PROPERTIES AND MECHANISM OF SYNTHESIS OF RIBOSOMAL RNA IN EVOLUTION

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ABSTRACT

The molecular weights, base compositions and nucleotide sequences of ribosomal RNA show differences which can be systematically related to the major phyla of plants and animals. The ribosomal RNA precursor, which is the direct product of the ribosomal genes in the nucleolus, has diverged more than the mature RNA; the differences are in the excess RNA which is degraded during maturation; the warm-blooded animals have at least twice as much of this excess RNA as any other species.

INTRODUCTION

The ribosome is one of the organelles which are fundamental to the structure and functions of all cells. The basic mechanism of protein synthesis is common to all prokaryotes and eukaryotes. The same genetic code is used throughout, as are similar methods of amino acid activation, chain initiation and translocation. The constraints on such a structure changing during the course of evolution are therefore likely to be severe. A study of differences between ribosomes of various species may, however, give some useful information about the mechanisms of protein synthesis as well as some picture of the possibilities of evolutionary development of such an essential organelle. This paper considers briefly some aspects of the structure of ribosomal RNA in eukaryotic cells that illustrate the extent to which different regions of the molecule have diverged during evolution. The paper discusses some of our thinking in this field; it is not intended to be a systematic review.

There is a further reason for studying ribosomal RNA: this is that at present it provides the only situation in which it is possible to isolate and study directly the DNA cistrons involved, the primary transcription product of these genes, the maturation of this primary product and its transport from nucleus to cytoplasm and the organisation of the mature cytoplasmic product. Of course, the product is not a protein but an RNA, but it may nevertheless provide a reasonable model for the corresponding synthesis of messenger RNA and protein. The paper, therefore, includes discussion of the ribosomal RNA precursor.

In all organisms, the ribosome consists of two nucleoprotein sub-particles, known by their approximate sedimentation coefficients as 50 *S* and 30 *S* in prokaryotes, and as 60 *S* and 40 *S* in eukaryotes. The complex of proteins in these particles is not considered further here, although a study of their

evolution would be equally interesting. The larger sub-particles contain two molecules of RNA per particle, 23 *S* in prokaryotes and 28 *S* in eukaryotes with a small molecule of 5 *S* in each case. In addition, in all eukaryotes examined, a small molecule of about 7 *S* is bound to the 28 *S* and can be removed by unfolding the molecule by heat or by denaturing agents. The smaller sub-particles contain one molecule of RNA: 16 *S* in prokaryotes and 18 *S* in eukaryotes. These sedimentation coefficients will be used in this paper only as names to identify the various molecules. The actual sedimentation coefficients vary in different species, and the measurements vary in different laboratories. The following paragraphs discuss the molecular weights, base composition and some comparisons of nucleotide sequence of the 28 *S* and 18 *S* rRNA of eukaryotes. We do not consider the 5 *S* RNA.

MOLECULAR WEIGHTS OF rRNA

The ease with which relative molecular weights of RNA can now be determined by gel electrophoresis has led to a comparison of the molecular weights of rRNA from a large number of species. It became apparent that the molecular weights of the rRNA of all prokaryotes, including blue-green algae and chloroplasts, were similar, namely 1.1 and 0.56 million. The smaller rRNA of most eukaryotes was shown to be close to 0.7 million and the larger one varies from 1.3 to 1.7 million according to species[1]. In general, in all the plants including fungi and algae (both unicellular and multicellular) and in all the protozoa, the larger rRNA has a molecular weight of 1.3 million. Among the animals there is a progressive increase from the sea urchin and insects with 1.4 million through the amphibia with 1.5, birds with 1.6 and mammals with 1.7 to 1.75 million. There are one or two striking exceptions to this general trend such as *Acanthamoeba castellani* (1.55 and 0.9 million) and *Euglena* (1.3 or 1.5 and 0.9 million). In both cases, the larger sub-unit or 28 *S* rRNA was unstable.

Since 1968[1] more results have been obtained in many laboratories and while the general trend has been confirmed, a number of minor exceptions have been found. Some of these could be due to errors in the determination of the molecular weights due to differences in the conformation of the RNA, such as changes in extent of folding according to the content of G + C.[2] It is clear, however, that there are some minor differences in the true molecular weights of rRNA from closely related species.

