Studies and Sequences of *Escherichia coli* 4.5 S RNA

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**SUMMARY**

4.5 S RNA, a biologically stable species with electrophoretic properties intermediate between 5 S and transfer RNAs, has been isolated from *Escherichia coli* and characterized. No function has yet been found for this molecule. Its primary structure and behavior suggests an unusually stable and possibly unique secondary structure. Even from single species of *E. coli*, there is some sequence heterogeneity within the molecule. The sequence of a major species from MRE 600 is:

\[
\begin{align*}
&\text{pG-G-G-C-U-C-U-G-U-U-G-U-C-U-C-C-G-C-A-A-C-} \\
&\text{C-U-C-A-C-U-C-G-G-U-U-U-C-A-C-A-G-G-U-C-A-G-G-G-C-C-} \\
&\text{C-C-G-A-A-G-A-G-C-C-A-G-C-A-G-U-A-C-C-G-C-C-G-U-G-G-C-C-G-A-A-A-G-G-C-C-C-} \\
&\text{C-A-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C-C
To isolate low molecular weight RNA, 2.5 volumes of cold ethanol were added to the aqueous supernatant and the mixture was stirred and allowed to stand for 30 min. The RNA was collected by centrifugation for 30 min at 10,000 rpm in the cold.

The precipitate (specific activity, about $1 \times 10^{6}$ cpm/µg) was dissolved in 150 µl of 0.01 M Tris-chloride (pH 7.5) and the products were separated by electrophoresis at 300 volts overnight at 4°C on a 10% acrylamide/0.5% bisacrylamide gel at pH 8.3, as described by Dingman and Peacock (12). Electrophoresis was stopped when the bromphenol blue dye marker was two-thirds the way down the gel. The positions of the gel bands were located by radioautography.

The 4.5 S band was cut out, and the RNA was extracted by homogenization in about 2 ml of 1 M NaCl (containing 1% phenol)/0.01 M Tris-chloride (pH 7.5). After centrifugation, the aqueous solution was removed and the gel was re-extracted to remove the rest of the radioactive material. Nonradioactive, carrier RNA (about 100 µg) and 2.5 volumes of cold ethanol were added to the combined extracts and the solution was left overnight at -20°C. The precipitated RNA was collected by high speed centrifugation.

In most experiments, the product (8 to 14 µg from this scale experiment) was solubilized in 0.01 M Tris-chloride (pH 7.5)/5 M urea and run on a 12.5% acrylamide/bisacrylamide stacking slab gel, as described by Vigne and Jordan (13).

**Time Course Study**—An inoculum of MRE 600 in 20 ml of low phosphate media was incubated at 37°C for 30 min with vigorous aeration, then 2 mCi of 32P-phosphate were added and growth was allowed to continue. At $A_{600}$ 0.40, half the sample was withdrawn, phenol-extracted, and the early log phase RNA was collected by alcohol precipitation. At $A_{600}$ 1.0, the rest of the sample was similarly treated to obtain late log phase RNA. The RNA samples were separated electrophoretically as described above, and products were located by autoradiography.

**Pulse Chase Study**—E. coli temperature-sensitive mutant AA-157 was grown as described by Griffin and Baillie (8). An inoculum of MRE 600 in 20 ml of low phosphate media (10 ml) containing 1 mCi 32P-phosphate and grown with vigorous aeration for 30 min at 30°C (permissive temperature); $A_{600}$ was 0.32. An aliquot (2 ml) was withdrawn for reference, then 0.1 M cold phosphate (1 ml, pH 7.2) was added, and the remaining solution was transferred to 42°C (nonpermissive temperature) and aeration was continued. Aliquots (2 ml) were withdrawn at 30, 60, 90, and 300 min and samples were worked up immediately as previously described. The RNA from each aliquot was separated by acrylamide gel electrophoresis (see above) and species containing radioactive label were located and counted by autoradiography.

