

Separation of Bacterial Ribosomal Ribonucleic Acid from Its Macromolecular Precursors by Polyacrylamide Gel Electrophoresis

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Electrophoresis on polyacrylamide gels was found to be a powerful technique for separating the mature from the precursor forms of bacterial ribosomal nucleic acid (rRNA). The separation of the 16S rRNA from its precursor was, for all practical purposes, complete; that of the 23S rRNA from its precursor was detectable but incomplete. When mature and precursor rRNA preparations were heated to randomize secondary structure, etc., and then cooled, it was found that electrophoretic mobility differences between mature forms of rRNA and their precursors persisted. This, in conjunction with the rather large electrophoretic mobility differences between mature and precursor forms, can be taken as strong evidence for a molecular weight difference between mature rRNA and its precursor forms of RNA. With the 16S rRNA, this difference could be as large as 130,000 daltons.

The 70S bacterial ribosomal particle contains three component ribonucleic acid (RNA) species: a so-called 23S RNA, of 1.1×10^6 daltons, a 16S RNA, of 0.55×10^6 daltons, and a more recently discovered 5S RNA, of 3.5×10^4 daltons (13, 15, 17). The first and last of these are parts of the 50S ribosomal subunit; the second is contained in the 30S ribosomal subunit. At least two of these forms of rRNA, the 16 and 23S varieties, are not formed directly by transcription from deoxyribonucleic acid (DNA), but are instead the result of post-transcriptional modification(s) of forms of RNA that are the primary transcription products. Such precursor or "immature" forms of 16S and 23S rRNA are present in the "eosomal" (nascent RNA) and "neosomal" (protein-poor particles) stages of ribosome biosynthesis (6, 9, 11). [Particles resembling or identical with neosomes and containing a precursor-type of rRNA can be caused to accumulate in large quantities in cells treated with agents or procedures that halt protein synthesis (7, 12, 13).]

For the bacteria, the nature of the differences between mature 16S or 23S rRNA species (to be called henceforth m16 or m23) and their precursor forms (to be called p16 or p23) is still unclear. It is known that the precursors are relatively sub-methylated (2), and there is evidence (18) which suggests that mature and precursor rRNA may differ in molecular folding (secondary or tertiary structure, or both), though not in primary struc-

ture (exclusive of methylation differences). These facts alone, however, do not definitively settle the matter. Likewise, the steps in the biosynthesis of the small 5S rRNA component have yet to be elucidated. One of the major problems in getting at these questions is a technical one: a mature rRNA and its precursor(s) differ little, if at all, in sedimentation characteristics, chromatographic column elution profiles, etc. (3, 8), making their separation extremely difficult if not impossible.

In the present investigation, we have devised a new technique for the separation of different forms of RNA by electrophoresis on polyacrylamide gels (1, 10). This technique provides by far the best separations yet attained between mature forms of rRNA and their precursors, and so should be of use in elucidating the relationships among the various forms of any given rRNA. Evidence from our studies strongly suggests a primary structural difference between p16 and m16 and between p23 and m23.

MATERIALS AND METHODS

The bacterial strain used throughout this study was *Bacillus subtilis* W23. The growth medium in all cases was 10^{-2} M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, containing 5×10^{-3} M $MgCl_2$, 9×10^{-2} M NH_4Cl , 7×10^{-4} M Na_2SO_4 , 3×10^{-4} M K_2HPO_4 , 5.5×10^{-2} M glucose, 2.5×10^{-3} M L-alanine, 2×10^{-2} M glutamic acid, 2×10^{-2} M asparagine, and all the remaining of the 20 amino acids at a final concentration of 4 μ g/ml.

Cells were grown in flasks shaken in a water bath at 37 C. In kinetic experiments, etc., growth was stopped rapidly by pouring the sample on finely crushed ice with simultaneous addition of sodium azide to a final concentration of approximately 10^{-2} M.

RNA extractions were done by grinding frozen cell pellets (obtained by centrifugation) in a mortar and pestle with 50- μ diameter glass beads (Superbrite, 3M Corp., St. Paul, Minn.) in the presence of a small amount of 20% water solution of sodium dodecyl sulfate; then, 5 volumes of 10^{-2} M Tris buffer, pH 7.4, containing 10^{-4} M $MgCl_2$, was added, and the whole mixture was immediately extracted by the phenol procedure of Kirby (5). The final alcohol precipitate of RNA was redissolved in 10^{-2} M Tris buffer, pH 7.4, containing 3×10^{-3} M ethylenediaminetetraacetate (EDTA), to a final concentration of approximately 0.2 mg of RNA per ml.

