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Evolution of the Transcription Unit of Ribosomal RNA*

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Abstract. In eukaryotes the two principal RNA components of the ribosomes are initially synthesized as a large complex precursor molecule, which may be thought of as a transcription unit. The precursor is converted, via intermediates, to the mature forms of ribosomal RNA (rRNA). In order to assess the extent of variation in the size of this rRNA transcription unit among different organisms, and to infer its possible mode of evolution, we have determined its molecular weight in several selected species. Pulse-labeled and long-term labeled RNA's were extracted from various types of cells, and analyzed by electrophoresis on acrylamide gels. Identification of particular components as rRNA precursors was made according to several stated criteria. Our results, together with an analysis of previously published data, suggest that in plants and lower animals, up to and including reptiles, the unit of transcription of rRNA is a 2.7–2.8 million dalton molecule, which is only about 25 per cent larger than its combined rRNA products. In contrast, birds, marsupials and placental mammals, exhibit a seemingly less economical form of rRNA synthesis. Their transcription units are 4.0–4.2 million daltons, about 80 per cent larger than the rRNA products. In the organisms with the smaller transcription unit the major intermediate precursor of rRNA is 1.5–1.6 million daltons, as compared to 2.0–2.2 million daltons in birds and mammals. The significance of these findings is discussed in relation to evolutionary changes in the base composition of the ribosomal RNA genes.

In eukaryotic organisms the two major ribosomal RNA's (rRNA's) are coded for by multiple genes located at the nucleolus-organizing regions of the chromosomes.[1] These genes are transcribed as complex precursor molecules[2, 3] which may be termed the rRNA transcription units (RTU's). These precursors are then subsequently converted in the nucleoli to the ribosomal components.[4, 5] In mammals, where this phenomenon has been studied in considerable detail, only slightly more than half of the RTU, or "45*S* component" as it is commonly called, is conserved in the maturation to rRNA: a precursor molecule of over 4 million daltons is reduced to two rRNA's, which together weigh about 2.4 million daltons.[6] By comparison, in an amphibian, *Xenopus laevis*, a much larger part of the precursor molecule is utilized: here two rRNA's with a combined weight of 2.2 million arise from an RTU of less than 3 million.[7]

In view of this striking difference and the possible limitations it might impose on a general model for rRNA production, we have investigated the sizes of the RTU's and their degree of conservation in other types of organisms, particularly in other vertebrates. Moreover, the possibility of discerning an evolutionary trend is also attractive. A similar analysis of the rRNA's themselves, indicated that in animal evolution there is a small progressive increase in the size of the large rRNA component and a constancy in the size of the small rRNA component as one ascends the phylogenetic scale.[8]

Our study has been greatly facilitated by the use of *in vitro* cultured cells of certain selected species. With cultured cells it is a relatively simple matter to label the RTU's and to extract them from nucleoli in an undegraded form, whereas with intact tissues and whole organisms this is not always the case.

Our results, taken together with data from the literature, suggest that a 2.7–2.8 million transcription unit, of which 70–80 per cent is conserved in the conversion to rRNA, is characteristic of plants, invertebrates, and lower vertebrates up to, and including, reptiles. Birds and marsupials resemble the placental mammals, insofar as they synthesize larger RTU's and, hence, exhibit a seemingly less economical form of rRNA processing.

Materials and Methods. Animal cell cultures—(1) Sources: Mouse L cells, grown in suspension culture at 37°C, were those used routinely in this laboratory.[2, 9] Marsupial kidney (*Potorous tridactylis*, *Ex* ATCC no. CCL35), grown in monolayer cultures, were kindly provided by Dr. D. A. Hungerford of this Institute. Primary cultures of chick fibroblasts were made by the conventional trypsinization procedure from 11-day-old embryos. Monolayer cultures of established lines[10] of iguana liver cells (*Iguana iguana*) and rainbow trout gonad (*Salmo gairdneri*) were kindly supplied by Dr. Fred Clark of the Wistar Institute, Philadelphia. Cells of a haploid embryo line of *Rana pipiens* were grown in plastic culture flasks at 25°C as described elsewhere.[11] The potoroo, chick, iguana, and trout cells were grown in plastic flasks in Eagle's minimal essential medium—10% fetal calf serum. The potoroo and chick cells were grown at 37°C, the trout cells were grown at 25°C, and the iguana cells were grown at both 30° and 37°C.

