Small Ribonucleic Acids of Escherichia coli

I. CHARACTERIZATION BY POLYACRYLAMIDE GEL ELECTROPHORESIS AND FINGERPRINT ANALYSIS*

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SUMMARY

Escherichia coli RNA molecules in the size range of 70 to 400 nucleotides have been characterized by polyacrylamide gel electrophoresis and by RNase T₁ (EC 2.7.7.26) fingerprinting. RNA labeled by ³²P₀₄⁺⁻ for 20 min is separated into about 20 bands by electrophoresis in 5, 10, and 20% polyacrylamide gels; many of them represent pure RNA species. Some correspond to known molecules such as tRNAs, 5 S RNA, 4.5 S RNA, and 6 S RNA, but others have not been described previously. The amounts of these latter molecules are low, usually less than 10% of the level of 5 S RNA. One molecule, with electrophoretic mobility similar to 5 S RNA, has a 5′-terminal guanosine triphosphate. Another, with a mobility similar to that of 6 S RNA, contains dihydrouracil. Several different RNA preparation methods give essentially the same results. Two-dimensional polyacrylamide gel electrophoresis (10% → 20% polyacrylamide) was used to purify RNA in the 4 S to 5 S region. This technique is capable of resolving at least 30 different RNA species; many of them are pure tRNA molecules. It allows for direct comparison of the quantities of purified tRNAs and the other molecules described in this paper. Such analysis shows that during 20 min labeling the newly characterized molecules accumulate at levels comparable to many individual tRNAs.

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purchased from Eastman Organic Chemicals. Acrylamide (catalogue Number 5521) and N,N'-methylene-bisacrylamide were also obtained from Eastman Organic Chemicals and used after filtration of a solution (usually 38% acrylamide, 2% bis-acrylamide in glass-distilled water) through Millipore filters (HAWP04700). Diethylylcarbonate was purchased from Naftone Inc., New York. Carrier-free 32PPO4 was purchased from New England Nuclear Corp., Boston. Fluorocide for fluorocarbon coating (S9020) was obtained from Scientific Products, Evanston, Illinois, and urea (catalogue Number 9200) was from Schwaren Mann Biochemicals, Orangeburg, N. Y. All test tubes and centrifugation tubes were siliconized (rinsed in 1% solution in toluene of dichlorodimethylsilane from Aldrich Chemicals, Milwaukee) and baked 3 hours at 150°. All heat-stable reagents which were used during sample preparation were autoclaved at 115° for 20 min. Carrier RNA, purchased from Sigma Biochemicals, was phenol extracted at 80° and ethanol precipitated before use.

**Methods**

**Bacterial Strain and Culturing**—E. coli strain CP78 (obtained from Dr. R. Lazzarini) was used; it requires arginine, leucine, histidine, threonine, and thymine for growth (20). Cultures (normally 2 ml) were grown at 37° in a low phosphate (0.25 mM PO₄₋) minimal Tris-glucose medium (21) supplemented with 10 µg per ml of thymine, 30 µg per ml of the four required amino acids, and 25 µg per ml of each of adenosine, guanosine, HCl, uracil, and thymine. Under these conditions the generation time is 1 hour. When the cell density reached 1.5 to 2.5 X 10^8 bacteria per ml, the cultures were labeled by the addition of neutralized 32PPO₄₋ to a final concentration of 0.3 mCi per ml. After 20 min labeling, 32P-labeled RNA samples were prepared by one of the following four different methods.

**RNA Preparation**

**Method 1**—Method 1 is primarily that described by Altman (22). After 32P labeling, the culture (2 ml) was poured into 2 ml of water-saturated phenol to which were added 0.1 ml of 2 M neutralized 32PPO₄ to a final concentration of 0.3 mCi per ml. Cultures (normally 2 ml) were grown at 37° in a low phosphate (0.25 mM PO₄₋) minimal Tris-glucose medium (21) supplemented with 10 µg per ml of thymine, 30 µg per ml of the four required amino acids, and 25 µg per ml each of adenosine, guanosine, HCl, uracil, and thymine. Under these conditions the generation time is 1 hour. When the cell density reached 1.5 to 2.5 X 10^8 bacteria per ml, the cultures were labeled by the addition of neutralized 32PPO₄₋ to a final concentration of 0.3 mCi per ml. After 20 min labeling, 32P-labeled RNA samples were prepared by one of the following four different methods.