The chloramphenicol particles used to obtain chloramphenicol particle RNA were obtained from log-phase cultures treated for 25 min with 200 μ g of chloramphenicol per ml. The exact procedures for isolating chloramphenicol particles are described elsewhere (N. B. Hecht, Ph.D. Thesis, Univ. of Illinois, Urbana, 1967).

The preparation of polyacrylamide gels and all other techniques associated with acrylamide gel analysis are fully documented in the literature (1, 10). The gel used for separation of RNA with a 15S to 30S size range was 2.4% polyacrylamide; that used for separation of RNA of less than 10S size was 6% polyacrylamide. The actual gel is cylindrical, 8 cm in length, and 0.9 cm in diameter. The running buffer was the E buffer described by Bishop et al. (1). The RNA was applied to the "top" of the gel in a 0.1-ml volume. The electrophoresis was done at constant current of 10 ma per tube (approximately 30 v). The running time was 3 hr for both the 2.4 and 6% gels.

For assay, the gels were immediately frozen and cut into 0.5-mm slices with a CO_2 -cooled microtome (1). Each slice was placed into a vial, 0.5 ml of 30% H_2O_2 was then added, and the vials were kept at 75 C for 12 hr, a procedure which disperses the gel matrix. A 10-ml amount of Kinard's scintillation fluid was then added to each vial (4). Samples were counted in a model 6860 scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.). 3H - and ^{14}C -labeled uridine was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Figures 1 and 2 show both the excellent separation polyacrylamide gel electrophoresis gave between m16 and (what will be shown to be) p16, and the kinetics of appearance of labeled uridine in the various forms of the 16S and 23S forms of rRNA. In Fig. 1, m16 and m23 appear as the two large 3H "steady-state" label peaks (m16 being on the right); p16 and p23 are the corresponding peaks in the ^{14}C pulses which "shadow" the 3H peaks. The separation of the

m-p16 couple is, for all practical purposes, complete; that for the m-p23 couple is detectable but slight. Although there appears to be but a single electrophoretically distinct form of p16, p23 may exist in two distinct forms.

The kinetics of appearance of labeled uridine in the p16 and m16 rRNA are in accord with the precursor role for the former. If p16 is a primary transcription product, the initial appearance of isotope in this fraction should be first power in time (11), assuming transcription time to be reasonably short; Fig. 2 shows this to be the case. (However, if there was a macromolecular precursor to p16 that was converted to p16 extremely rapidly, it would not be detected in such an experiment.) On the other hand, isotope moves into the m16 fraction with a time dependence of a higher order; thus, this fraction cannot be a primary transcription product, but must come about from the post-transcriptional modification of some primary transcription product.

The ribosomal development sequence—eosome \rightarrow neosome \rightarrow ribosome—suggests that m16 and m23 are each preceded by two major precursor stages. As can be seen in Fig. 2, the initial third (or higher)-order time dependence for the appearance of label in m16 is consistent with this notion (11). This suggests, then, that the forms of rRNA in both the eosomal and neosomal stage of ribosome biosynthesis are identical, at least electrophoretically. [Either a one or a two serial precursor model presents essentially the same initial kinetics of incorporation for the precursor (11).] This conjecture is verified by the demonstration below (Fig. 3) that eosomal p16 (short pulse label) and "neosomal" p16 (from chloramphenicol particles) are actually indistinguishable electrophoretically.

There are two possible explanations of the difference between p16 and m16 (and thus p23 and m23). Either p16 and m16 have identical primary structures but somehow differ in molecular folding (the molecule being able to assume at least two quasi-stable forms), or the two have primary structural differences. With *Escherichia coli*, it has been found that, upon heating to "melt out" tertiary and secondary structure, both mature rRNA and precursor ("relaxed particle") rRNA give a similar elution pattern from a methylated albumin column, the pattern being that characteristic of the (unheated) precursor rRNA (18). Although this shows that tertiary structural changes result upon heating mature rRNA, it proves nothing with regard to the question of possible primary structural differences between mature and precursor forms. [For certain organisms, the methylated albumin column