(2) **Radioactive labeling and harvesting:** In a typical experiment replicate cultures of a particular cell type, each containing about 10^6 proliferating cells, were incubated in 20 ml of medium containing 1–5 μCi/ml [^{3}H]uridine for 0.5 or 1 hr and 2.5 hr. A third culture was treated for 1.5 hr with 0.04–0.08 μg/ml actinomycin D, and then labeled 0.5–1 hr with [^{3}H]uridine. In some experiments additional cultures were labeled 0.5 or 1 hr with 5 μCi/ml [^{3}H]methyl-methionine in the presence of unlabeled adenosine and guanosine.[6] After incubation the radioactive medium was drawn off and the cells removed from the plastic surface by gentle agitation with glass beads in balanced salt solution lacking divalent cations and containing 1 mM ethylenediamine tetraacetate (EDTA). Each sample of [^{3}H]-labeled cells was mixed with 2×10^7 unlabeled L cells, freshly obtained from an exponentially growing suspension culture, and pelleted. The L cells served as a carrier and also as an internal indicator for the effectiveness of subsequent extraction procedures in isolating undegraded RTU (*vide infra*).

(3) **Extraction of RNA:** The pellet of mixed cells was fractionated into nucleoli, nucleoplasm, and cytoplasm as described by Penman[5] with slight modifications detailed previously.[12] RNA was extracted from each fraction with phenol-sodium dodecyl sulfate (SDS).[12]

Other organisms: Twenty to fifty *Drosophila virilis* or *D. melanogaster* adult females were harvested 20 min after injection with 2.5 μCi [^{3}H]uridine/fly or 3 hr after injection of 45 μCi $^{32}PO_4^{=}$/fly. RNA was extracted from whole flies with phenol-SDS in the cold as described previously.[13, 31] Pith tissue of tobacco (2 *N* hybrid of *Nicotiana*

glauca × *N. langsdorfia*) was grown in culture in a modified Murashige and Skoog medium (G. Hagen, unpublished) at 24°C and labeled for 0.5–1.0 hr with 20 μCi/ml [^{3}H]uridine. After the bulk of the rRNA was extracted from the tissue with phenol the interphase was re-extracted with 0.5% SDS-phenol at 55°C to yield the RTU.

Analysis of RNA's: The RNA's were analyzed by electrophoresis on 8 cm, 2.8% acrylamide gels.[14] To insure that the RNA samples did not contain any large DNA strands which might clog the gels, they were incubated briefly with 100 μg/ml DNAase (DPFF, Worthington Biochemical Corp.) before layering on the gels. In most experiments the RNA sample applied to the gel was mixed with a small quantity of RNA extracted from the nucleoli of L cells that were labeled for 3.3 hr with $^{32}PO_4^{=}$. This RNA served as a set of reproducible and precise radioactive mobility markers since it contained 45*S*, 41*S*, 36*S*, 32*S*, 28*S*, and 18*S* components (cf. Fig. 1). After electrophoresis for 5–5.5 hr, the gels were scanned at 260 mμ on a Gilford recording spectrophotometer, frozen, sliced with a manifold of razor blades, and counted.[12]

Determination of molecular weights: Molecular weights were determined graphically using the relationship between mobility and logarithm of molecular weight.[8, 15] On the basis of a literature consensus[16] the 28*S* and 18*S* rRNA of L cells were assigned values of 1.70 and 0.65 × 10^6 daltons, respectively. From these values and data from 20 separate gel fractionations of 12 different samples of L cell nucleolar RNA the molecular weight of the 45*S* component was calculated to be 4.19 million daltons with a standard error of the mean of 0.05. A similar determination of the molecular weight of the L cell 32*S* component gave 2.16 ± 0.01 million daltons. The 45*S*, 32*S*, and 28*S* L cell RNA's then served as calibration markers for the RTU's and intermediates of the other organisms. The molecular weights of the cytoplasmic rRNA's of the various organisms were calculated similarly using L cell rRNA as a standard.