**Method 2**—This method is based on the hot SDS treatment described by Brenner and Yuan (23). After 32P labeling, the culture (2 to 20 ml) was poured into an equal volume of hot sodium dodecyl sulfate solution (1% SDS, 200 mM sodium chloride, 40 mM EDTA, and 20 mM Tris-HCl, pH 7.5) which had been kept in a boiling water bath. Ninety seconds later, the cell lysate was cooled to room temperature. Diethylylcarbonate was added to a final concentration of 0.1%, as well as carrier RNA (to make 25 µg per ml) and potassium phosphate buffer pH 7.0 (to make 1.5 mM). The samples were shaken at 37° for 10 min. After addition of sufficient concentrated sodium chloride to make the samples 0.4 M sodium chloride, the samples were chilled in an ice bath for 10 min and then centrifuged at 10,000 X g (0-2°) for 15 min. The supernatant fluid was poured into 2.5 times its volume of ethanol and stored overnight at -20°. After centrifugation (10,000 X g, 0.2°, 20 min) the RNA precipitates were resuspended in 2 ml of 0.25% SDS, 50 mM sodium chloride, 10 mM EDTA, and 5 mM Tris-HCl pH 7.5, and extracted at room temperature with an equal volume of buffer-saturated phenol. The RNA was then precipitated twice with ethanol from (0.5 to 1.0 ml) 300 mM sodium acetate (pH 5).

**Method 3**—In this method, the culture was poured on an equal weight of crushed ice containing sodium acetate (1.5 mg per g of ice) and centrifuged at 15,000 X g (0-2°) for 3 min. The cell pellet was suspended at 0-5° in medium containing 1 mM sodium acetate and then poured into the hot SDS solution as in Method 2. More modifications were introduced. Unless otherwise noted, the electrophoresis buffer was Buffer A (25) which consists of 21.6 g of Tris base, 1.86 g of disodium EDTA, and 11.0 g of borax acid per liter, giving a final pH of 8.3. Buffer was re-circulated between the top and bottom buffer reservoirs during electrophoresis. The 10% polyacrylamide gel was polymerized in Buffer A containing 9.5% acrylamide, 0.5% N,N'-methylene-bisacrylamide, 0.4% 3-dimethylamipropionitrile, and 0.1% ammonium persulfate, at room temperature. In the case of 5% or 20% gels, the concentrations of acrylamide and N,N'-methylene-bisacrylamide were halved or doubled, respectively. Before application to the gel, the samples (about 100 µg) were heated to about 60° for 3 min in 10 µl of a solution containing 50% urea, 25 mM sodium chloride, and 4 mM EDTA (pH 7). After cooling to room temperature, the samples were mixed with 10 µl of a solution containing 20% sucrose, 1 mM EDTA (pH 7), and 0.1% bromphenol blue, and applied to the gel. During electrophoresis (at about 10 volts per cm) the cells were cooled by circulation of coolant maintained at 15°. Essentially the same results were obtained with or without heat and urea treatment. When eluted samples were further purified by re-electrophoresis they were heated to about 80° in urea, as above, before application to the second gel.

When 20% gel was used, a few modifications were introduced. Because the 20% gel sticks very tightly to the Plexiglas of the electrophoresis apparatus, it was necessary to coat with fluorocarbon both the slot former and a border of about 2 cm around the two sides and bottom of the coolant plates that are in direct contact with the gel. Unless the cell is treated in this way, it is very difficult to remove the slot former after polymerization or to dismantle the apparatus after electrophoresis. Even after

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.
fluorocarbon coating, however, it was occasionally necessary to apply rather strong force during dismantling. The polymarization of the 20% gel was done while coolant was circulating at 15°, to prevent the trapping of gas bubbles between the gel and cell surfaces. This type of bubble distorts electrophoresis patterns.