Among these are the differences between the urodeles and anurans. The 28 *S* rRNA of *Triturus* and of the other urodeles examined has a weight of 1.4 million compared to about 1.5 million in *Xenopus* and other anurans[3].

The 18 *S* rRNA of Planarians (*Dugesia*) is larger than expected, with a molecular weight of about 0.8 million, while the 28 *S* rRNA compares to that of other lower metazoans with a molecular weight of about 1.4 million[4]. The rRNA of most of the fungi compares to that of other plants and Protozoa, but the Oomycetes and the slime moulds (*Physarum* and *Dictyostelium*) compare to the lower animals in which the 28 *S* rRNA has a weight of about 1.4 million[5].

We have examined some other species of amoeba in order to make a comparison with the anomalous *Acanthamoeba*. We found that the rRNA

of *Nigleria* is 1.3 and 0.7 million in common with that of other protozoa. This amoeba has a flagellated stage; maybe one could suggest that it is a flagellate with an amoeboid stage. The parasitic *Entamoeba* seems to have rRNA of approximately 1.6 and 0.67 million (samples kindly given by Dr. D. Barker;[6]): thus its larger rRNA compares to that of *Acanthamoeba* but the smaller one is like that of other protozoa. Other species are being examined by us at present; it is clear that there are likely to be large variations among some of these protozoa, and further investigations are needed.

THE STRUCTURE OF THE RIBOSOMES

The total size of the ribosome and of the ribosomal sub-units is related to that of the RNA, which accounts for approximately 50 per cent of the total weight. The overall structure of the ribosomal sub-units seems to have been conserved in evolution despite the large changes in the composition and sequence of the RNA. Cammarano *et al*[7] determined the bouyant densities in caesium chloride of fixed ribosomes and of their sub-particles from a range of species; this density is directly related to the ratio of protein: RNA of the particles. In all eukaryotes the buoyant density of the ribosome is between 1.580 and 1.590 $g\,ml^{-1}$, the latter value being characteristic of the higher animals, birds and mammals. Thus the proportion of protein to RNA is much the same throughout; the total weight of the larger sub-particle is correspondingly larger in the higher animals. Cammarano *et al* have confirmed that hybrid 80 *S* ribosomes prepared from pea and mouse or rat sub-units in either combination are active in the synthesis of polyphenylalanine coded by poly-U. Thus the structures have been conserved sufficiently to allow sub-units from different species to combine in an active form, although perhaps some of the more subtle details are distinct. Cammarano *et al* recently investigated the ribosomes of the *Acanthamoeba*. They found that the monoribosome is made up of a small sub-particle of molecular weight 1.76 million (as compared with 1.50 million for other eukaryotes) and a large sub-particle of 2.92 million. The latter is considerably more than the 2.40 million of the ribosome sub-particle of pea seedlings and other plants and is similar to that of the rat liver large sub-particle of 3.00 million. Unfortunately, however, the cell-free system from *Acanthamoeba* was extremely weak and the activity of the hybrid sub-particles could not be distinguished from the control rat liver large sub-particle. (Cammarano, personal communication).

BASE COMPOSITION OF rRNA

Larva Sanchez and his colleagues[8] have shown that the base compositions of the smaller and the larger rRNA sub-units can be correlated with the major groups in the plant and animal Phyla. In general, in the 'higher' plants and 'higher' animals, there is an increase in the proportion of G + C in rRNA compared to the 'lower' species; in some species there is a considerable difference in base compositions between the 18 *S* RNA and 28 *S* RNA. Amaldi and his colleagues have plotted the ratio of the content of (G + C):(A + U) for the 18 *S* rRNA against that for the 28 *S*. In the plant