**Nucleotide Composition and Fingerprint Analysis**—The nucleotide composition of 4.5 S RNA was determined after digestion of RNA overnight at 37°C in 0.5 M KOH. The 3′- and 5′-terminal nucleotides were determined by size exclusion chromatography using a Sephadex G-100 column. The products from the pancreatic A digest were digested again with the same RNase, and the resulting fragments were treated in the same manner. In most cases, the identity of any fragment was evident by a comparison of its position on the ionophoretogram with that of the fragments from a complete digest of the whole molecule. In cases of ambiguity, the fragments were cut out, eluted, and further digested by the procedures mentioned above for characterization of fragments.

In a similar experiment, a sample of 4.5 S RNA, containing a large excess (280 µg) of carrier RNA, was digested with pancreatic ribonuclease A at 4°C for 30 min in 20 µl of 0.01 M Tris-chloride/0.001 M MgCl2 using an enzyme to substrate ratio of 1:20000. The mixture was put immediately into a narrow slot (15 mm) in a 10% acrylamide/0.5% bisacrylamide gel and electrophoresis was carried out as described above in borate buffer (pH 8.3). The vertical band containing the separated oligonucleotides was cut out, digested with RNase (1 mg/ml) for 30 min at 37°C, and the products were treated again with pancreatic ribonuclease A at 4°C for 30 min. The products from spots corresponding to separated oligonucleotides were further digested with pancreatic ribonuclease to complete digestion and analyzed by the two-dimensional fingerprinting technique (14).

**RESULTS**

4.5 S RNA was found in the supernatant fraction of the cells after removal of the ribosomes. In a “time course” study which monitored the appearance of low molecular weight RNAs during the growth cycle of the cell, it appeared in early log phase in yields approximately equivalent to a single tRNA species (Fig. 2). An examination of the nucleotide composition showed a G + C content of 62% (Table I), with pG as the 5′-terminal nucleotide, thus suggesting that the molecule might be related to the tRNA species. Studies with the Escherichia coli mutant, AA-157, temperature-sensitive with respect to RNA synthesis...
Figs. 1-4
the T1 fragments, the use of polyethylenimine-cellulose thin layer chromatography (Fig. 3) for a second dimensional separation was particularly valuable. This technique separated the three T1-octanucleotides found to be present in 4.5 S RNA (ionophoresis on DEAE-cellulose paper (Fig. 7) did not) and resolved the 3' end oligonucleotide (which streaked badly on DEAE-cellulose paper) as a discrete spot. The sequences of the small oligomers obtained by complete digestion with ribonucleases are summarized in Table II. In all cases, the 5'-terminal nucleotide in a sequence was determined by comparing the composition of a complete venom phosphodiesterase digest with either Tt or pancreatic A ribonuclease digestion, gave sufficient information to order the sequence. The structure (I-C-C-A-C-C-C-G) was felt to be a function of the large number of cytidine residues in the fragment. The structure C-C-C-A-C-C-C-G was assigned to the 3'-terminal fragment because it contained the same ratio of A:C (1:5) whether it was digested with Tt-ribonuclease (to nucleoside 5'-phosphates), and a fragment obtained from U2-ribonuclease digest had an A:C ratio of 1:3.

Because of the streaking of the Tt fragment containing the 3' end (Table II, Spot 21) on a DEAE-cellulose ionophoretogram (cf. Fig. 7), it was originally felt that this species might be modified on its terminal nucleotide. Precedence for this resides with the transfer RNAs and with the finding of a blocked 3' end in one of the 4.5 S RNA molecules (4.5 S RNA3M) in mammalian cells (18). Treatment of E. coli 4.5 S RNA with aqueous ammonia before digestion, under conditions which would readily remove any acyl group, failed to change the properties of the 3' end fragment. Moreover, this fragment did not streak on polyethyleneimine cellulose (Fig. 3) and was susceptible to periodate oxidation (19). Therefore, the observed streaking on DEAE-cellulose was felt to be a function of the large number of cytidine residues in the fragment. The structure C-C-C-A-C-C-C-G was assigned to the 3'-terminal fragment because it contained the same ratio of A:C (1:5) whether it was digested with Tt-ribonuclease (to nucleoside 3'-phosphates) or with venom phosphodiesterase (to nucleoside 5'-phosphates), and a fragment obtained from U2-ribonuclease digest had an A:C ratio of 1:3.