In two-dimensional polyacrylamide gel electrophoresis, the first dimension was carried out in a small slot (1.5 mm thick and 3.5 mm wide) of 10% slab gel at about 20 volts per cm for 3 hours in one-half diluted Buffer A. Samples were pretreated by heat and urea, as described above. After the run, a gel slice containing the 4 S and 5 S RNA region was cut out with a microne tome blade. The lateral position of the sample was determined from the positions of dye markers that were added to the sample slot at periodic intervals during the run. The gel slice was put into the electrophoresis cell at right angles to the first dimension in a procedure similar to that described by others for separations of some other RNA species (26-29). Twenty per cent acrylamide solution was poured into the cell and it was polymerized around and below the 10% strip. Electrophoresis in the second dimension was about 20 volts per cm for 17 hours in one-half diluted Buffer A.

**Autoradiography and Elution of RNA from Gel**

After polyacrylamide gel electrophoresis the RNA was located by autoradiography. This was accomplished by placing the gel on a sheet of moist filter paper which was secured to a large piece of Saran Wrap in a thick paper folder. A piece of Saran Wrap was placed over the gel and the edges were sealed with tape. Spots of radioactive ink (PO₄³⁻ added to fountain pen ink) were placed on the paper around the gel, for later alignment.

Often, before removal of the gel from the electrophoresis apparatus, the top 1 cm of gel was cut off and removed. The lower part of the gel was then removed and briefly rinsed in water before being placed on the filter paper. The top of the gel was then placed in the corresponding position on the paper. This process greatly reduced the level of contamination of the gel caused by leaking of ribosomal RNA (16 S and 23 S), which cannot enter into the gel, out of the slot at the top.

The part of the gel corresponding to each RNA band or spot was cut out and the radioactivity was measured by Cerenkov radiation. The piece of gel containing the RNA was then ground with a glass rod in a centrifuge tube. After addition of 1 ml of 300 mm sodium chloride containing 0.2% SDS, the gel suspension was shaken on a Vortex mixer for 1 hour at room temperature. The gel was pelleted by centrifugation at 4000 × g for 30 min and re-extracted two more times with 0.5 ml of the same solution. The supernatants were pooled, 80 µg of carrier RNA were added, and RNA was precipitated by the addition of 5 ml of ethanol and overnight storage at −20°. RNA was resuspended in 300 mm sodium acetate (pH 5) and re-precipitated two more times by ethanol before fingerprinting.

**Fingerprint Analysis of RNA**

Two-dimensional fingerprints after RNase T₁ digestion of the RNA (1:20, w/w, enzyme to RNA) were made according to the methods described by Sanger and his collaborators (12, 13). Purified oligonucleotides in the fingerprint were analyzed by pancreatic RNase digestion after elution from the paper (10 µl of 0.1 mg per ml of enzyme and 2 mg per ml of carrier RNA in 10 mm Tris-tHCl, pH 7.6; 37° for 30 min) and electrophoresis on DEAE-cellulose paper as described by Adams et al. (32). Analysis of minor bases in tRNA was done by paper electrophoresis and chromatography as described by Barrell (33).

**RESULTS**

**Ten Per Cent Polyacrylamide Gel Electrophoresis of E. coli**

Small RNAs—Polyacrylamide gel electrophoresis is an efficient technique for the separation of RNA molecules of similar sizes. We used this technique both to characterize and to purify the various small RNAs of E. coli.

