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 suggest 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:


6 S RNA has been discovered (8). To date, no precursor of 4.5 RNA has been found.1

4.5 S RNA has been shown to be a component of a number of strains of E. coli studied by this author. It is found in the supernatant fraction, after sedimentation of ribosomes, and use of a temperature sensitive mutant shows it to be a stable species. It is composed of 107 nucleotides and has no detectable minor bases. Its primary structure and properties indicate a G,C2 content similar to that of the transfer RNAs, a very high proportion of paired bases, and a stability greater than 20 kcal/mol.

EXPERIMENTAL PROCEDURE

Materials and Strains

Pancreatic ribonuclease A and snake venom phosphodiesterases were obtained from Worthington Biochemical Corp. T1-, T2-, and U1-ribonucleases were obtained from Sankyo Co., and N1-ribonuclease from Miles Laboratories, Inc. Spleen phosphodiesterase was purchased from Boehringer Mannheim GmbH (Germany). Acrylamide and N',N'-methylenebisacrylamide were obtained from British Drug House Chemicals Ltd.; the former was recrystallized from ethyl acetate and the latter from acetone before use. Polyethyleneimine (PEI)-cellulose thin layer chromatography plates were purchased from Schleicher and Scholl, Dassel (Germany). Yeast carrier RNA (Sigma Biochemicals) was phenol-extracted and ethanol-precipitated before use. [32P]Phosphate (carrier-free) was obtained from the Radiochemical Centre, Amersham, England. Strains studied were MRE 600 and Escherichia coli B, CA 265, and CA 276. A temperature sensitive mutant of E. coli, AA-157, was obtained from Dr. A. G. Atherley.

Methods

Preparation of 4.5 S RNA—32P-labeled RNA was obtained from MRE 600 grown in the presence of [32P]phosphate, essentially as described by Brownlee and Sanger (11). In a typical preparation, 20 mCi of [32P]phosphate were added to a low density cell culture in 200 ml of low phosphate media and the bacteria were grown with vigorous aeration at 37° to A660 N 1.2. The bulk of the RNA in the cells was isolated by standard procedures. It was dissolved in 0.01 M Tris buffer (pH 7.5)/0.01 M EDTA, made 1 M with respect to NaCl, and left at 4° for several hours, then centrifuged for 30 min at 10,000 rpm in the cold. Examination of the resulting precipitate by acrylamide gel electrophoresis showed it to contain mainly high molecular weight material, probably ribosomal RNA. This step resulted in a considerable purification of low molecular weight RNA at this stage, with negligible loss of desired material.

1 In a personal communication, Dr. S. Altman and A. Bothwell report that they have isolated and are studying a precursor of 4.5 S RNA.

2 The abbreviations used are: G, guanosine 3'-phosphate; C, cytosine 3'-phosphate; A, adenosine 3'-phosphate; U, uridine 3'-phosphate; CMCT, N'-cyclohexyl-N'-(p-morpholinyl)-4-ethylcarbodiimide p-toluenesulfonate.

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To isolate low molecular weight RNA, 2.5 volumes of cold ethanol were added to the aqueous supernatant and the mixture was left at -20°C overnight. 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 x 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 slab gel (20 x 40 cm) 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 90 to 95% homogeneous, as determined by a fingerprint assay and by accurate and complete digestion with T1-ribonuclease (Fig. 3). This purity was sufficient for most of the subsequent experiments. When higher purity was desired, the RNA was dissolved in 0.01 M Tris-chloride (pH 7.5)/7 M urea and rerun on a 12.5% acrylamide/bisacrylamide stacking slab gel, as described by Vigne and Jordan (13).

**Pulse Chase 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 A660 0.40, half the sample was withdrawn, phenol-extracted, and the early log phase RNA was collected by alcohol precipitation. At A660 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.

**Time Course Study**—An inoculum of MRE 600 in 20 ml of low phosphate media (pH 7.2) was added, and the remaining solution was transferred to 32P-labeled 4.5 S RNA (cf. Fig. 1) was isolated from a number of other strains and the T1-ribonuclease digestion (Fig. 3). This purity was sufficient for most of the subsequent experiments. When higher purity was desired, the RNA was dissolved in 0.01 M Tris-chloride (pH 7.5)/7 M urea and rerun on a 12.5% acrylamide/bisacrylamide stacking slab gel, as described by Vigne and Jordan (13).

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 1:200. The mixture was put immediately into a narrow slot (15 mm) in a 12.5% acrylamide gel band were run on the same strip of cellulose acetate used in the first dimensional separation, the first sample being applied at one end of the strip, and the other in the middle. Electrophoresis was stopped when the yellow dye from the marker dye mix (14) of the first sample reached the origin of application of the second sample. Band spots were excised and eluted immediately to the top of a 12.5% acrylamide/0.5% bisacrylamide stacking slab 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, agitated gently in water at 4°C for several hours and then in 7 M urea, and subsequently was applied horizontally to the top of a 12.5% acrylamide/0.5% bisacrylamide stacking slab gel (20 x 20 cm) (15). Electrophoresis was carried out again with borate buffer and stopped when the blue dye marker was halfway down the gel.