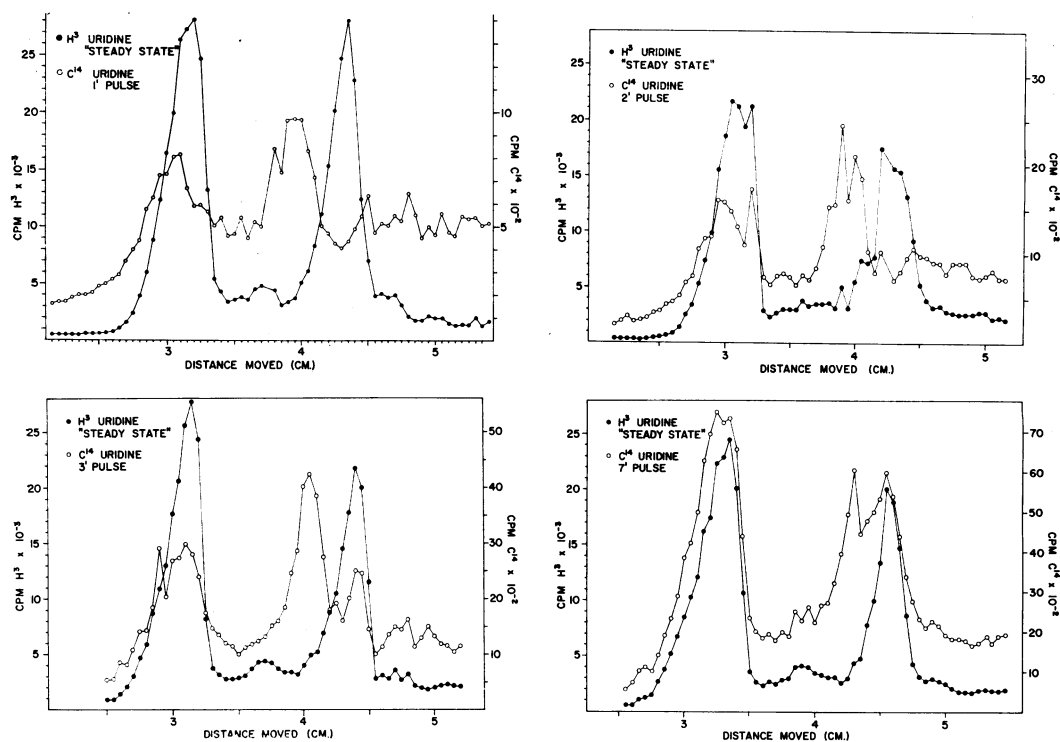


FIG. 1. Polyacrylamide gel electrophoretic separations of *Bacillus subtilis* RNA. An 800-ml log-phase culture of *B. subtilis* was prelabeled with 250 μC of ^3H -uridine (specific activity $\sim 5 \text{ mc}/\mu\text{mole}$) for approximately two generations. Then 100 μC of ^{14}C -uridine (specific activity 24 $\mu\text{C}/\mu\text{mole}$) was added. As a function of time thereafter, 200-ml portions of the culture were rapidly removed, and growth abruptly halted as described under Materials and Methods. The cells were then collected, and RNA was extracted and analyzed on polyacrylamide gels, as described above. The "steady-state" ^3H peaks correspond to m23 (left-hand one) and m16 (right-hand one) forms of RNA. The two dominant peaks seen in the ^{14}C profiles for early times are referred to as p23 (possibly a multiple peak) and p16. Position of any given slice in the gel is plotted on the abscissa; radioactive count in that slice is plotted on the ordinate.

cannot even separate the two major rRNA species, i.e., forms of RNA differing twofold in molecular weight (14, 16).] However, the very large electrophoretic mobility differences we observe between m16 and p16 do suggest primary structural differences between the two, since gel electrophoretic mobility is a strong function of RNA molecular weight (1, 10).

To test this possibility further, preparations of precursor and mature rRNA were heated to melt out secondary and tertiary structure. Given no primary structural difference, all forms of a given rRNA species upon cooling should assume the same configuration and thus exhibit no electrophoretic mobility differences.

The results of the heating study are shown in Fig. 3. Three types of labeled rRNA were heated, cooled, and analyzed as indicated. [Mature rRNA carried a ^3H -uridine label, eosomal rRNA carried a ^{14}C -uridine (1.5-min pulse) label, and

the "neosomal" RNA from chloramphenicol particles carried a ^{32}P (20-min) label.] It is clear from Fig. 3 that, even after a treatment that certainly melts out all secondary and tertiary structure, the mature and precursor forms of rRNA still do not travel with the same electrophoretic mobilities, thus making an explanation of their different characteristics in terms of differences solely in molecular folding most unlikely. (It might be added that, by use of an appropriate marker, we have determined that the absolute electrophoretic mobilities of the mature and pulse-labeled rRNAs do not change upon heating, a fact not apparent from the data as presented in Fig. 3. Further, longer times of heating at 100 C do not alter the pattern of peak positions as seen in the figure, though considerable disappearance of all labels from the various peaks, particularly those in the general 23S region, and a rise in general background label are noted for