Fig. 1a is an autoradiogram of a 10% polyacrylamide slab gel analyzing small E. coli RNAs labeled by ³²PPO₄⁻ for 20 min at 37°. In order to test whether the gel pattern depends upon the method of RNA preparation, samples were made by several methods in which cellular RNA metabolism was believed to be stopped rapidly. The four methods which were used to make the following samples were described under “Experimental Procedure.” Sample 1 was prepared by direct phenol extraction of the culture medium as described by Altman (29). Sample 2 was made by the hot SDS treatment described by Bremer and Yuan (23), in which the culture was poured directly into a hot SDS solution. In Sample 3, the culture was poured on crushed ice containing sodium azide, and then centrifuged. The pellet cells were then treated with the hot SDS solution. Sample 4 was prepared by a modification of that used for Sample 2, with the addition of mild alkaline treatment to remove aminoacyl and peptidyl residues from tRNAs (24). These four samples gave similar electrophoretic patterns. This is a good evidence that the RNA patterns are not dependent on the specific method of RNA preparation.

The RNA in each numbered band was identified by RNase T₁ fingerprint analysis. Band I RNA gave the very complicated but characteristic fingerprint of E. coli tRNA mixtures which was obtained by Sanger et al. (12). Band I actually is resolved into at least two regions, containing different tRNA species. This double band nature is evident only after very short exposure of the autoradiogram.

Band II also can be resolved into two parts, but again, this is best seen after short exposure. The slow part of Band II, called IIₕ, consists of two RNA species, as shown by further re-electrophoresis in a 13.5% gel (Fig. 2a). Both of these RNA bands have minor bases such as dihydrouracil, thymine, and pseudouracil. The faster, and faster, part (called as IIₕ in Fig. 2a) has the same fingerprint as tRNA₅₅.export published by Dubé et al. (19, 34). The fingerprint of the minor, and slower, part of IIₕ (IIₕₕ) is shown in Fig. 3a. This does not correspond to any published fingerprint. However, compositional analysis of each of T₁ oligonucleotide shows that this is tRNA₅₅.export which has recently been sequenced by Ishikura and Nishimura. The faster part of Band II (called IIₕ in Fig. 1a) contains an undermethylated form of tRNA₅₅.export and two or three other tRNAs, as yet unidentified. We shall discuss the mature and immature forms of tRNA₅₅.export in more detail in the accompanying paper (19).

The fingerprints of the RNAs in Bands III and IV were made after re-electrophoresis in 11.5% or 13.5% gels, although the RNAs are essentially pure after the 10% gel electrophoresis. The fingerprint of Band III RNA, shown in Fig. 3b is the same as that published by Griffin (17) for the 4.5 S RNA. Therefore, we conclude that Band III RNA is 4.5 S RNA. Griffin showed that this RNA does not have any minor bases. She also pointed out that this molecule probably corresponds to one of the RNAs which Hindley (14) described as being slightly smaller than 5 S RNA.

2 II. Ishikura and S. Nishimura, personal communication.
The RNA of Band IV has a fingerprint which does not correspond to any published one (Fig. 3c). This RNA has no minor bases. Pulse-chase experiments described in the accompanying paper (19) showed that this RNA is unstable. Sequence analysis of this molecule indicates that it has a triphosphate pppG at the 5' end since one RNase T₁ product co-electrophoresed on DEAE-cellulose paper at pH 3.5 with pppGp after alkali treatment, but with pGp after snake venom phosphodiesterase digestion. The sequence at the 3'-OH end is U-U-U-U-U-A since pancreatic RNase digestion of the terminal oligonucleotide yields only Up, whereas snake venom phosphodiesterase produces pA and pU in the ratio 1:5; up to half the molecules lack the 3'-OH terminal A₁.

RNAs of Bands V, V', and V'' have the fingerprint of 5 S RNA, as reported by Brownlee and Sanger (13), although V'' was heavily contaminated by other RNAs. Re-electrophoresis of these three bands after heat treatment in the urea solution showed that they are heat interconvertible conformational isomers of each other. Aubert et al. (35) and Forget and Weissman (36) reported multiple conformations of 5 S RNA, as assayed by methylated albumin kieselguhr column chromatography.