The products from the pancreatic A digest 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 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 1:200. The mixture was put immediately into a narrow slot (15 mm) in a 10% acrylamide/0.5% bisacrylamide stacking slab 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, agitated gently in water at 4°C for several hours and then in 7 M urea, and subsequently was applied horizontally to the top of a 12.5% acrylamide/0.5% bisacrylamide stacking slab gel (20 x 20 cm) (15). Electrophoresis was carried out again with borate buffer and stopped when the blue dye marker was halfway down the gel.

Isolated 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).
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 that obtained either from alkali or Ts-ribonuclease. In many cases, this, together with T1 plus pancreatic ribonuclease A digestion, gave sufficient information to order the sequence. The legend to Table II shows the other methods and enzymes used.

Sequence Analysis

Products of Complete Digestion with Ribonucleases—As a first step in sequence determination, the fragments obtained from complete digestion with either T1 or pancreatic A ribonuclease were separated, isolated, and quantitated and their base compositions and ultimately their sequences were determined. For 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 that obtained either from alkali or T1-ribonuclease. In many cases, this, together with T1 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.

In the cases of the T1-octanucleotides U-U-U-A-C-C-A-G and U-C-A-C-U-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-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.)

Because of the streaking of the T1 fragment containing the 3' end (Table II, Spot 21) on a DEAE-cellulose ionophoregram (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 RNA14) 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 polyethylenimine 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-COH was assigned to the 3'-terminal fragment because it contained the same ratio of A:C (1:5) whether it was digested with T1-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 quantities 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 T1 plus pancreatic ribonuclease A digestion), in samples from MRE 600, remained constant at 3.0

Fig. 1 (top left). An autoradiogram of the separation of low molecular weight 32P-labeled RNA from Escherichia coli by gel electrophoresis. A slab gel (20 X 40 cm) composed of 10% acrylamide/0.5% bisacrylamide was used and electrophoresis was carried out at 4° using barate buffer, pH 8.3 (12). The position of 4.5 S RNA relative to other low molecular weight RNAs and to a bromphenol blue (B) dye marker was determined by autoradiography, one-half of the gel is shown. Gel bands were excised and counted by Cerenkov radiation. From a number of similar experiments, the yield of 4.5 S was found to be roughly equivalent to that of a single species of transfer RNA.

Fig. 2 (top right). An autoradiogram of the separation by gel electrophoresis of low molecular weight 32P-labeled RNA isolated from Escherichia coli cells during early (E) and late (L) log phase growth. The gel conditions used were the same as those described in Fig. 1. At E the Azex of the cells was 0.40; at L Azex was 1.0. The yield of 4.5 S RNA relative to tRNA is (17) was relatively constant with time, whereas the yield of 6 S RNA increased, being much greater at late than early log phase.

Fig. 3 (bottom left). An autoradiogram of a two-dimensional separation of the ribonuclease T1-terminal digestion products of 32P-labeled 4.5 S RNA from MRE 600. Separation in the first dimension was by electrophoresis on cellulose acetate, pH 3.5 (14), and in the second by thin layer chromatography on a sheet (20 X 40 cm) of PEI-cellulose (4). For the second dimensional separation, the thin layer chromatography plate was eluted up to 10 cm using 1.5 m pyridinium formate, then transferred to 2.2 m pyridinium formate and eluted up to 30 cm. This step-wise technique allowed for maximum separation of the larger oligonucleotides without loss of resolution of the smaller oligonucleotides. The identification of each product is shown. 4.5 S RNA from Escherichia coli CA 265 gave, in addition to products shown, another molar equivalent of C-A-G. B is the position of the xylene cyanol blue dye marker.

Fig. 4 (bottom right). An autoradiogram of a two-dimensional separation of the pancreatic ribonuclease A terminal digestion products of 32P-labeled 4.5 S RNA from MRE 600. The techniques are the same as those described in Fig. 3 except that the entire second dimensional separation was carried out in 1.0 m pyridinium formate. Missing from this photograph is cytidine 3'-phosphate (C); it runs slower than all other products in the first dimension and faster in the second. 4.5 S RNA from Escherichia coli CA 265 gave, in addition to products shown, 1 molar equivalent of A-G-G-G-C; its position is indicated. B, position of the xylene cyanol blue dye marker.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>% (experimental) a</th>
<th>% (theoretical) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>28.9</td>
<td>28.3</td>
</tr>
<tr>
<td>AMP</td>
<td>18.7</td>
<td>17.9</td>
</tr>
<tr>
<td>CMP</td>
<td>32.0</td>
<td>34.0</td>
</tr>
<tr>
<td>UMP</td>
<td>19.4</td>
<td>19.8</td>
</tr>
<tr>
<td>CMP + GMP</td>
<td>61.8</td>
<td>62.3</td>
</tr>
</tbody>
</table>

a Average of a number of experiments.  
b Based on the sequence shown in Fig. 10.