kingdom, the values range from about 0.8 to about 1.5 (*Figure 1*). The lower algae and yeast start near the bottom with the lowest G + C ratios and representatives of the various groups stretch diagonally across the graph to a ratio of approximately 1.2. *Euglena*, which has anomalous rRNA also has a higher ratio for the 18 *S* than the 28 *S* rRNA. The dicotyledonous plants are all in a group with a higher ratio for their 28 *S* rRNA than for the 18 *S*; the monocotyledons have the highest ratio for 28 *S* RNA. This accords with the commonly held view that the monocotyledons are the most highly evolved of the plants. It is interesting to reflect that this evolution is correlated with a higher G + C content mainly of the larger or 28 *S* rRNA, in contrast to the situation in *Euglena* and the fungi. Interestingly, the cycads, ferns and gymnosperms all occur in a group between the tops of the fungi and the dicotyledonous plants with (G + C):(A + U) ratios of about 1.1 and 1.2 for the smaller and larger rRNA molecules.

Among the animal kingdom, the spread of the ratio (G + C):(A + U) is much wider, from about 0.5 to 2.0, (*Figure 2*); the protozoa form a diagonal line across the graph with *Tetrahymena* at the bottom; many other protozoa as a group are in the middle with a ratio for both rRNA sub-units of about 1.0 and the anomalous *Euglena* and *Acanthamoeba* near the top with ratios of around 1.3. The insects fall into two or three groups along a similar line. Above these the molluscs from *Murex* to the *Octopus* fall on a steep line in which the (G + C):(A + U) ratio of the 18 *S* rRNA increase as much as that of the 28 *S*. In contrast, the vertebrates from the fish and urodeles to the mammals form a spread-out group in which most of the increase in (G + C):(A + U) is only in the larger rRNA; man is at the extreme tip. It is interesting that *Triturus* (a urodele) and *Xenopus* and *Rana* (anurans) have a similar composition of 18 *S* rRNA but the G + C content of the 28 *S* rRNA is higher in *Xenopus*, which may be correlated with its higher molecular weight. It is also interesting that the echinoderms, which could be considered a stem from which the vertebrates evolved, are near the lower end of the spread of values for the vertebrates.

These results from both the plant and animal kingdoms make it amusing to speculate about the requirements for the highest evolution. It seems essential that the G + C content of the larger ribosomal sub-unit should be higher than that of the smaller, as in the case of the mammals and the monocotyledonous plants. We might say that the highly evolved octopus has failed to become a dominant species because it also increased the G + C content of its 18 *S* rRNA. It is clear that some of the minor variations in molecular weight of the rRNA such as between the anurans and the urodels, are correlated with differences in base composition; the exceptional rRNA of *Euglena* and *Acanthamoeba* also fit this idea. Thus, the changes that seem to have taken place during evolution are by no means random and suggest that it is worthwhile looking in more detail at different regions of the sequence of nucleotides in rRNA.

EVOLUTION OF THE NUCLEOTIDE SEQUENCE OF rRNA

Many questions would be solved if we knew the whole nucleotide sequence of the rRNA of a number of species. Although good progress has been made

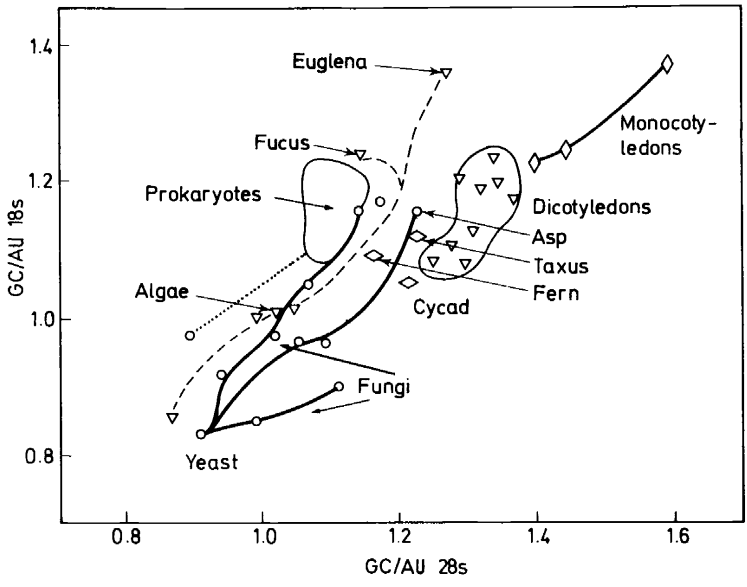

Figure 1. Base compositions of ribosomal RNA of fungi, algae and higher plants; (redrawn from Larva Sanchez., Amaldi and La Posta).