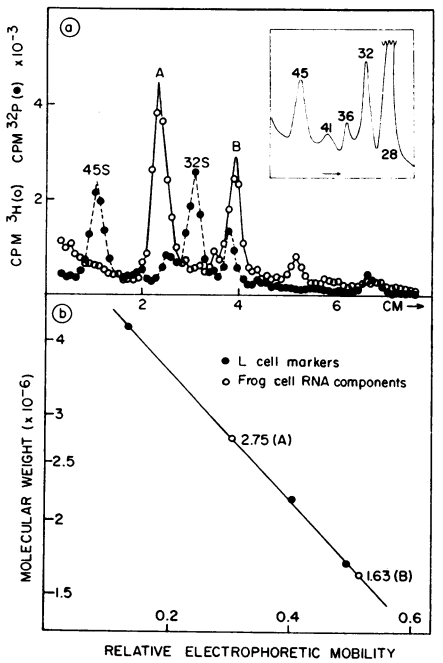
An analysis of bandwidth on the gels and of the variance encountered in the determination of the sizes of the various RNA components indicates that the uncertainty in a single measurement is of the order of 2%.

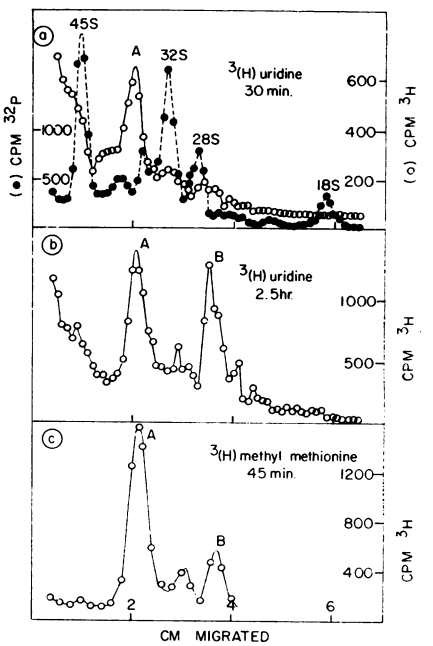
With the electrophoretic buffer used (0.04 *M* Tris, 0.02 *M* sodium acetate, pH 7.2, 10^{-3} *M* EDTA, 0.2% SDS) the effect of base compositional differences on relative mobilities is, for the most part, negligibly small. In an extreme case, e.g., *Drosophila* vs. mammals, it leads to a slight overestimation of the molecular weights of the *Drosophila* components relative to those of mammals.[17]

Results. One or more of the following criteria were used for identifying the RTU's and the rRNA intermediates of the various organisms. (*a*) They must be homogeneous, well defined components, which are uniquely nucleolar constituents, i.e., they are present in the RNA extracted from isolated nucleoli, and not in RNA extracted from nucleoplasm or cytoplasm.[2, 5] (b) The components are methylated, i.e., they are labeled when cells are incubated under appropriate conditions with [^{3}H]methylmethionine.[5] (c) The synthesis of the components is selectively inhibited with a low dose of actinomycin D.[2]

The first RNA component to be labeled after incubation of the cells with radioactive uridine is considered to be the RTU. A nucleolar RNA component, in which label subsequently appears, is considered to be an intermediate.

