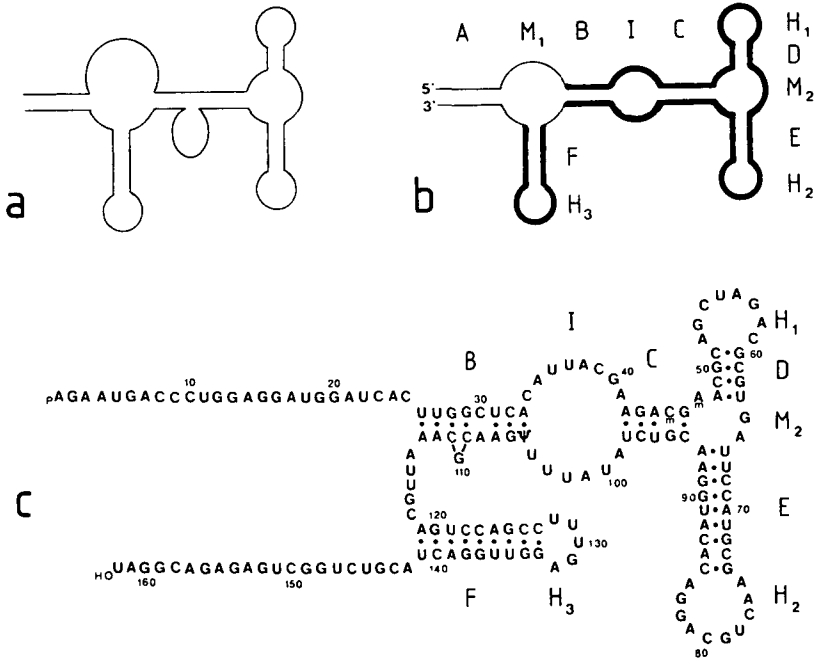could be found for the nucleotides preceding position 15 and following

Fig. 3.   5.8 S rRNA secondary structure models

a) Nazar model[4]

b) Our model, with the structural parts lettered as in Fig. 2. The essential
   difference with (a) is a modified base pairing scheme in area B. As a
   result, loop M_1 shrinks in size and the bulge in (a) becomes an internal
   loop I. In the 5.8 S - 28 S rRNA complex, only the heavy line structure is
   thought to exist, the rest being involved in 28 S rRNA binding.

c) Our model illustrated with the Artemia 5.8 S rRNA sequence. Areas corre-
   sponding with thin lines in (b) are shown unpaired and helix F is tilted
   to emphasize the similarity with the S. cerevisiae 5.8 S - 26 S inter-
   action model (Fig. 5).   The subscript m denotes 2'-0-methylribose
   residues.

position 157 in the alignment. In general, the complementarity A-A' is much less conserved than in areas B-B' to F-F', in the sense that more bulges, interior loops and odd base pairs have to be assumed. Although helix A may exist in free 5.8 S rRNA, it is probably absent in the 5.8 S - 28 S complex (see below). In Fig. 3c, we have therefore represented Artemia salina 5.8 S rRNA in a secondary structure lacking helix A. As illustrated in Fig. 3a and b the main feature distinguishing our model from Nazar's consists in helix B, which connects multibranched loop M_1 with helix C via interior loop I. In Nazar's model the connection M_1 - C consists of a different helix ($GUGC_{36} \cdot GCAC_{114}$ in Xenopus) separated from C by a large bulge. Although the Nazar models, for the sequences where they are available, show a super-ficial similarity in this region, a drawing of the proposed pairing schemes on the alignment showed that they are much less conserved in position than helix B. Moreover, we have not been able to extend the Nazar pairing scheme for this area satisfactorily to the arthropod sequences. Calculated free energy changes of secondary structure formation are listed in Table 2. In order to allow a comparison with two other models, we calculated the energies for our model with areas A-A' base-paired (Fig. 3b). With the exception of Xenopus laevis and the two plant sequences, our model is more favourable energetically than Nazar's. Luoma and Marshall[31] have proposed a four helix model that shares only areas A-A' and F-F' with that of Nazar. The free energies are listed in Table 2 for 4 cases where such a model has been published. Apart from the fact that is is energetically less favourable, we have been unable to transpose this base pairing scheme satisfactorily to other sequences.

Model for 5.8 S - 28 S rRNA interaction

Having ascertained that the base pairing scheme defined in Fig. 2, with some reservations for helix A, applies to all known sequences, is energeti-cally favourable and highly conserved, we will now examine if it is compat-ible with the current knowledge on 5.8 S - 28 S rRNA interaction. Pace et al.[6] concluded from a partial RNAse digestion study on the 5.8 S - 28 S complex that the binding involves the 3'-terminal sequence of 5.8 S rRNA extending beyond loop F. Pavlakis et al.[7], found that in Drosophila ribosomes both the large and the small 5.8 S rRNA fragment bind independently to the 28 S rRNA as well as to each other. They concluded that both the 5'- and the 3'-terminal sequence of 5.8 S rRNA are involved in binding to 28 S.