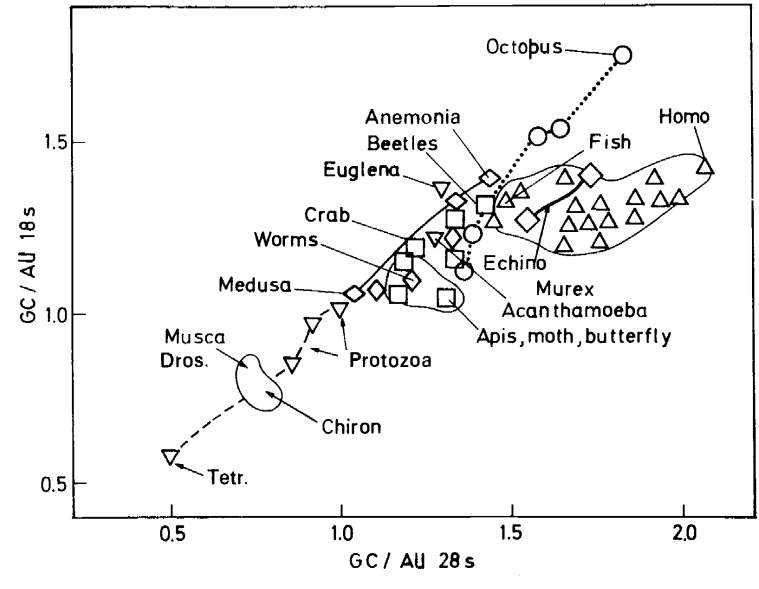

Figure 2. Base compositions of ribosomal RNA of animals; *Euglena* is included to provide a comparison to *Figuel.* (redrawn from Larva Sanchez, Amaldi and La Posta).

in the elucidation of the sequence of *E. coli* 16 *S* RNA and although fingerprint comparisons of digests of the RNA of higher organisms have been obtained, other approaches towards comparing sequences must be used at present. The determination of the extent and kinetics of hybridization of rRNA to DNA should in principle provide a large amount of data about the extent of homology between species. The problem with this approach is one of interpretation; the conditions and techniques of hybridization must be defined so that the hybrid lengths of poorly matched sequences can be determined and distinguished from hybrid of a shorter but perfectly matched region. Birnstiel and Grunstien[9] have used the techniques developed by Melli *et al*[10] in which the hybridization is done with a vast excess of DNA; the kinetics of renaturation of sequences of the DNA are followed by using trace amounts of very highly radioactive RNA. The time of half-hybridization of RNA to the DNA sequences gives a measure of the number of copies of these DNA sequences; the extent to which the RNA becomes absorbed into the hybrid gives a measure of the proportion of the RNA which is homologous to the DNA. Any heterologous or very poorly matched sequences of the RNA will not hybridize under suitably stringent conditions. The method requires large amounts of unlabelled DNA from a few species and small amounts of very radioactive RNA from the species to be investigated. Birnstiel and Grunstien showed that the time of half-renaturation of the ribosomal DNA was a characteristic of the DNA and was the same using RNA from a wide variety of species, even as heterologous as sea urchins and mammals[9]. The extent of hybridization, however, varied with the extent to which the RNA was related to the DNA; thus sea urchins RNA hybridize poorly to human DNA whereas the more homologous systems such as *Xenopus* RNA hybridized to human DNA to a greater extent. Even with the most heterologous systems, 10 to 13 per cent of the RNA hybridized to the DNA with kinetics characteristic of the DNA. Competition experiments showed that this represented the same small region of all species of rRNA. It thus appears that about 10 to 13 per cent of the sequence of ribosomal RNA has been strongly conserved in evolution. The remainder of the molecule appears to have evolved and the extent of change increases with the divergence of the species. The changes in base compositions which Amaldi and his colleagues described would therefore probably be more striking if they were concerned only with the 85 per cent of the molecule which diverged.