The other difficulties encountered in fragment sequencing could be attributed to the heterogeneity of 4.5 S RNA. Working with a single strain of E. coli, the quantitations of the fragments containing A-A-G seemed to vary from preparation to preparation. There were also strain variations. Although the yield of A-A-G itself (obtained from Tt plus pancreatic ribonuclease A digestion), in samples from MRE 600, remained constant at 3.0

In the cases of the T1-octanucleotides U-U-U-A-C-C-A-G and U-C-A-C-U-C-U-G, it proved necessary to cleave the molecules with U2-ribonuclease and separate the products (U-U-U-A, C-C-A, G, and U-C-A, C-U-C-U-G, respectively) before definitive sequences could be determined. In the case of U-U-C-U-C-C-G, the CMCT modification plus pancreatic ribonuclease digest (14) gave partial sequence information but still failed to distinguish between U-U-C-U-C-C-G and U-U-C-U-C-C-G. In this case, a partial venom phosphodiesterase digestion was carried out and the products were analyzed using the method set out by Ling for DNA fragments (15). The evidence obtained supported the sequence U-U-C-U-C-C-G but could not be called absolutely conclusive due to the very small amounts of product being analyzed at the end of the experiments. (With all the elaborate methods available for sequencing oligoribonucleotides, the sequence of a long pyrimidine chain still presents considerable difficulties.)

Sequence Analysis

Products of Complete Digestion with Ribonucleases—As a first step in sequence determination, the fragments obtained from complete digestion with either Tt or pancreatic A ribonuclease were separated, isolated, and quantitated and their base compositions and ultimately their sequences were determined. For the Tt fragments, the use of polyethylenimine-cellulose thin layer chromatography (Fig. 3) for a second dimensional separation was particularly valuable. This technique separated the three T1-octanucleotides found to be present in 4.5 S RNA (ionophoresis on DEAE-cellulose paper (Fig. 7) did not) and resolved the 3' end oligonucleotide (which streaked badly on DEAE-cellulose paper) as a discrete spot. The sequences of the small oligomers obtained by complete digestion with ribonucleases are summarized in Table II. In all cases, the 5'-terminal nucleotide in a sequence was determined by comparing the composition of a complete venom phosphodiesterase digest with that obtained either from alkali or Tt-ribonuclease. In many cases, this, together with Tt plus pancreatic ribonuclease A digestion, gave sufficient information to order the sequence. The legend to Table II shows the other methods and enzymes used.

Additional elaboration was necessary in a few cases, however, to complete the sequences.
Escherichia coli 4.5 S RNA

Underlined regions of fragments show di- and trinucleotides obtained by combined T1 and pancreatic A ribonuclease digests. 5’-Terminal nucleotides were identified by complete digestion with venom phosphodiesterase. The high yield of the T1 product, U-G, may be attributed to secondary breaks by the enzyme.

| Spot no. | Sequence | Molar yield |
|----------|----------|-------------|
| 1        | U-U-C    | 0.8         |
| 2        | U-U-C-G  | 1.0         |
| 3        | U-C-A    | 0.9         |
| 4        | U-G      | 1.0         |
| 5        | C-U-C    | 1.1         |
| 6        | G        | 0.5         |
| 7        | A-C      | 1.0         |
| 8        | U-G      | 1.0         |
| 9        | C-U-C    | 1.2         |
| 10       | C-C-C    | 1.1         |
| 11       | C-U-C    | 0.9         |
| 12       | A-C-G    | 1.0         |
| 13       | C-A-A-G-C | 1.2       |
| 14       | C-C-A    | 0.7         |
| 15       | A-A-G    | 0.7         |
| 16       | U-G      | 1.7         |
| 17       | C-C-A    | 1.7         |
| 18       | C-G      | 1.1         |
| 19       | C-G      | 1.3         |
| 20       | G        |             |
| 21       | C-C-C-A-C-C-C-G | 0.7 |

† Represents an average of several measurements.

‡ Indicates that U1-ribonuclease was used to get information about the position of A in the fragments. Products of digestion were separated by one-dimensional electrophoresis on Whatman DE81 paper with 7% formic acid. Positions relative to xylene cyanol blue dye marker were: U-U-C-U-C-C-C-G (undigested) = 0.15; U-U-U-A (Spot 1) = 0.25; C-U-C-U-G (Spot 3) = 0.3; C-C-C-out (Spot 21) = 1.0; U-C-A (Spot 2) = 1.45; C-C-C-A (Spot 21) = 1.3; and C-C-A (Spot 1) = 1.6.