Hypotheses concerning the precise 28 S rRNA sequences engaged in the in-teraction had to await data on the primary structure of these molecules.

Table 2. Free energy of secondary structure formation for different models

Species, references (a)	ΔG (kcal/mole)			
	Luoma-Marshall model	Nazar model	Our model	
			Complete (b)	Area B to F'(c)
Xenopus laevis[8,29]	—	-67.4	-65.3	-50.1
Drosophila melanogaster[7]	—	—	-23.0	-23.8
Sciara coprophila[14]	—	—	-33.9	-31.1
Artemia salina (this paper)	—	—	-16.0	-21.8
Crypthecodinium cohnii[30]	—	+ 3.5	- 9.4	-12.0
Acanthamoeba castellanii[32]	—	- 3.3	- 8.0	-12.0
Neurospora crassa[33,9,33]	-16.2	-17.9	-21.8	-25.0
Saccharomyces cerevisiae[34,1,31]	-24.0	-20.8	-26.0	-28.6
Thermomyces lanuginosus[35]	-10.0	-14.8	-20.9	-25.3
Triticum aestivum[36,36,37]	-12.0	-30.2	-21.1	-13.3
Vicia faba[38]	—	-28.0	- 9.9	- 1.9
Chlamydomonas reinhardtii[39]	—	+11.8	+ 2.7	+ 0.2

(a) If the sequence and the secondary structure model(s) are taken from different papers the references are given in the order : sequence, Nazar model, Luoma-Marshall model. If necessary, models were adapted according to subsequent sequence corrections.

(b) The complete model is defined by the boxes in the sequence alignment (Fig. 2).

(c) This is the part of the molecule that would remain if area A-A' and the pieces of loop M_1 connecting it to the rest of the molecule were cut off. It corresponds to the bold line structures in Fig. 3b, plus a small piece of connecting loop M_1.

Three different proposals have been made, illustrated in Fig. 4 (models 1-3). The first model[9] is based on Neurospora crassa 25 S rRNA 3'-end sequencing and assumes that both ends of the 5.8 S rRNA are bound to nearby sequences at the 3'-end of the large RNA. According to the second model[27,13] 5.8 S and 28 S rRNA are bound heads to tails, in other words, the 5.8 S forms a bridge between the extremities of the 28 S. In the case of Xenopus, specific sequences were designated[28] for the interaction (3'-end of 5.8 S)·(5'-end of 28 S). The third model[10] is based on the complete primary structure of the two RNAs in the yeast ribosome large subunit and postulates binding of the 5.8 S termini to two sequences, both near the 5'-end of 26 S rRNA, but separated
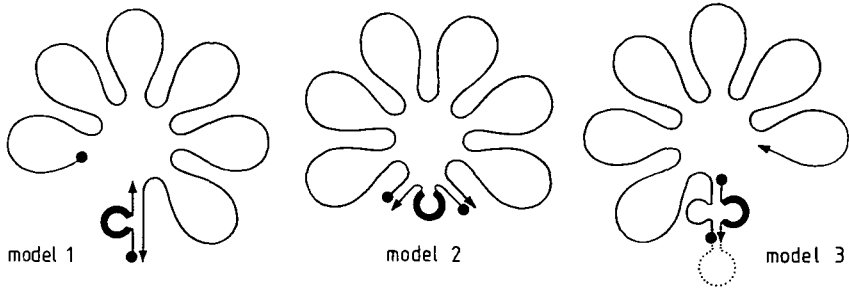
Fig. 4. Models for 5.8 S - 28 S RNA interaction

The two RNA chains are represented by lines of different length, the termini by a circle (5') and an arrowhead (3'). The petals of the 28 S rRNA flower symbolize its 7 secondary structure domains[10]. The heavy line-loop in the 5.8 S rRNA symbolizes the part of the structure not engaged in 28 S-binding and folded back upon itself.

model 1 : both ends of 5.8 S rRNA bind to the 28 S rRNA 3'-end.
model 2 : the 5.8 S rRNA ends bind to the 28 S rRNA ends heads to tails.
model 3 : both ends of 5.8 S rRNA bind to sequences, separted by approximately 300 nucleotides, near the 28 S rRNA 5'-terminus. The spacer in the primary transcript is drawn as a dotted line.
model 4 : a structure, analogous to model 3, can be drawn for E. coli 23 S rRNA, but the termini extend further and are base-paired.