In the region between 5 S and 6 S RNA, there are at least three distinct RNA molecules, although they can barely be seen in Fig. 1a because of their low intensities. The wide region between Bands V' and VI was eluted and repurified in a 11.5% gel after Hindley (14) also observed a small separation of conformational isomers of 5 S RNA during polyacrylamide gel electrophoresis. In our experiments, the relative ratios of the three isomers, especially the ratio of V'' to the others, is variable. We would like to stress the fact that we find no evidence of similar conformational isomers for any other small RNAs.

The fingerprint of the RNA of Band VI is shown in Fig. 3d. This is the fingerprint of 6 S RNA obtained by Brownlee, who determined the primary sequence of this molecule (16). Analysis of each T₁ oligonucleotide by pancreatic RNase digestion confirms this conclusion. Brownlee (16) noted that this 6 S RNA corresponds to the one described both by Hindley (14) and by Goldstein and Harewood (15). The RNA in a weak band, VI', has a fingerprint only slightly different from that of the 6 S RNA in Band VI. The differences, as well as radioactivity pulse-chase experiments (19), indicate that the RNA in Band VI' is most likely a precursor of 6 S RNA. These two correlated molecules are not heat interconvertible.

In the region between 5 S and 6 S RNA, there are at least three distinct RNA molecules, although they can barely be seen in Fig. 1a because of their low intensities. The wide region between Bands V' and VI was eluted and repurified in a 11.5% gel after Hindley (14) also observed a small separation of conformational isomers of 5 S RNA during polyacrylamide gel electrophoresis. In our experiments, the relative ratios of the three isomers, especially the ratio of V'' to the others, is variable. We would like to stress the fact that we find no evidence of similar conformational isomers for any other small RNAs.
The RNAs of both Bands VIII and X have rather complex fingerprints in lower percentage gels. The electrophoresis pattern of an E. coli RNA species. Re-electrophoresis of each band in 7% polyacrylamide gel gives broad and partially separated bands. In contrast to the two Bands VIII and X, Band IX RNA has a relatively simple fingerprint (Fig. 3e). We have been unable to detect any minor nucleotides in this RNA. Radioactivity pulse-chase experiments show that this RNA is stable. From its electrophoretic mobility and the complexity of the fingerprint, we estimate that it is 300 to 400 nucleotides long.

Approximately 15 different small RNA species of E. coli are purified to a nearly homogeneous stage by electrophoresis in 10% and 5% polyacrylamide gels after re-electrophoresis in different concentrations of gel. However, there is only poor resolution in and near the bulk tRNA region. In order to obtain better resolution of the RNAs in that region, 20% polyacrylamide gel electrophoresis was carried out. A typical electrophoresis pattern is shown in Fig. 1c. The assignment of bands was made by RNase T1 fingerprinting. The RNA band numbers corresponding to those in the 10% gel are shown in the figure. The bulk tRNA region is resolved better here than on the 10% gel, but still only a few of tRNA species were found to be pure at this stage.

Several RNA molecules have rather unexpected mobilities in 20% polyacrylamide gels. 6 S RNA, which is 184 nucleotides long (16), co-migrates with the conformational isomers of 5 S RNA which are 120 nucleotides long (37). A second unusual feature is that the relative mobilities of the mature and immature forms of tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} are reversed from those seen on the 10% gel, as discussed above and elsewhere (19), in 10% polyacrylamide gel electrophoresis the immature form migrates faster, but in 20% gels the mature form migrates faster. In addition, the immature form of tRNA\textsuperscript{Leu}{\textsuperscript{UAA}}, which co-purifies on 10% gels with tRNA\textsuperscript{Leu}{\textsuperscript{UAA}}, migrates on the 20% gel free of tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} but with several different RNAs. This is shown in Fig. 2c which is the 10% gel pattern of RNA that co-migrated with a marker sample of mature tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} in the 20% gel. The mobility marker in the 10% re-electrophoresis is immature tRNA\textsuperscript{Leu}{\textsuperscript{UAA}}. RNA in Band \(\omega\) has the mature tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} fingerprint, and the RNA of Band \(\psi\) has a fingerprint different from either (mature or immature) tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} or tRNA\textsuperscript{Leu}{\textsuperscript{UAA}}. Compositional analysis of the T\textsubscript{1} oligonucleotides of this RNA (Fig. 3j) indicates that it is pure tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} which was sequenced by Blank and Soll (38).