Figure 1*a* illustrates the acrylamide gel electrophoretic pattern of nucleolar RNA of frog cells that were incubated 1 hr with [^{3}H]uridine. One notes two major components (A and B) which are distinct from those of a [^{32}P]-labeled sample of L cell nucleolar RNA which was run on the same gel. Nucleolar RNA from frog cells that were labeled for 2.5 hr gave a similar pattern except that the relative amount of the A component was diminished. RNA extracted from the nucleoplasm was very heterogeneous and gave no evidence of containing com-





(*Left*) FIG. 1.—Estimation of molecular weights of frog nucleolar RNA's. (*a*): Acrylamide gel electrophoresis of a mixture of nucleolar RNA from frog cells labeled 1 hr with [^{3}H]uridine (—○—) and mouse L cells labeled 3.3 hr with $^{32}PO_4$ (- -●- -). The RTU and major intermediate are designated A and B, respectively, for frog cells and 45*S* and 32*S*, respectively, for L cells. *Inset:* A_{260} trace of same gel showing profile of L cell nucleolar RNA which was co-extracted with frog RNA. (*b*) Semilogarithmic plot of molecular weight vs. relative electrophoretic mobility, based on the data shown in (*a*). Molecular weight values for L cell RNA's were determined as described in *Methods* and used to establish calibration line. Abcissa represents distance from origin to a particular component divided by total length of gel.

(*Right*) FIG. 2.—Acrylamide gel electrophoresis of iguana nucleolar RNA. Nucleolar RNA from iguana cells incubated at 37°C for 30 min (*a*) and 2.5 hr (*b*) with [^{3}H]uridine or 45 min with [^{3}H]methyl-methionine (*c*). (- -○- -) [^{3}H] iguana nucleolar RNA; (- -○- -) mouse L cell nucleolar RNA (same preparation as that used in the experiment of Fig. 1). Peaks are labeled as in Fig. 1.

ponents A and B. The fact that the nucleolar RNA from the unlabeled L cells which had been mixed with the frog cells contains normal proportions of the rRNA precursors (inset to Fig. 1*a*) strongly indicates that no degradation occurred during nucleolar isolation or RNA extraction, and hence that component A is not a breakdown product of a larger precursor. A similar verification was made in the experiments with trout, iguana, chicken, and potoroo cells.

Using the semilogarithmic relationship between molecular weight and electrophoretic mobility, and values for the L cell nucleolar RNA components determined as described in the *Materials and Methods* section, the molecular weight of frog RNA components A and B were calculated to be 2.75 and 1.63 $\times$ 10^6 daltons, respectively (Fig. 1*b*). The value for component A is in relatively good agreement with one reported by Birnstiel and coworkers for the RTU of another amphibian, *Xenopus laevis*.[7]

A similar set of gel profiles from experiments with reptilian cells is illustrated in Figure 2. After a 30-minute incubation with [^{3}H]uridine a labeled component, A, with essentially the same mobility as that of the analogous component in frog cells, is observed (Fig. 2*a*). When the incubation is prolonged for an additional two hours (Fig. 2*b*), this component is still prominent and in addition the component B is evident. Both of these components are labeled when the cells are incubated with [^{3}H]methyl-methionine (Fig. 2*c*), substantiating the contention that they are rRNA precursors. The slow-moving material near the origin of the gels shown in Figures 2*a* and *b* is not methylated, and might represent very large nucleoplasmic RNA's contaminating the nucleolar RNA preparation. There were no detectable mobility differences in the RNA's from iguana cells cultivated at 30° and 37°C.

A further indication that component A is indeed an RTU is that its synthesis is selectively inhibited by low doses of actinomycin D. This is seen in Table 1

TABLE 1. *Selective effect of actinomycin D on RTU synthesis.*

Organism	Percentage of Residual Synthesis $\left(\frac{\text{actinomycin}}{\text{control}}\right)$ RTU	Nucleoplasmic RNA
Mammal (mouse L cells)	0	100
Marsupial (potoroo)	0	82
Reptile (iguana)	0	62
Amphibian (frog)	20	70

Cells were exposed to 0.08 μg/ml actinomycin D 30–90 min, and then labeled for 0.5–1 hr with [^{3}H]uridine in the presence of drug. Control cells, not treated with actinomycin, were labeled for equivalent periods. Nucleolar RNA was analyzed on acrylamide gels and the inhibition of the synthesis of RTU (45*S* component for L cells and potoroo, component A for iguana and frog) was calculated by comparing the extent of labeling of these components in control and treated cultures. Values for nucleoplasmic RNA were obtained from measurements of the total radioactivity in the nucleoplasmic fraction. For mammals and amphibians, portions of the control nucleoplasmic RNA's were analyzed on acrylamide gels to verify that they did not contain detectable amounts of RTU, and hence were not contaminated with nucleolar RNA.

where the inhibition of labeling of the 45*S* component in mouse and potoroo cells, and component A in frog and iguana cells, is compared with the inhibition of synthesis of the respective nucleoplasmic RNA's. Although the differential effect in the organisms with the smaller RTU was not as pronounced as that found in mammals, it was nevertheless readily detectable.