(8), showed that 4.5 S RNA was a stable species, and therefore an unlikely candidate for a simple tRNA precursor. Similar results have been found by Ikemura and Dahlberg (3). Preliminary attempts to charge 4.5 S RNA with an amino acid mixture were not successful. Careful examination revealed no minor bases in the molecule.

FIG. 3

TABLE I

Nucleotide composition of 4.5 S RNA

<table>
<thead>
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</tr>
</tbody>
</table>

a Average of a number of experiments.  
b Based on the sequence shown in Fig. 10.
TABLE II

*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.

<table>
<thead>
<tr>
<th>T1 Ribonuclease Digestion Fragments</th>
<th>Pancreatic Ribonuclease-A Digestion Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spot No.</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>1</td>
<td>U-U-G</td>
</tr>
<tr>
<td>2</td>
<td>U-A-G-A</td>
</tr>
<tr>
<td>3</td>
<td>C-C-A-A-C-C-G</td>
</tr>
<tr>
<td>4</td>
<td>U-A-C-G</td>
</tr>
<tr>
<td>5</td>
<td>G-C-U-C-G</td>
</tr>
<tr>
<td>6</td>
<td>G-U-G-U-G</td>
</tr>
<tr>
<td>7</td>
<td>A-A-G</td>
</tr>
<tr>
<td>8</td>
<td>U-A-G-A</td>
</tr>
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<tr>
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<tr>
<td>11</td>
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<td>18</td>
<td>C-C-G</td>
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<td>19</td>
<td>G-G</td>
</tr>
<tr>
<td>20</td>
<td>C</td>
</tr>
<tr>
<td>21</td>
<td>C-C-C-A-C-C-G</td>
</tr>
</tbody>
</table>

† Represents an average of several measurements.

a 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-OH (Spot 21) = 1.0; U-C-A (Spot 3) = 1.45; C-C-C-A (Spot 21) = 1.3; and C-C-A (Spot 1) = 1.6.

b 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-C + G + G + A-A. Spot 5 gave G-G + G-U, and Spot 6 gave A-A + G-G + G-G-C.

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. Products 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 3 and the penultimate nucleotide from U1-ribonuclease digest of Spot 3 a 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).

molar equivalents, considerable variation occurred within the A-A-G-containing T1 or pancreatic A digestion fragments. This is not reflected in the molar yields given (Table II, Spots 13 to 15 in T1 digest, and Spots 2 and 6 in pancreatic A digest) as they represent averages from several preparations.

The determination of the sequences of pancreatic ribonuclease A fragments (Fig. 4) GaA2C and GaA2C 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 GaA2C 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 GaA2C, and in *E. coli* CA 265 some evidence of a species GaA2C. The sequence given, G-G-A-A-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-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 digests 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 T1 fingerprint, but with two unique products missing: U-C-C-G and C-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 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 were strong enough for them to remain associated 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. 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.
into two fragments were, however, unsuccessful. Fig. 6 shows the separation of the two products from gel Bands D and E. In all cases (Bands B to F), the 5'-terminal strand migrated faster than the 3'-terminal in the first dimension and in most cases slower in the second. The spots corresponding to separated strands were eluted; one-half of the RNA from each spot was digested completely with T\(_1\)-ribonuclease and the other half with pancreatic ribonuclease A, and fingerprints made to allow analysis of the separated strand fragments. Fig. 7 shows T\(_1\)-ribonuclease fingerprints of whole 4.5 S RNA and of products from the separated strands of Bands D and F; Fig. 8 shows pancreatic ribonuclease fingerprints of whole 4.5 S RNA and the products from separated strands of Band D. Similar fingerprints were made for separated strand products from Bands B to F obtained by partial digestion with either T\(_1\)- or N\(_1\)-ribonuclease, and a band (C') obtained only from the latter (Fig. 5). 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 (N\(_1\)) and is not found with T\(_1\)-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 subsite 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 come from the “non-stem” portion of the molecule and to contain the overlaps missing from the above T\(_1\)-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 T\(_1\) and N\(_1\)-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 (23). 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
FIGS. 7 and 8
portion of the cells. tRNAs, was found exclusively in the non-ribosomal, supernatant analyzed by gel electrophoresis. 4.5 S RNA, together with the natant and the RNA from both fractions was isolated and notable aspects of this study was that another small RNA, suggested that if 4.5 S RNA has a precursor, maturation occurs early in the cell cycle. MRE 600, 4.5 S RNA was found in relatively high yield in cells very low yields at early log phase, only appearing in "normal"ments, no precursor of 4.5 S RNA was found. The experiments (NaCl)-washed ribosomes were separated from of terminal T1 ribonuclease digestion products of whole 4.5 S RNA; it would be the fastest RNA from MIRE 600 and the separated 5' and 3' stem fragments from gel Band D, and similarly from gel Band E. and F (Fig. 5). The results are summarized in Table II. B, position of the xylene cyanid blue dye marker. Similar two-dimensional analyses (not shown) were carried out on the T1 products from the separated 3' and 5' associated fragments of gel Bands B, C, C', and E (Fig. 5). The results are summarized in Table III. 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-3' and D-5', and F-3' and F-5', 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 photograph of products from Bands D and F, and those belonging to the 5' portion in the left half. Numbers 1 and 2 show the positions of the 3'- and 5'-terminal T1 products (C-C-C-A-C-C-C-G and pG, 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 cyanid 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