INSTABILITY AND HIDDEN BREAKS IN THE 28*S* RNA

It is a peculiar property of 28 *S* rRNA, especially from lower organisms, that the molecule is easily degraded into two pieces. For many years there was some doubt about whether the 28 *S* rRNA was in fact a single polynucleotide chain *in vivo*, and whether the cleavage was an artifact produced by ribonuclease action during isolation, or whether the molecule consisted of two parts *in vivo*. In addition to this probable break in the molecule, all eukaryotic 28 *S* rRNA has bound to it a small RNA molecule originally called 7 *S*;[11,12] its true sedimentation coefficient is closer to 5.6 *S*.

Several studies have now suggested that a break in the 28 *S* rRNA is

frequently formed *in vivo*, and that a large proportion of the ribosomes of some species are 'nicked' in this way. In the higher organisms, especially the amphibians and mammals which have been extensively studied, the 28 *S* rRNA appears to be more stable.

In the lower animals, it is clear that the newly synthesized 28 *S* RNA is a single stable polynucleotide chain (probably with 7 *S* RNA attached) and that the nick is formed some time after synthesis, so that only the older ribosomes contain broken RNA. The time at which this happens, and therefore the proportion of broken RNA, varies in different species from a few per cent to almost all the ribosomes. Also, the extent of unfolding of the RNA required to allow the two pieces to separate varies in different species, probably according to the content of G + C and the secondary structure of the RNA. Hidden breaks in 23 *S* rRNA of prokaryotes have also been found; the 23 *S* RNA of chloroplasts is largely broken in this way[13]. Some examples of 28 *S* RNA in eukaryotes, in which breaks into two or more pieces are revealed when the RNA is denatured in low salt concentrations, by heat or by urea or similar agents, are as follows: *Tetrahymena* 28 *S* is nicked near the middle of the molecule and is so readily melted that some care is needed during extraction to maintain the secondary structure of the 28 *S* intact[14]. In *Drosophila* and *Chironomous*, the 28 *S* RNA is obtained by normal methods of RNA extraction, but is readily melted by heat, and yields RNA with the same weight as 18 *S*. Rubinstein and Clever showed that in *Chironomous*, the nick is formed sometime after the synthesis of the 28 *S*, so that there is a small percentage of young intact 28 *S* molecules in the cell[15]. The snail, (*Ilyanassa*) similarly has unstable 28 *S* rRNA[16].

The rRNA of the higher animals (and plants) seems to be more 'stable', in the sense that it contains few nicks and is not so easily melted. The low proportion of nicks in the 28 *S* rRNA of rat liver seem to occur entirely in old ribosomes[17].

Since the original finding that the 28 *S* rRNA of *Acanthamoeba* is unstable, both we[18] and Stevens and Pachlar[19] have found that the instability is due to the presence of one nick near the centre of the molecule. The 1.55 million RNA on being melted yields two pieces of 0.9 and 0.7 million approximately, as well as the small 28 *S*-associated RNA, ("7 *S*"). Almost the whole of the 28 *S* RNA is cleaved in this way when melted but a very small amount, visible on polyacrylamide gel electrophoresis, remains and is stable. We have shown (Ref. 18 and Urquhart and Loening, in preparation) that the 28 *S* obtained after labelling for less than two hours, does not have this nick and is stable to melting. Thus, we can be fairly confident that the nick occurs *in vivo*. In the case of this amoeba and some other lower organisms, almost all ribosomes are nicked; it is clear that such ribosomes must be active in protein synthesis *in vivo*.

It is striking that in most species the nicks in the 28 *S* rRNA are in or near the centre of the molecule, and that its molecular weight is always about twice that of the 18 *S* RNA. The result is that the cleaved fragments of the 28 *S* RNA always have about the same size as the 18 *S* RNA. It is perhaps worth speculating about whether there is any significance in this. One could contemplate the idea that 28 *S* rRNA has evolved by gene duplication of a sequence similar to that of 18 *S* RNA, followed by divergence of the two