Table 2 presents a summary of molecular weight data for the various organisms studied. The values for the rRNA's are in very good agreement with those reported previously by Loening.[8] Whereas the small rRNA's do not differ significantly among organisms, the large rRNA's vary from about 1.3×10^6 daltons (plants) to 1.7×10^6 daltons (mammals). The RTU's seem to be essentially of two major size classes: those of approximately 4 million daltons, found in birds and mammals, and those of 2.7–2.8 million daltons, found in the plant and lower animals. The RTU of mammals is approximately 0.27×10^6 daltons larger than that of birds. This difference is more than three times the limits of error, and is thus significant.

In birds and marsupials, as in the placental mammals, the nucleoli contain a "32*S*-type" intermediate which gives rise to the large rRNA component by a

TABLE 2. *The comparative size of rRNA's and their precursors in selected organisms.*

Organism	Number of determinations	Criteria*	Molecular Weights (daltons × 10^{-6}) RTU	Intermediate†	rRNA Large	rRNA Small	Per cent of RTU conserved
Rodent (mouse)	(20)	(a, b, c)	4.1_9	2.1_6	1.7_0	0.65	56
Marsupial (potoroo)	(2)	(a, c)	4.1_9	2.1_6	1.7_0	0.65	56
Bird (chicken)	(2)	(a, c)	3.9_2	1.9_8	1.6_1	0.63	57
Reptile (iguana)	(3)	(a, b, c)	2.7_4	1.5_8	1.5_1	0.62	78
Amphibian (frog)	(2)	(a, c)	2.7_6	1.6_5	1.5_8	0.61	79
Fish (trout)	(1)	(a)	2.7_0	1.6_0	1.5_5	0.65	81
Insect (*Drosophila*)	(2)	(b)	2.8_5	1.6_0	1.4_0	0.65	72
Plant (tobacco)	(4)		2.7_6	1.5_0	1.2_9	0.66	71

Values represent the arithmetical mean of the molecular weights determined graphically as in Fig. 1*b*. In all cases duplicate determinations agreed to within 2%.

* (a), (b), and (c) refer to the criteria used in identification of rRNA precursors, discussed in text at beginning of *Results*. In the case of tobacco, identification is based on experiments involving pulse labeling with [^{3}H]uridine followed by a chase with actinomycin D.

† Intermediate: Signifies the component analogous to the 32*S* RNA of mammals, characterized by its being the major nucleolar RNA which is labeled subsequently to the RTU.

further molecular weight reduction of some 400 thousand daltons. In comparison, the other organisms exhibit intermediates that are only about 50–200 thousand daltons larger than the respective large rRNA's. Thus in the higher vertebrates, each step in the maturation of rRNA may entail a larger reduction in molecular weight. The over-all proportion of the RTU which can be accounted for in the mature rRNA is only about 56 per cent in birds and mammals, whereas it is 70–80 per cent in the other organisms (Table 2).