§ Indicates that products from partial U2-ribonuclease digests, separated by one-dimensional electrophoresis on Whatman DE81 at pH 3.5, were used in determining complete sequences: Spot 2 gave G-A-G + G-G + A-A. Spot 5 gave G-G + G-U, and Spot 6 gave A-A + G-G + G-G-C.

¶ Indicates that CMCT blocking, followed by pancreatic ribonuclease A digestion, was used to locate the positions of U in the fragments. Products of digestion were separated by one-dimensional electrophoresis on Whatman 3MM paper. At pH 3.5, CMCT-modified products migrated toward the cathode and are given a positive sign relative to the origin; unmodified products migrated toward the anode and are given a negative sign. Product identity (after removal of the blocking group) is given in parentheses. Migration of products were Spot 1 at +12.0 (U-U-U-A-C), +0.5 (A-G) and -3.5 cm (C); Spot 2 at +5.0 (U-U-C), +2.5 (U-C), -3.5 (C), and +3.5 and -7.0 cm (G); Spot 5 and the pancenucleotide from U2-ribonuclease digest of Spot 3 at +11.0 and +5.3 (U-G), and -3.5 cm (C); Spot 12 at +2.5 (U-C), -3.5 (C) and +3.5 and -7.0 cm (G).

The determination of the sequences of pancreatic ribonuclease A fragments (Fig. 4) G-A-A-C and G-A-A-C was complicated by the heterogeneity present in individual samples of 4.5 S RNA. Analysis of a partial spleen phosphodiesterase digest of both fragments using the two-dimensional procedure of Ling (15) showed that G-A-A-C appeared to have the sequence A-A-G-G-C regardless of its molar yield; in some species of E. coli there was only 1 molar equivalent, but in most it was nearer 2. There was, however, some indication of ambiguity in G-A-A-C, and in E. coli CA 265 some evidence of a species G-A-A-C. The sequence given, G-G-A-A-G-G-C, can only be said to represent the major species with this composition in MRE 600. Moreover, in E. coli CA 265, a base change (A → G) was found in the stem portion of the molecule, which resulted in the appearance of A-G-G-G-G-C (rather than A-A-G-G-C) among the pancreatic ribonuclease A digests and C-A-G among the T1 products. The results obtained on analysis of the pancreatic A complete digestion products of 4.5 S RNA are summarized in Fig. 4 and Table II. The heterogeneity of 4.5 S RNA will be considered in more detail below.

Fragment Overlaps in Sequence Determination—In an attempt to get large oligoribonucleotides for fragment overlaps, enzymatic digestions at low temperature, with low concentrations of either T1, or pancreatic ribonuclease A, were carried out. Analysis of the products obtained under a variety of conditions, using the homochromatography method (20) showed that 4.5 S RNA, unlike most other low molecular weight RNAs studied, gave mainly extremely large fragments (not separable by the method used) or products expected from complete enzymatic digestion. There were a few molecules of intermediate size, but in very low concentrations. The identity of molecules obtained by this method is shown schematically in Fig. 10. It seemed unlikely that this technique could be made to yield a complete sequence for 4.5 S RNA.

When partial digests with T1-ribonuclease, however, were examined by electrophoresis on acrylamide gels, a series of discrete bands was observed, as shown in Fig. 5. The slowest migrating band (A) was found to give a fingerprint corresponding to that of whole 4.5 S RNA. The next band (B) gave a similar fingerprint, but with two unique products missing: U-C-C-G and A-A-G. The most notable feature of Band B was that it contained both the 3’ and 5’ end products. Continued analysis of other bands (C to F) showed this phenomenon to persist: the fingerprints became increasingly simpler as the bands decreased in size, but each band contained the 3’ and 5’-terminal fragments. This suggested that the secondary structure, possibly in conjunction with tertiary structure, left only one area (or a very few areas) open to enzymatic attack, and further digestion proceeded from this area (or areas) in the directions of the termini, and that the terminal portions of the molecule possessed an unusually stable structure. A less likely assumption was that if "randomly" digested to small fragments, their secondary structures interacted with one another in a specific manner during separation by gel electrophoresis.