parts. The structure of the larger ribosomal sub-particle (60 *S*) could be such that the two halves from which it may have originated are linked by the 28 *S* RNA chain, but the linking region is still vulnerable to nuclease attack; it may be readily cleaved by nucleases either *in vitro* during isolation or *in vivo*; the cleavage would be in the same position as required to produce a piece of 18 *S* rRNA from a polycistronic precursor molecule. This hypothesis would predict that the nucleotide sequences of the 28 *S* RNA should bear some resemblance to that of the 18 *S*. There is in fact a considerable amount of data which suggests that there is some sequence homology between the two rRNA sub-units, but most of it is difficult to evaluate for the reason that many preparations of 18 *S* RNA were contaminated by the nicked pieces of 28 *S* RNA. However, Retel and Planta[20], for example, have found convincing evidence that the two rRNA components of yeast are related, and we (Grierson and Loening in preparation) have found the same for plants. The extent of the competition observed between 28 *S* and 18 *S* RNA during hybridization to DNA of course varies with the stringency of the conditions of hybridization used. There seems to be no homology between the RNA subunits among the higher animals, such as *Xenopus* and mammals[21].

Perhaps the position of the nick in the 28 *S* rRNA of *Acanthamoeba* is significant: of the fragments produced, one has the size of the 18 *S* rRNA of most eukaryotes, and the other the size of its own smaller sub-unit rRNA. Either this is a coincidence, or it means that the 28 *S* rRNA evolved from a combination of two 18 *S* type sequences; one of these would have increased in size from the usual 0.7×10^6, to provide the 0.9 million rRNA as well as part of the larger rRNA sub-unit. On this hypothesis, the cleavage of the precursor molecule which was previously required to produce 18 *S* rRNA still occurs in the present 28 *S* rRNA molecule. Thus it would be interesting to find out if this cleavage in *Acanthamoeba* is the result of normal nuclease action, or takes place in the nucleus with the unidentified enzyme used for processing.

EVOLUTION OF THE RIBOSOMAL RNA PRECURSOR

Ribosomal RNA is synthesized in the nucleolus, as a large precursor molecule which contains the sequences of 18 *S* and 28 *S* RNA as well as some additional or excess RNA. During maturation to ribosomal subparticles, the precursor is cleaved in a series of steps to yield the rRNA molecules and the excess RNA is degraded. The ribosomal genes are reiterated 100 to 1000 times in different species, and are in the DNA of the nucleolar organizer[21]. This large number of genes is essential to maintain a rate of synthesis of up to 100 new ribosomes per second, to provide 1 to 5 million ribosomes per cell in rapidly dividing cells. There are stretches of DNA between the ribosomal cistrons, which seem not to be transcribed, and have been called spacer regions. The transcription and spacer regions can be seen in the electron microscope[22].

Thus the precursor RNA which is synthesized is longer than the mature molecules and the stretches of the DNA are longer than the transcribed lengths.

A probable structure of the ribosomal precursor is shown in *Figure 3*: the

distribution of the regions of excess RNA are based on studies on the Hela cell[23]. It is probable that after synthesis, the 45 *S* precursor, with a molecular weight of about 4.2 million, loses about 1.0 million of excess RNA from one end (shown in *Figure 3* adjacent to the 18 *S* region, but it is not known at which end of the molecule it actually occurs); the remaining molecule of 3.2 million is then split to give immediate precursors of 2.2 million to the 28 *S* RNA and of 1.0 million to the 18 *S* RNA; each of these then lose pieces of excess to give the mature ribosomal RNA.

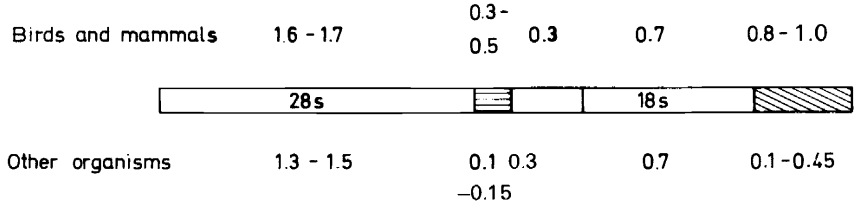

Figure 3. The structure of the ribosomal RNA precursor. The molecular weights of the different regions of the molecule are indicated in millions (from Grierson *et al.*).