Discussion: Although in the present study we have compared only one species from a particular group of organisms, there is reason to believe that the sizes of the RTU's and the relative amounts of nucleotide conserved in the RTU→rRNA transition are typical of the groups which these species represent. For example, among cells derived from placental mammals, the size of the 45*S* RTU of mouse L cells seems to be indistinguishable from that of human HeLa cells[6] or from that of rat liver tissue.[18] As for amphibia, our value for the RTU of *Rana* is close to that reported for another anuran, *Xenopus*,[7] and very similar to what one would estimate for the RTU of urodeles on the basis of the 38–40*S* sedimentation coefficient reported by Gall.[19] Moreover, a comparison of published sedimentation analyses indicates that there is no appreciable difference in size between the 37-38*S* RTU's of the flies *Drosophila*,[13] *Chironomus*,[20] and *Rhynchosciara*.[21] Finally, according to recently published sedimentation profiles, it appears that the RTU's of yeast,[22] the protozoans *Tetrahymena*,[23] *Amoeba*,[24] and *Gyrodinium*,[25] and the annelid *Urechis*[26] are also of the 38*S* type. Since these comparisons encompass a wide range of experimental material that includes intact organisms as well as cultured cells, it seems safe to conclude that the size of the transcription unit is a characteristic of the organism, and is independent of whether a cell is growing in culture or not.

This analysis makes it reasonable to consider two major events in the evolution of the mechanism for rRNA production. The earliest event involves a change from the transcription of individual rRNA components, as occurs in prokaryotes,[27] to the production of a complex precursor molecule containing

both rRNA components, as occurs in eukaryotes. The transcription unit, a molecule of about 2.7 million daltons, appears to have remained relatively uniform in size throughout long periods of plant and animal evolution. The second event involves an abrupt increase in the size of the RTU from 2.7 million daltons (20–30% larger than the sum of the rRNA's) to about 4 million daltons (about 80% larger). This event could have occurred in early reptilian evolution, and have been stabilized, with a minor variation, in the separate branches which led to birds and mammals. It should be noted that the multiplicity of rRNA genes, which is a common property of all organisms, requires that any mutational event which changes the size of the transcription unit be propagated or magnified so as eventually to embrace all the rRNA gene copies.

A central question, which for the moment remains in the speculative realm, is whether vertebrate evolution of the RTU entails an enlargement of the genes to include additional nucleotide sequences, or alternatively whether these sequences are present in the lower forms as "spacers" which are not transcribed. One piece of evidence that might support the latter contention is the electron microscope observation of Miller and Beatty[28] suggesting the possible existence of nontranscribed portions of amphibian rRNA genes. These spacer segments correspond to about 40 per cent of a ribosomal cistron or roughly 1.1 million daltons. This correlates reasonably with the 1.2–1.4 million difference between bird or mammalian and amphibian RTU's. If such were the case, the evolutionary event could be explained in terms of a change in an initiator or terminator signal that would allow full transcription of the entire segment, including the spacer.

Although there appears to be homology in base sequences of the rRNA genes among various organisms,[29] there is also a great deal of variability in sequences as evidenced by the extreme differences in base composition between the high G-C rRNA's of animals such as mammals or amphibians and the high A-U rRNA's of *Drosophilia* or *Tetrahymena*. Moreover, among different organisms the base compositions of the nonconserved portion of the RTU's are even more disparate than those of the rRNA's (Table 3). Thus it seems clear that during the course of evolution substantial changes in the DNA base sequences determining the RTU have occurred in both conserved and nonconserved regions without appreciably affecting the size of the transcription unit. This in turn suggests that strong selective pressures were responsible for maintaining the size of the RTU throughout the early periods of evolution. These pressures

TABLE 3. *Base composition of conserved and nonconserved portions of RTU.*

Organism	Per Cent Guanylic Plus Cytidilic Acid		References
	Conserved*	Nonconserved†	
Mammal (HeLa cells)	64.6	76.8	30
(Rat liver)	64.8	71.7	18
Amphibian (*Xenopus*)	59.9	78.4	7
Insect (*Drosophila*)	39.0	10.9	31
Protozoan (*Tetrahymena*)	43.1	28.3	23

* Over-all value for both rRNA components.

† Calculated by difference from the C-G contents of RTU and rRNA, using published molecular weights and those given in this paper.

were probably as strong as those preserving the size of the small rRNA component, and perhaps greater than those governing variations in the large rRNA component (cf. also ref. 8). As to the causes for selection in birds and mammals of the larger RTU and the less conservative processing mechanism, one can imagine these to be related to some property shared by all the cells of such organisms, for example, maintenance at a constant elevated temperature, an obligatory condition in homeothermic animals.

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