<table>
<thead>
<tr>
<th>Band</th>
<th>5'-STRAND</th>
<th>3'-STRAND</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>same as B</td>
<td>same as B</td>
</tr>
<tr>
<td>C'</td>
<td>same as C</td>
<td>same as C</td>
</tr>
<tr>
<td>F</td>
<td>same as E</td>
<td>same as E</td>
</tr>
</tbody>
</table>

* In some experiments, only 2G-U were found, suggesting an initial cleavage after G7 instead of G7 (Fig. 11).

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, 1:20,000) 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 T1 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 T1-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 2 belonged mainly to the 5' end of the molecule. They were not, however, fully characterized. Spots 3 to 5 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 87, and Spot 7 from position 41 to 58 (Fig. 11). B, position of the bromphenol blue dye marker.
or it may be the ribonuclease-sensitive species responsible for serving as a template for DNA synthesis (Okazaki fragments), in the regulation of protein synthesis, it may be the RNA which RNA, it may be a direct transcription product which has a part piece of RXA excised during the maturation of a large precursor followed by UAG.

Since it has been found to be a biologically stable species from which the 3' terminal cytidine residues had been removed.) Since it has been found to be a biologically stable species that under all growth conditions is a highly ordered structure—over 70% of the bases can form hydrogen bonds—with one region, a purine rich region in the center of the molecule, more susceptible than other regions to the action of endonucleases.

The secondary structure proposed for this molecule (Fig. 11) attempts to account for the unusual physical stability observed for 4.5 S RNA (in many of its characteristics, it behaves more like a double- than a single-stranded RNA), its apparent “compactness” (for its molecule weight, it migrates abnormally fast during electrophoresis on acrylamide gels under nondenaturing conditions) (17), and its positions of susceptibility to endonucleases. Using the data of Tinoco et al. (28), the molecule shown (Fig. 11) for 4.5 S RNA from MRE 600 would have $\Delta G > -20$ kcal/mol. (The major 4.5 S species from Escherichia coli CA 265, which has a base change (A → G) in the stem of the molecule, would be even more stable.) The structure shown fits the observations that the molecule has a highly ordered structure—over 70% of the bases can form hydrogen bonds—with one region, a purine rich region in the center of the molecule, more susceptible than other regions to the action of endonucleases.

No function for 4.5 S RNA has yet been found. Like the transfer RNAs, it has a high G,C content, but neither the primary nor proposed secondary structure indicates any further similarity. (Preliminary attempts to charge 4.5 S RNA with amino acids were unsuccessful, but no attempts were made to charge a species from which the 3'-terminal cytidine residues had been removed.) Since it has been found to be a biologically stable species, 4.5 S RNA is probably neither a precursor of a yet smaller molecule nor a small messenger RNA. The two AUG residues in the molecule are not in phase, but are both located near the 3' end of the molecule, one of them being immediately followed by UAG.

Several possible roles remain for 4.5 S RNA: it may be a piece of RNA excised during the maturation of a large precursor RNA, it may be a direct transcription product which has a part in the regulation of protein synthesis, it may be the RNA which serves as a template for DNA synthesis (Okazaki fragments), or it may be the ribonuclease-sensitive species responsible for holding 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 T,-ribonuclease fragments from 4.5 S RNA with those shown 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 Dahlgberg 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 -(CpCpCpA)pApCpCpC, 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 folded 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 Dahlgberg (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 present in amount approximately 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

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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 x 40 cm) thin layer plates in the second (23). 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 C was obtained from CA 265 which corresponded to a base change in that species of G → A at position 98.
attached to ribosomes, it should be capable of the loose attachment found for tRNAs.

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Fig. 11. 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 sites susceptible to enzymatic cleavage. This structure should be more stable than that suggested previously (22).
Studies and sequences of Escherichia coli 4.5 S RNA.
B E Griffin


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