Two laboratories have investigated the sizes of the precursor of ribosomal RNA in a number of different species[23–25] and the results are summarized in *Figure 3*. The very high molecular weight 45 *S* precursor, which contains about 40 per cent of excess RNA, was found only in the warm-blooded animals, the mammals, birds and marsupials. All the cold-blooded animals and the plants have ribosomal precursors which contain only 10 to 25 per cent of excess RNA. Also, the immediate precursors to 28 *S* RNA contain more excess RNA in the warm-blooded animals than in any other organisms. No species has been described which contains an intermediate amount of excess RNA. Since in *Xenopus*[22] the ribosomal genes also contain lengths of DNA between the reiterated sequences which appear not to be transcribed, both Perry and Loening and their collaborators have suggested that the variations found in the molecular weights of the precursor result from a shift in the positions of initiation or termination of transcription[24,25]. This would provide a simple mechanism for the evolution of different sizes of precursor without any drastic alteration in the lengths of the DNA. Even closely related species could then have ribosomal RNA precursors which differ in weight. Examples of these have been given previously and were reviewed by Grierson *et al*[23,26]. We also noticed that the ribosomal precursor as analyzed by polyacrylamide gel electrophoresis appears to be heterogeneous. It is possible that this heterogeneity is due to a true difference in the transcribed lengths of the RNA, although conformational differences may contribute. If there are such differences, then it is conceivable that different tissues of the same organism may transcribe different sizes of precursors. Evidence for this has been obtained[26] although we think now that there may be other interpretations of this result. A heterogeneity in the 45 *S* RNA of Hela cells has also been reported by Tiollais *et al*[27], from a different point of view.

The difficulties of determining the true molecular weight of the ribosomal RNA precursor and the various reasons why it could appear to be heterogeneous even if the molecules may in fact all have the same chain lengths, were briefly discussed previously[18]. Despite the difficulties of measurement, it is very likely that the differences in weight between precursors from different organisms are very much greater than the differences between the final product of processing i.e. the ribosomal RNA. There seems, therefore, to have been less stringency in the conservation of the precursor during evolution than there has been for the ribosomal RNA. These results correlate very well with similar conclusions reached by Brown *et al*[28]; they show that the sequences of the spacer regions in the ribosomal DNA of *Xenopus laevis* and *Xenopus mulleri* had diverged, while the transcribed (40 *S*) precursor RNA molecules were similar.

Perhaps the peculiar properties of the RNA of *Acanthamoeba* could again be used to give us a clue about the mechanism of evolution of the sizes of ribosomal RNA. This amoeba, as stated above, has ribosomal RNA each of whose components are 0.2 millions heavier than would be expected in comparison with most other protozoa. If we make the assumption that most of the interior of the precursor has been slow to evolve and that the evolution in length has been through a shift in initiation or termination, we could explain the existence of higher molecular weight rRNA in amoeba by suggesting that the immediate precursors to the mature molecules fail to be cleaved, and become wrapped up in the finished ribosome. We therefore investigated the ribosomal RNA precursors in this amoeba and the results were briefly described at another symposium[18]; we found that this idea was completely wrong. There is a precursor to the amoeba 1.55 million rRNA which has a molecular weight of about 1.85 million. This difference of about 0.3 million in the amount of excess RNA compares to that in the warm-blooded animals rather than to that in other organisms. We do not know whether there is a precursor to the amoeba 0.9 million rRNA. The ribosomal RNA precursor which we assume to be the primary transcription product, was found to have a molecular weight of about 3.5 million and this is cleaved to a molecule of about 2.9 million losing about 0.6 million. This amount of excess RNA is also much larger than that found in any other cold-blooded organism. Thus while the components found in this amoeba would fit the scheme of processing of the precursor proposed for the Hela cell, the molecular weights of all the components are larger than would be expected for a protozoan.

The interpretation of all these results depends on detailed knowledge of the topology of the components of the precursor; at present it is not known whether the structures and schemes of cleavage of the precursors are similar in all species. If there is a universal structure, then one can conclude that the amount of excess RNA at one end of the molecule has diverged very much more than the rRNA; but exceptional species, such as the amoeba, indicate that all parts of the precursor can evolve in size.

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