PRECURSORS OF STABLE RNA ACCUMULATED IN A MUTANT OF E. COLI

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1. Introduction

Some stable RNAs in *E. coli* appear to be transcribed as longer units which are subsequently cleaved to give the functional species [1, 2]. This phenomenon *may* occur with all stable RNA molecules. In this study an *E. coli* mutant (AA-157), which is temperature sensitive with respect to RNA synthesis, was investigated and several new precursor species were found. The technique described might offer a useful method for studying precursors of other stable RNA species.

2. Materials and methods

E. coli mutant AA-157 (isolated and characterised by A.G. Atherly) was grown in 70 ml low phosphate media at permissive temperature (30°C) to A_{550} of 0.30. The culture was shifted to the restrictive temperature (42°C) for 1 hr, then rapidly collected by centrifugation at room temperature. The cells, after being washed once with a modified TPG2A media [3] containing *no* phosphate, were resuspended in 5 ml of this media (pre-warmed to 30°C) to which 5 mCi of [^{32}P]phosphate were added. The culture was then incubated with vigorous aeration for 1 hr at 30°C. The entire suspension was extracted with hot (65°C) phenol, and the RNA precipitated from the aqueous layer at −20°C by the addition of 2.5 vol of ethanol. In the experiment, 1−2% of the label was incorporated into RNA.

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The products were separated on a 10% acrylamide—0.5% bis-acrylamide slab gel at pH 8.3, as described by Dingman and Peacock [4]. The results of a radioautograph are shown in fig. 1. The seven bands, labelled A−F (fig. 1) were cut out and a portion of each band homogenised in M NaCl to recover the RNA. Bands which did not prove to be homogeneous, on the basis of initial fingerprint evidence, were further purified by washing the gel band at 4°C first with water, then with 7 M urea, and re-running the band on a 12.5% acrylamide stacking slab gel, as described by Vigne and Jordan [5]. Bands E and F were each separated into two major components (designated E′, E″, F′ and F″, respectively, in fig. 1) using this procedure.

2.1. 5′-Termini of RNAs

A T_2-ribonuclease hydrolysate of RNA from each band was applied to a polyethyleneimine (PEI) thin layer chromatography plate (20 × 20 cm, Schleicher and Schüll, F1440 PEI, Dassel, W. Germany). This was developed with 1.5 M KH_2PO_4 as described by Cashel and Kalbacher [6], together with a reference sample of pppGp (a generous gift of G. Rubin), and the plates subsequently radioautographed to reveal 5′-terminal nucleotides.

2.2. Fingerprints of T_1-ribonuclease digests of RNA

The RNA's from appropriate acrylamide gel bands were digested with T_1-ribonuclease and examined by a two-dimensional fingerprinting technique on PEI-cellulose essentially as described by Griffin [7]. The thin layer plates (20 × 40 cm, Schleicher and Schüll) were washed before use [8]. For the second dimensional separation, they were developed with 1.5 M pyridinium formate up to 10 cm, and then with
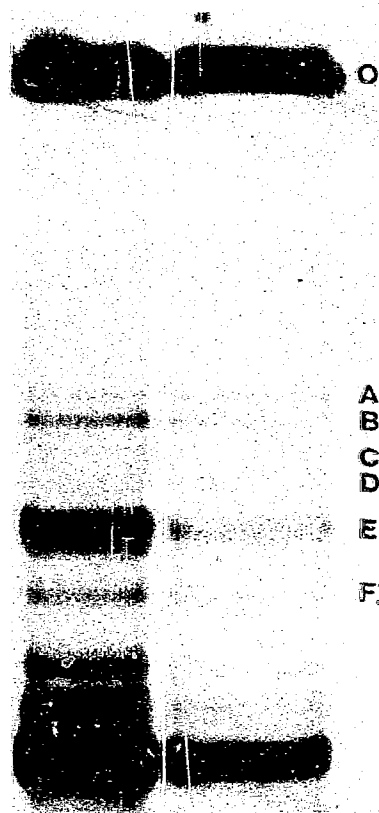
Fig. 1. Separation of the RNA from *E. coli* mutant AA-157 by acrylamide gel electrophoresis using 10% acrylamide—0.5% bis-acrylamide [4]. The origin is labelled O.

either 2.0 M or 2.2 M pyridinium formate, as indicated on the fingerprints (fig. 2, 3, and 4).