Two-dimensional Separation of RNA in 4 S to 5 S Region—The preceding results show that changes of acrylamide concentration affect the relative electrophoretic mobilities of RNAs with similar sizes. Thus we concluded that two-dimensional electrophoresis should give good separation of RNA molecules, if the first and second dimensions were carried out in different percentage gels. Because of the strikingly anomalous behavior of RNA in 20% gels, relative to that seen in 10% gels, we elected to use a 10% → 20% system, as described under “Experimental Procedure.” Much of this procedure was adopted from the work of others in the purification of other RNA species (26-29).

An autoradiogram of a two-dimensional polyacrylamide gel analysis of the small E. coli RNAs is shown in Fig. 4a. In this experiment, the region of 10% gel containing bulk tRNA and 5 S RNA was transferred to 20% gel for the second dimension. In the autoradiogram it is possible to see about 30 well resolved spots. Fingerprint analysis of each of the spots in this characteristic and reproducible pattern is now in progress. Most of the spots represent pure RNA molecules, although several contain mixtures of molecules. A schematic diagram of the two-dimensional gel is presented in Fig. 4b. The spots that contain four pure tRNAs, 4.5 S RNA, 5 S RNA, and Band IV RNA, are indicated by symbols placed above the respective spots in the dia-

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**Fig. 2.** Re-electrophoresis of eluted RNA in different concentrations of polyacrylamide gel. a, re-electrophoresis, in 13.5% acrylamide, of Band II\textsuperscript{F} from the 10% gel shown in Fig. 1a. b, re-electrophoresis, in 11.5% polyacrylamide, of RNA obtained from the region between V and VI (Fig. 1a). c, the region containing mature tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} in a 20% polyacrylamide gel (Fig. 1c) which had been determined by co-electrophoresis with mature tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} and by fingerprinting, was repurified in a 10% polyacrylamide gel with a marker of pure undermethylated tRNA\textsuperscript{Leu}{\textsuperscript{UAA}}. The undermethylated tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} migrates slightly faster than the mature tRNA\textsuperscript{Leu}{\textsuperscript{UAA}} in 10% gel, as mentioned in the text.
FIG. 3. Autoradiograms showing two-dimensional paper electrophoresis fingerprints of RNase T1 digests of RNAs purified by polyacrylamide gel electrophoresis. Electrophoresis in the first dimension, on cellulose acetate at pH 3.5 in 6 M urea, 1 mM EDTA, was from right to left; in the second dimension, on DEAE-cellulose paper in 7% formic acid, it was from top to bottom as described by Sanger et al. (12, 13). a to k correspond to the autoradiograms of individual RNAs mentioned in the text. For reference, the positions of G, U-G, and U-U-G are indicated in every fingerprint, whether or not the corresponding spots are there. The oligonucleotide positions of C-G, (U, C) G, and (U, U, C) G are also shown in every fingerprint, by the arrow (↑) near G, U-G, and U-U-G, respectively. The position of a blue marker dye (Xylene cyanol F.F.) is shown by the letter b. The origin of the second dimension is indicated by O— at the top of each fingerprint. I is a schematic diagram, patterned after that published by Sanger and co-workers (12), for orientation of the various oligonucleotides shown in a to k. The numbers and lines indicate increasing numbers of Ap (—) or Cp (—) in the oligonucleotides.

DISCUSSION

Using polyacrylamide gel electrophoresis, we have characterized and purified a large number of E. coli small RNAs. Many of the RNA species were purified by electrophoresis in three different percentages of polyacrylamide gel, by successive re-electrophoresis, or by two-dimensional polyacrylamide gel electrophoresis.