For further analysis, the gel bands obtained from similar partial enzymatic digests (Fig. 5) were excised, the RNA was eluted, and the 3’- and 5’-terminal “stem strands” were separated. The separation procedure used was the two-dimensional homochromatography method of Brownlee and Sanger (20). For the larger products (B to C), the separation was essentially accomplished by the first dimension, but for the smaller products (D to F), both dimensions afforded separation. It was hoped that Band A would be composed of two “halves” of the entire molecule, isolation of which would reveal the site most vulnerable to enzymatic digestion; all attempts to separate Band A...
B, position of the xylene cyan01 blue dye marker. The questions posed in the study of the molecule from E. COli illustrated by D-(6') and E-(6'), and D-3' and E-S', respectively, weight RNAs found in eukaryotic cells. A review by Weinberg (26) discusses the low molecular weight RNAs found in eukaryotic systems (18). 4.5 S RNA found in the cytoplasm of Novikoff hepatoma ascites cells has been sequenced (25). The findings are summarized in Tables III and IV.

Two points seem worthy of note in conjunction with these results. One is that gel Band C', and the digestion pattern it represents, is peculiar to the Neurospora crassa guanosine-specific ribonuclease (N1) and is not found with Tt-ribonuclease. Pinder and Gratzer (21) found that although the primary specificity of these two enzymes is the same, the cleavage patterns of rabbit reticulocyte RNA under limiting digestion conditions with the two enzymes was different. Both results point to different substrate affinities for these enzymes. The second point to note is that the enzyme cleavage leading to the 3' strand portion in gel Band C is due to cutting in what is normally a minor 4.5 S species, one in which the cytidine at position 58 has been replaced by guanosine. Attempts to obtain large oligonucleotides under limiting digestion conditions with pancreatic ribonuclease A were only successful under extreme conditions: in the presence of large excess of carrier RNA with minute amounts of enzyme. Two fragments, obtained under these conditions and separated by a two-dimensional acrylamide gel electrophoretic procedure, were found to be from the “non-stem” portion of the molecule and to contain the overlaps missing from the above Tt-ribonuclease results. They are labeled 6 and 7 in Fig. 9.

A primary structure for 4.5 S RNA is proposed in Fig. 10, based on the sequences of the products obtained by complete digestion with ribonucleases (Table II) and the overlaps observed by digestion under limiting conditions (Figs. 5 to 10). The secondary structure (Fig. 11) attempts to account not only for the unusual stability of this molecule but also for the sites of cleavage by Tt- and N1-ribonucleases.

DISCUSSION

An early report of an RNA molecule in E. coli intermediate in size between 5 S and transfer RNAs was made by Hindley in 1967, when he isolated an impure species from MRE 600 (22). Subsequently, a number of strains of E. coli have been examined (as indicated) and been found to contain 4.5 S RNA; it has also recently been reported present in some stringent and relaxed strains studied by Ikemura and Dahlberg (24). Molecules of similar size, although probably unrelated, have been isolated from eukaryotic systems (18). 4.5 S RNA found in the nucleus of Novikoff hepatoma ascites cells has been sequenced (25). A review by Weinberg (26) discusses the low molecular weight RNAs found in eukaryotic cells.

The questions posed in the study of the molecule from E. coli designated 4.5 S RNA were: where is it found in the cell, when
TABLE III

T1-ribonuclease products from partial digestion fragments after strand separation

| BAND | 5' STRAND | 3' STRAND |
|------|-----------|-----------|
| B    | U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, pGp, 4-5G |
| BAND C | U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, pGp, 3-4G |
| BAND C' | same as C |
| BAND D | U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, pGp, 3-4G |
| BAND E | U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, U-U-U-4C-C-G, pGp, 3-4G |
| BAND F | same as F |

The E. coli mutant AA-157 shows a complete shut-off of stable RNA production within minutes of being shifted from permissive to nonpermissive temperature, whereas most messenger RNA production is unimpaired at the latter temperature (8, 27). In a pulse-chase experiment, aimed at studying the stability of 4.5 S RNA, it was found that label 32P was not chased out of this molecule when cells were given excess nonradioactive phosphate and left at the nonpermissive temperature for times exceeding several cell growth cycles. Thus, 4.5 S RNA was found to be related to other low molecular weight RNAs such as 5 S, 6 S, and transfer RNAs in its stability.