After elution with 30% triethylammonium carbonate (pH 10), information about partial sequences and base composition of the large oligoribonucleotides was obtained by pancreatic ribonuclease or alkaline digestion [9].

The base composition of the product migrating in the position expected for U_2CG, in the T_1-ribonu-

clease fingerprints of bands C, D, and E', was determined by chromatography of a T_2-ribonuclease hydrolysate on a thin layer cellulose plate (20 × 20 cm, E. Merck, Darmstadt, W. Germany). The plate was developed with propan-2-ol (68 ml)—conc. HCl (18 ml)—H_2O (14 ml). Reference samples were run as markers.

3. Results and discussion

The mutant AA-157 used in this study shows a complete shut-off of stable RNA production within minutes after the shift up from 30°C—42°C, whereas some (if not all) messenger RNA production, as assayed by β-galactosidase induction, is unimpaired at the restrictive temperature [10]. This mutant is defective in fructose-1,6-diphosphate aldolase and behaves in a manner similar to a mutant previously described by Böck and Neidhardt [11]. We have used this mutant of *E. coli* as a tool to block stable RNA production, prior to addition of [^{32}P]phosphate, so that the cells might become depleted of their stable RNA complement. In order to obtain sufficient material for study, and be able to isolate it under conditions which would minimise the maturation of any precursors present [2], we used the high-density cellular technique described in the previous section. We expected that when stable RNA synthesis was restarted (at the time of shift down to permissive temperature), the cells would transiently accumulate precursors of various RNA species. Moreover, as the mutant has a doubling time of approximately 90 min under the conditions used, we hoped that by harvesting cells at times less than this, to be able to obtain and study such precursors.

Separations of the total RNA, from cells grown for 1 hr at permissive temperature, on acrylamide gels (fig. 1) seemed to confirm our predictions. Bands A, C, and D are not normally seen in preparations of *E. coli* RNA, and bands E and F, which normally contain 5 S and 4.5 S RNA respectively, were found by subsequent purification to contain more than one species. That some of these bands contain uncleaved or partial precursor species of stable RNA's was demonstrated by obtaining a T_1-ribonuclease fingerprint of materials from each of the bands labelled in fig. 1.
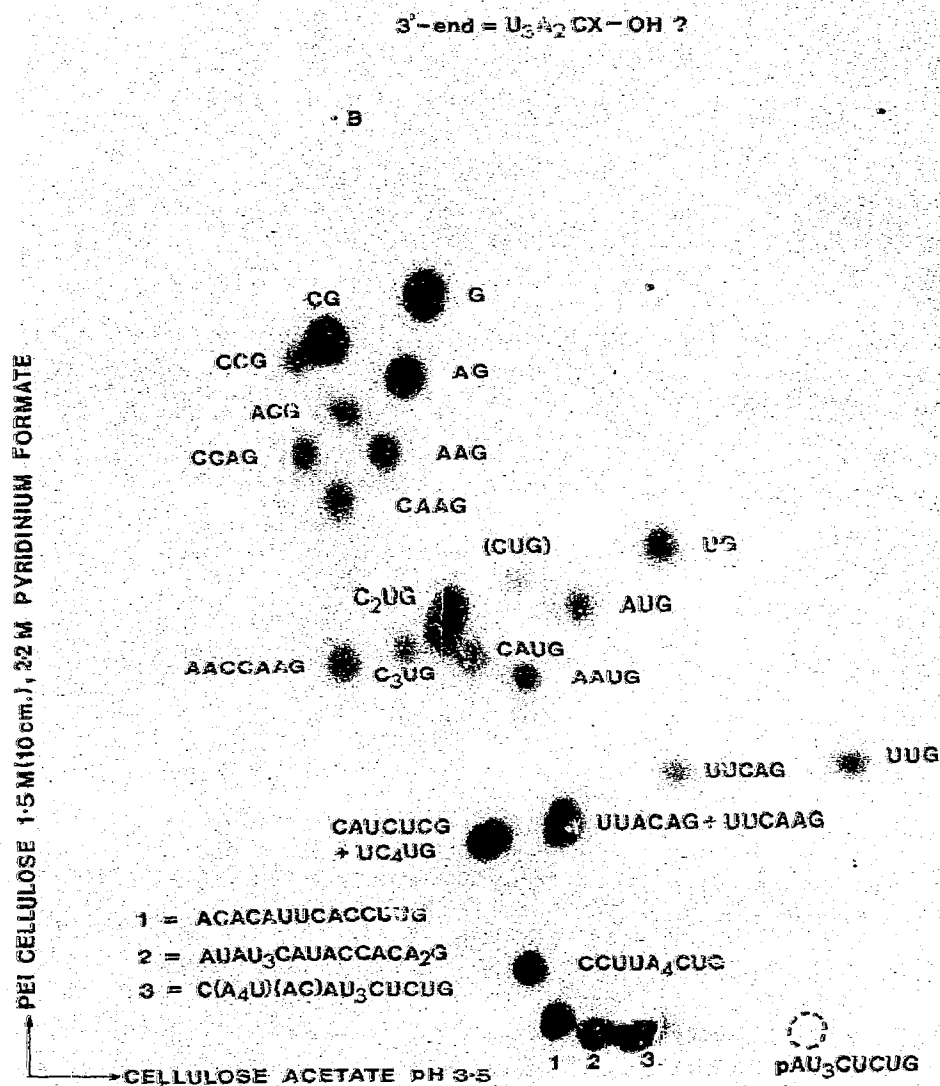
$$3'-end = U_3 A_2 CX - OH \ ?$$