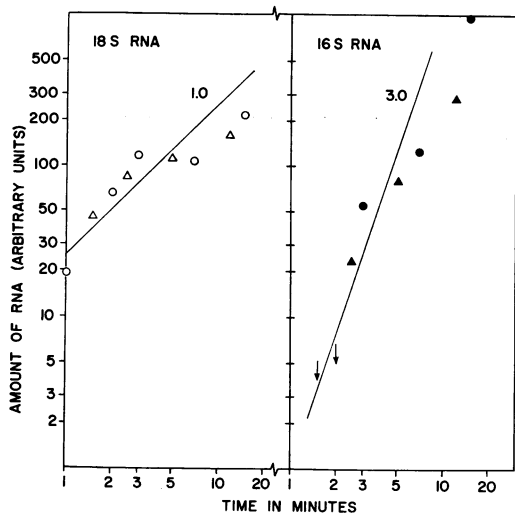


FIG. 2. Kinetics of appearance of label in the electrophoretically defined p16 and m16 rRNA. The total amounts of ^{14}C -uridine incorporated into the p16 and m16 electrophoretic peaks for experiments of the type described in Fig. 1 are plotted as a function of labeling time. The areas under the peaks have been corrected for general background (message) RNA, and corrected for variations in amount of RNA applied to the gel by the areas of the ^3H steady-state peaks. The arrows designate upper bounds for these particular values. The numbers 1.0 and 3.0 are the slopes of the lines drawn. First-order kinetics of synthesis would yield an initial slope of 1.0 on a log-log plot, second-order kinetics an initial slope of 2.0, and so on (11).

these longer heating times—the result of cleavage of the RNA backbones.)

DISCUSSION

Our results show that polyacrylamide gel electrophoresis yields essentially complete separation between m16 and p16, and detects differences between m23 and p23 (which latter cannot be accomplished centrifugally). This excellent separation should now permit definitive answers to questions regarding the changes in rRNA during ribosome biosynthesis.

The experiment involving heating to melt out secondary and tertiary structure is itself a strong indication that significant primary structural differences exist between p16 and m16 (and p23 and m23). Preliminary results from experiments in which nucleic acid hybridization studies were used to determine the point more definitively (Hecht and Woese, unpublished data) confirm this interpretation.

If we assume that the major determinant of gel electrophoretic mobilities is molecular weight [for which there is some precedent (1, 10)], one

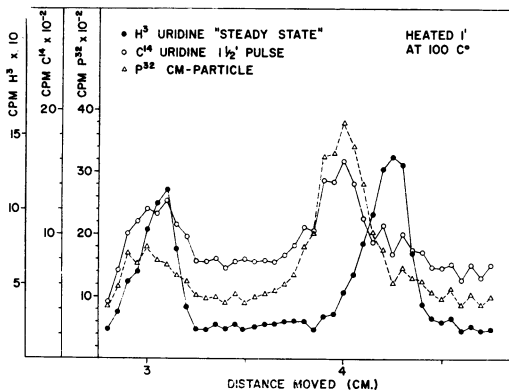


FIG. 3. Effect of heating on the relative electrophoretic mobilities of various forms of rRNA. ^3H steady-state and 1.5-min ^{14}C -labeled RNA were prepared as described above. ^{32}P -labeled chloramphenicol-particle RNA was obtained as described elsewhere (Hecht, Ph.D. Thesis, Univ. of Illinois, Urbana, 1967). A mixture of all three was heated to 100 C for 1 min in 10^{-2} M Tris buffer, pH 7.4, containing 3×10^{-3} M EDTA. After cooling rapidly (in an ice bath), the RNA was analyzed directly by gel electrophoresis, as described. Ordinate and abscissa are as in Fig. 1.

can calculate that the conversion p16 \rightarrow m16 should involve cleavage of a piece of roughly 130,000 daltons from p16. Similarly, the p23 \rightarrow m23 conversion could involve removal of a fragment of roughly 50,000 to 100,000 daltons. Existence of such cleavage products would then raise the question of their fate in the cell. It is conceivable that 5S rRNA, for example, is (or is derived from) one such split fragment. In a separate publication (Hecht, Bleyman, and Woese, *in press*), we show that the 5S rRNA, like its m16 and m23 counterparts, is not a primary transcription product, but, unlike its larger counterparts, the 5S rRNA is not derived from a precursor similar in electrophoretic mobility to itself. These facts are consistent with the concept that 5S may be derived from p23 or possibly p16. Nucleic acid hybridization studies designed to settle this matter definitively are in progress.

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