Having thus defined 4.5 S RNA as a stable species which appears in the supernatant fraction of the cell early in the growth cycle, an attempt was made to sequence this molecule with the hope that its structure might shed some light on its function. Sequence analysis was complicated by the fact that several related species were found in material isolated from whole cell MRE 600. Attempts to separate these species by biological or chemical methods were without success, although purification of the RNA to a high degree led to the predominance of two

**Fig. 7** (top). Autoradiograms of two-dimensional separations of terminal T1-ribonuclease digestion products of whole 4.5 S RNA from MRE 600 and the separated 5' and 3' stem fragments from gel Bands D and F (cf. Fig. 5). Separation in the first dimension was by electrophoresis on cellulose acetate strips, pH 3.5, and in the second by electrophoresis on sheets of DEAE-cellulose (45 X 85 cm, DE81) in 7% formic acid (14). For accurate comparison of the position of oligonucleotides, the digestion products from the separated 5' and 3' fragments of Band D, and similarity of Band F, were each put on the same strip of cellulose acetate and applied at one end and in the center (D-5' and D-3', and F-5' and F-3', respectively, as shown) electrophoresis was carried out and the products were separated further by a second dimension on the same sheet of DE81. The oligonucleotides belonging to the 3' portion of the molecule can be seen in the right half of the photographs of products from Bands D and F, and those belonging to the 5' portion in the left half. Numbers 1 and 6 show the positions of the 3'- and 5'-terminal T1 products (C-C-C-A-C-C-CON and pGp, respectively). The identities of the other numbered products are given in Table II. All products were isolated and identified as described in the text. B, position of the xylene cyanol blue dye marker. Similar two-dimensional analyses (not shown) were carried out on the products from the 3' and 5' associated fragments of gel Bands B, C, C', E, and F (Fig. 5). The results are summarized in Table III.

**Fig. 8** (bottom). Autoradiograms of two-dimensional separations of terminal pancreatic ribonuclease A digestion products of whole 4.5 S RNA from MRE 600 and the separated 5' and 3' stem fragments from gel Band D (Fig. 5). The procedures used were identical with those described in Fig. 7. (Uridine-3'-phosphate (Spot 19) does not appear on the photograph of the pancreatic ribonuclease A digest of whole 4.5 S RNA; it would be the fastest running spot in the second dimensional separation.) The oligonucleotides belonging to the 3' portion of the separated fragments from gel Band D can be seen in the right half of its photograph and those belonging to the 5' portion in the left half. Number 1 shows the position of the 5'-terminal product, pGp-G-G-G-C. The identities of all the other numbered products are given in Table II. B, position of the xylene cyanol blue dye marker. Similar two-dimensional analyses (not shown) were carried out on the products from the 3' and 5' associated fragments of gel Bands B, C, C', E, and F (Fig. 5). The results are summarized in Table IV.
TABLE IV

Pancreatic ribonuclease A products from partial digestion fragments after strand separation

| BAND | 3'-STRAND |
|-------|------------|
| B     | G-G-G-A-U, A-G-A-U, 2A-A-G-G-C, G-G-C, 3G-U, 2G-C, G-A-C, A-G-C, A-C, 6-C, 5-6C, U |
| C     | same as B  |
| C'    | G-G-G-A-U, A-G-A-U, 2A-A-G-G-C, G-G-C, 3G-U, 2G-C, G-A-C, A-G-C, A-C, 6-C, 5-6C, U |
| D     | G-G-G-A-U, A-G-A-U, 2A-A-G-G-C, G-G-C, 3G-U, 2G-C, G-A-C, A-G-C, A-C, 6-C, 5-6C, U |
| E     | G-G-G-A-U, A-G-A-U, 2A-A-G-G-C, G-G-C, 3G-U, 2G-C, G-A-C, A-G-C, A-C, 6-C, 5-6C, U |
| F     | same as E  |

* In some experiments, only 2G-U were found, suggesting an initial cleavage after G" instead of G" (Fig. 11).