by some 300 nucleotides. The latter model seems the most plausible because the proposed binding sites consist of stable helices, and because the resulting hybrid structure resembles that of the 5'-terminal domain of the bacterial 23 S rRNA[28]. An important difference remains : in the 23 S rRNA the two ends are joined by base-pairing (Fig. 4, model 4) but in yeast (Fig. 4, model 3), the 5'-end of 5.8 S rRNA does not reach the 3'-end of the 26 S rRNA. Interestingly, when Nazar[11] aligned trout 5.8 S rRNA with the 5'-terminus of E. coli 23 S rRNA, he had to leave an unmatched tail of 12 nucleotides at the 5'-end of the bacterial sequence. Precisely this tail is responsible for binding to some 10 nucleotides at the 3'-end. Hence the 5'-end of 5.8 S rRNA cannot bind to the 3'-end of 28 S rRNA because a short piece of sequence is lacking.

     The question now arises, how much of Nazar's 5.8 S rRNA secondary structure model (Fig. 3a), or our variant of it (Fig. 3b) remains intact after it has been stripped of the sequences necessary for binding to the large RNA ?

In Fig. 2 these sequences, as postulated by different authors, are underlined. In the case of Xenopus laevis and Neurospora crassa, the sequences interfere only with helix A. This is not surprising, since helix A is not very con- served in position, nor, with some exceptions, favourable energetically. The latter point is demonstrated in the last column of Table 2. If the sequences consisting of area A-A' and the parts of loop M_1 joining it to the rest of the molecule were cut off, a stabler structure would result in 8 out of 12 cases. However, the sequences of yeast 5.8 S rRNA that participate in inter-
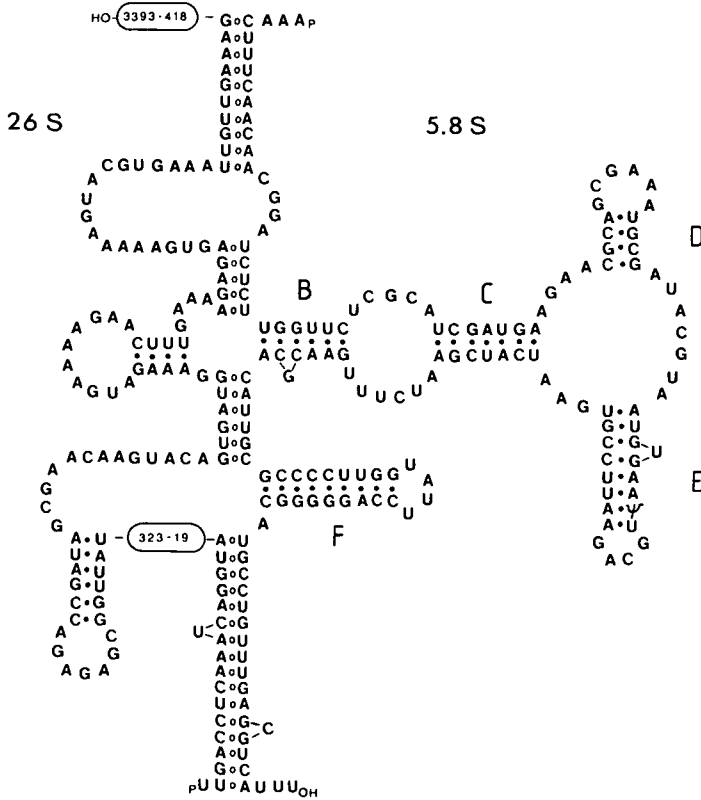
Fig. 5. Model for 5.8 S - 26 S rRNA binding in yeast.

The model is modified from Veldman et al.[10] who present a structure where the 5.8 S rRNA moiety retains only helices C,D,E and F, the sequences of helix B participating in 26 S rRNA binding. In our model, this role is taken over by the sequence linking B to F. The secondary structure of residues 19-323 and 418-3393 can be found in Veldman et al.[10]. Intermolecular base pairs are denoted by circles, intramolecular ones by dots.

action model 3, which we advocated above, interfere not only with helix A, but also with helix B. This potential helix is quite conserved in position and favourable energetically. We have therefore examined if the structure proposed[10] for the 5.8 S - 26 S rRNA interaction in yeast can be redrawn to an equally stable structure saving helix B. This is actually possible, as demonstrated in Fig. 5. The difference in free energy between the two schemes is only 2.6 kcal/mole in favour of the one originally proposed, which is negligible for such a large structure. This proves that the type of interaction postulated in model 3 (Fig. 4) is compatible with the existence in 5.8 S rRNA of helix B as well as helices C,D,E, and F. It does not prove, of course, that helix B actually exists. This will require at least some more comparative sequencing evidence, especially of 28 S rRNAs.

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