The purified molecules were further characterized by RNase T1 digestion and fingerprint analysis. Such analysis was also used as a measure of purity of a particular molecule, since inhomogeneous preparations have complex and irregular finger-
prints in which the relative intensities of the different oligonucleotides are distributed over a wide range. However, purity as assayed by fingerprinting does not necessarily mean the existence of only a single species. Such conclusions are obtained only after determination of total primary sequence. For example, in a purified sample two molecules may exist which differ by only one nucleotide substitution or by minor differences at the 5' or 3' ends. Even such minor changes, however, often lead to noticeable mobility differences, giving either separate or oblong spots in the two-dimensional gel electrophoresis.

Purification of individual molecules by re-electrophoresis of RNA mixtures is successful because mobility is determined not only by molecular weight but also other factors such as conformation. For example, conformation isomers of 16 S rRNA and 23 S rRNA have different mobilities and are clearly separated in appropriate concentrations of polyacrylamide gel (40, 41). Thus, because of the complex nature of the separation, changes in electrophoresis conditions such as acrylamide concentration or temperature could have nonuniform effects on the mobilities of different RNA molecules with similar molecular weights. Preliminary experiments of electrophoresis in 14 different concentrations of polyacrylamide gels (between 5 and 20%) confirmed these expectations. As shown above, some RNA molecules which migrate together in one concentration of gel were well separated in another concentration.

There are two pieces of evidence which indicate that the RNA pattern that we observe reflects the kinds of molecules in the cell. First, the bands probably do not result from RNA breakdown during isolation, since we obtain the same pattern regardless of the method of RNA preparation or the way in which cell metabolism was stopped (Fig. 1c). Of course, we cannot rule out a very fast acting nuclease that works in all cases before metabolism is completely halted. However, we should point out that 16 S rRNA, precursor 16 S rRNA (40, 42), and 23 S rRNA can be reproducibly recovered in our RNA preparations with no evidence of degradation.

Secondly, the bands are not degradation artifacts created during electrophoresis nor are they composed of RNA aggregates, since the eluted RNA does not change its

\[ T. Ikemura and J. E. Dahlberg, unpublished observations. \]
Fig. 1. Separation of 32P-labeled RNAs by two-dimensional electrophoresis in polyacrylamide gel. The first dimension of electrophoresis was in 10% polyacrylamide. The region of that gel containing 4 S and 5 S RNA was subjected to further electrophoresis in 20% polyacrylamide, at right angles to the first dimension, as described under “Methods.” The roman numerals, arranged in the direction of first dimension, show the regions corresponding to the bands in the 10% gel of Fig. 1a. a, an autoradiogram of the gel. b, a schematic diagram of a. The positions of 5 S RNA, 4.5 S RNA, Band IV RNA, tRNA\textsuperscript{\textalpha}, tRNA\textsuperscript{\textbeta}, tRNA\textsuperscript{\textgamma}, and tRNA\textsuperscript{\textdelta} are indicated in b. c, an autoradiogram of the same gel, exposed for a longer time to permit visualization of the less intensely labeled spots. Electrophoresis was done in one-half diluted Buffer A and at the voltage of about 20 volts per cm in a gel. In this electrophoresis condition, Band IV RNA migrates with Band V in the 10% gel of the first dimension.

Although this study is useful in describing a number of small RNA molecules other than tRNA and 5 S RNA, it has no direct bearing on the function of these other molecules. Most of the molecules that we have studied were isolated on the basis of physical characteristics, such as discrete size or conformation. Since the method of RNA preparation does not seem to affect the pattern of bands on the gels, we feel that these bands are representative of the kinds of molecules that exist in cells as well as their relative rates of accumulation. The actual numbers of various molecules per cell are not reflected in the intensity of the gel bands since we are studying relatively short labeling periods. This short term labeling does permit visualization of several molecules that are metabolically unstable (19) and have not been described previously. Information about the location of these molecules within cells would undoubtedly be of great use in determining their functions. Characterization of these RNA molecules by polyacrylamide gel electrophoresis and fingerprinting should facilitate correlation of physical, chemical, and physiological studies. One such study on the accumulation of these RNAs when cells are in various physiological states, is presented in the accompanying paper (19).

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