The products of complete digestion of 4.5 S RNA with either T₄ or pancreatic A ribonucleases were examined (Figs. 3 and 4). The characterization of each product of complete digestion was determined using methods previously described (cf. Table II). Sequence analysis was complicated by what in hindsight can be seen as the secondary structure of the molecule. Under limiting enzymatic digestion conditions, it proved to be difficult to get oligonucleotides of intermediate size, necessary for determining the overlaps of the products of complete enzymatic digestion. Most digestion conditions gave either the latter products themselves, or products of size too large for analysis on the usual two-dimensional systems (14). Oligonucleotides of intermediate size, some of which were isolated and partly characterized, were generally present in yields too small to allow for absolute identity determination. This problem was solved when the affinity for association of stem portions of the molecules was recognized. 4.5 S RNA was digested under limiting digestion conditions with T₄-ribonuclease and the associated stem fragments were separated by electrophoresis on acrylamide gels (cf. Fig. 5). The isolated fragments were then further separated into 3'- and 5'-terminal species under denaturing conditions and each fragment subsequently was digested and analyzed. The largest associated stem fragment contained 98 of the products belonging mainly to the 3' end of 4.5 S RNA (Table IV) whereas those from Spot 3 belonged mainly to the 5' end of the molecule. They were not, however, fully characterized. Spots 6 to 9 were not pure enough for accurate characterization but contained products expected from associated 3' and 5' stem fragments. Spots 6 and 7 were found to contain products belonging to the center of the molecule and were fully characterized by the two-dimensional procedure shown in Figs. 7 and 8. Spot 6 contained sequences corresponding to a region of the molecule extending from position 52 to 57, and Spot 7 from position 41 to 58 (Fig. 11). B, position of the bromphenol blue dye marker.

Fig. 9. An autoradiogram of a two-dimensional acrylamide gel electrophoretic separation of the products from a partial pancreatic ribonuclease A digest (enzyme to substrate ratio, 1:23000) of 4.5 S RNA. The conditions for the first dimensional separation were the same as those given in Fig. 1. The gel band (2 cm wide) containing the separated products was soaked in 7 M urea, then applied horizontally to the top of a 12.5%-acrylamide stacking gel (20 X 20 cm) essentially as described by Vigne and Jordan (13) and electrophoresis was carried out in borate buffer, pH 8.3. Spots 1 to 7 were eluted, further digested with pancreatic ribonuclease A, and analyzed by the two-dimensional methods described in Figs. 7 and 8. The results on Spot 1 showed it to contain species differing by only a single C → G base change. A corollary of this finding is that there must be at least two gene copies, and possibly more, for 4.5 S RNA.

The products of complete digestion of 4.5 S RNA with either T₄ or pancreatic A ribonucleases were examined (Figs. 3 and 4). The characterization of each product of complete digestion was determined using methods previously described (cf. Table II).