Fig. 2. A two-dimensional separation of the T_1-ribonuclease products of band A RNA. The seventeen long oligonucleotide peculiar to 6 S precursor is labelled 3. All other oligonucleotides are common to both precursor (band A) and mature (band B) RNA. The position of the normal 5'-end of mature 6 S RNA (pAU$_3$CUCUG) is shown by a dotted circle. The extent of electrophoresis necessary to separate spots 1,2, and 3 (first dimension) would result in the loss of pppGp; it would appear as the fastest spot in the first dimension and the slowest in the second.

Bands B and F″ showed fingerprints identical with those expected for the stable 6 S [12] and 4.5 S [7] RNA's of *E. coli*, respectively. Band E″ was identified as the 5 S RNA precursor described by Forget and Jordan [13].

RNA from band A gave the T_1-ribonuclease fingerprint shown in fig. 2. In addition to the expected products of 6 S RNA [12], an oligonucleotide 17 residues long (fig. 2, spot 3) was found which contained the 9 residues from the normal 5'-end of 6 S RNA as well as C, A_4U and AC. A T_2-ribonuclease digest of band A material showed it to contain pppGp, together with some ppGp, at its 5'-end. Preliminary experiments indicated that the 3'-end also
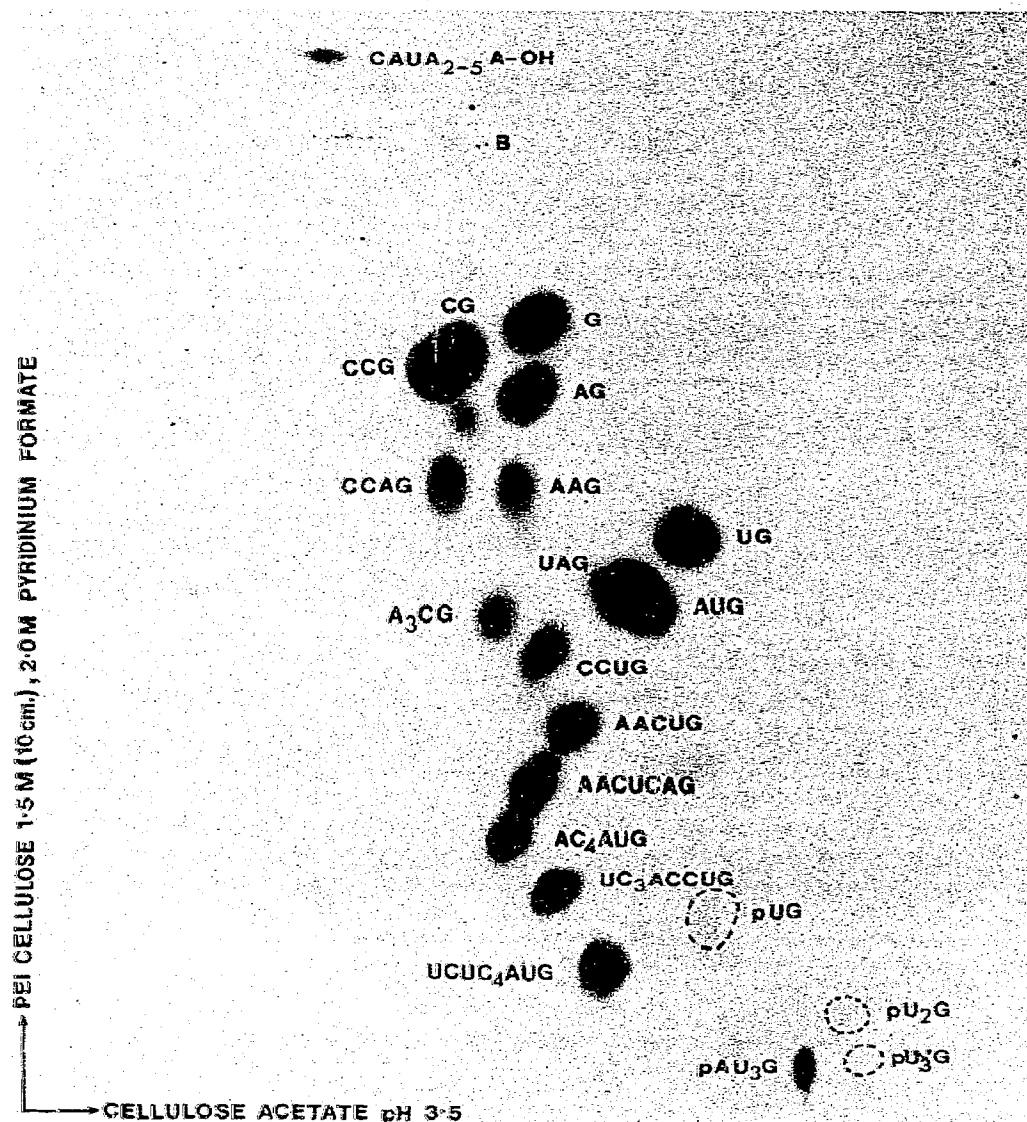
Fig. 3. A two-dimensional separation of the T_1-ribonuclease products of band E' RNA. The 5'-end product, pAU_3G, and the 3'-end product, $CAUA_{2-5}A$—OH, are peculiar to precursor 5 S RNA. The 5'-end products of the partially matured 5 S RNA from band E'' are indicated by dotted circles.

contained a few more nucleotides than normally found in 6 S RNA; its sequence, based on a quantitation of the products obtained by alkali or ribonuclease digestion, and the 3'-end products reported by Brownlee [12], is tentatively proposed to be—AUUCAUX—OH.

From this evidence, it seems likely that RNA from band A represents a direct precursor of mature 6 S RNA. Since we have found no evidence of heterogen-

ity in the new 5'-oligomer, it would appear that cleavage to the mature form occurs via an endonucleolytic split, similar to that previously reported for the conversion of precursor to mature tyrosine tRNA [14].