Sequence analysis was complicated by what in hindsight can be seen as the secondary structure of the molecule. Under limiting enzymatic digestion conditions, it proved to be difficult to get oligonucleotides of intermediate size, necessary for determining the overlaps of the products of complete enzymatic digestion. Most digestion conditions gave either the latter products themselves, or products of size too large for analysis on the usual two-dimensional systems (14). Oligonucleotides of intermediate size, some of which were isolated and partly characterized, were generally present in yields too small to allow for absolute identity determination. This problem was solved when the affinity for association of stem portions of the molecules was recognized. 4.5 S RNA was digested under limiting digestion conditions with T₄-ribonuclease and the associated stem fragments were separated by electrophoresis on acrylamide gels (cf. Fig. 5). The isolated fragments were then further separated into 3'- and 5'-terminal species under denaturing conditions and each fragment subsequently was digested and analyzed. The largest associated stem fragment contained 98 of the products belonging mainly to the 3' end of 4.5 S RNA (Table IV) whereas those from Spot 3 belonged mainly to the 5' end of the molecule. They were not, however, fully characterized. Spots 6 to 9 were not pure enough for accurate characterization but contained products expected from associated 3' and 5' stem fragments. Spots 6 and 7 were found to contain products belonging to the center of the molecule and were fully characterized by the two-dimensional procedure shown in Figs. 7 and 8. Spot 6 contained sequences corresponding to a region of the molecule extending from position 52 to 57, and Spot 7 from position 41 to 58 (Fig. 11). B, position of the bromphenol blue dye marker.
Fig. 10. A diagrammatic representation of sequences of some of the small oligonucleotides obtained from partial enzymatic digestions, placed on the primary structure of 4.5 S RNA. The partial T₁ (—) and pancreatic A (---) ribonuclease products were obtained by digestion conditions described in Figs. 5 and 9 and were separated by a two-dimensional procedure using electrophoresis at pH 3.5 on cellulose acetate strips in the first dimension and homochromatography on DEAE-cellulose (20 × 40 cm) thin layer plates in the second (29). The products shown were present in very small yields, but were useful for confirming the sequence overlaps suggested by data from experiments described in Figs. 5 to 9 and summarized in Tables III and IV. The primary sequence given here is suggested by this data. The oligonucleotide (*) came from a partial pancreatic ribonuclease A digest of *Escherichia coli* CA 265; it was not found in digests of MRE 600. In addition, a partial digestion product, G G C A G G C, was obtained from CA 265 which corresponded to a base change in that species of G → A at position 98.

Several possible roles remain for 4.5 S RNA: it may be the ribonuclease-sensitive species responsible for maintaining *E. coli* DNA in a tightly folded conformation. There is as yet no concrete evidence to support or reject any of these suggestions, although some possibilities seem more plausible than others.

Ademik and Levinthal first demonstrated the existence in *E. coli* of precursors to the 16 S and 23 S ribosomal RNAs (29). The precursor for 16 S RNA is approximately 200 nucleotides longer than the mature species. A comparison of some of the unique T₁-ribonuclease fragments from 4.5 S RNA with those on the fingerprints of precursor 16 S RNA (30–33) suggests that the 4.5 S RNA is not the species released in the maturation process. The work of Dahlberg and Peacock implies that although slight changes in primary structure may accompany the maturation of precursor 23 S RNA, the major alterations are probably in secondary and tertiary structure (34).

To postulate that 4.5 S RNA is the RNA covalently linked to nascent DNA fragments in the discontinuous mode of DNA replication is an attractive idea, but it can probably be discarded. Sugino and Okazaki have examined the RNA-DNA linkage in these species (35) and their studies show that the 3′ end of the RNA species is -(Py)pAp(U or C). The 3′ end of 4.5 S RNA does contain a portion (italicized) of this sequence, being -(Cy)pAp(U or C), but no 3′ end fragment containing uridine has ever been found.

The work of Stonington and Pettijohn (7) suggests that an RNA molecule is probably involved in maintaining *E. coli* DNA as a tightly coiled core structure. If such is the case, a low molecular weight, highly structured RNA such as 4.5 S RNA would seem an ideal candidate for interacting with DNA and maintaining its structure. This must remain a possible role for this molecule.

Finally, the evidence of Ikemura and Dahlberg (24) suggests that 4.5 S RNA is under stringent control. Therefore, the possibility exists that it may serve a regulatory role for protein synthesis. This concept is particularly attractive since 4.5 S RNA fulfills the requirements for such a molecule: it is a biologically stable species that under all growth conditions is maintained in amounts equivalent to that of a single tRNA species (i.e. more than one copy/ribosome). Its heterogeneity suggests multiple gene copies, and although not tightly...
attached to ribosomes, it should be capable of the loose attachment found for tRNAs.

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Fig. 11. The primary structure of 4.5 S RNA and a postulated secondary structure. The base changes (C → G) at position 58 observed in MRE 600 and (A → G) at position 98 observed in CA 265 are shown. The letters A to E indicate main cleavage sites using partial digestion conditions with T1-ribonuclease and correspond to letters used in Fig. 5. Numbers 6 and 7 show sites of cleavage obtained under partial pancreatic ribonuclease A digestion conditions and correspond to numbers shown in Fig. 9. The secondary structure suggested takes into account thermodynamic considerations (28), the general behavior of the molecule, and the site susceptible to enzymatic cleavage. This structure should be more stable than that suggested previously (22).
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