The other precursor species studied in detail was that found in band E. When band E (fig. 1) was separated into two components using a stacking gel procedure, E'' RNA was shown by fingerprinting techniques to be the partially matured 5 S RNA [13]; it
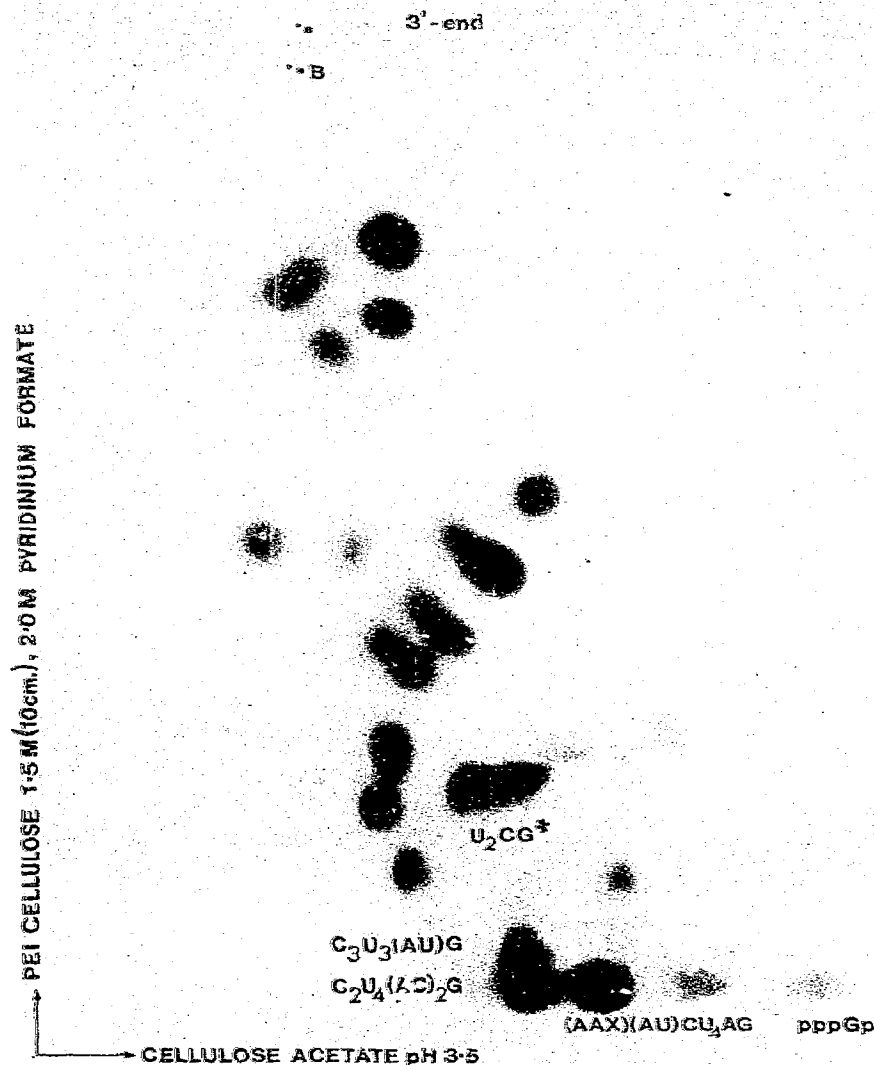
Fig. 4. A two-dimensional separation of the T_1-ribonuclease products of band F' RNA. The simplicity of the fingerprint indicates that it is probably a single species, and the 5'-end product, pppGp, suggests that it is a primary transcription product. It is possebly a precursor of a transfer RNA.

contained the heterogeneous 5'-ends pAU$_3$G, pU$_3$G, pU$_2$G and pUG. Band E', on the other hand, had as a 5'-end only the larger oligomer, pAU$_3$G. No pppGp or other tri- or tetra-phosphate species was found in this material. The results are summarised in fig. 3. A more interesting feature of band E'RNA was the apparent presence of additional nucleotides at the 3'-end. When the 3'-end fragment was treated with pan-

creatic ribonuclease, and the products separated by ionophoresis on DEAE paper (pH 3.5), adenylyl-3' 5'-uridine-3' phosphate (ApUp) was found; in mature 5 S RNA of *E. coli* the 3'-end is CAU-OH [15], which would give rise to ApU—OH under these conditions. Moreover, treatment of the 3'-end fragment with venom phosphodiesterase gave a ratio of mono-nucleotides equal to 4—5 A:1 U in one experiment.
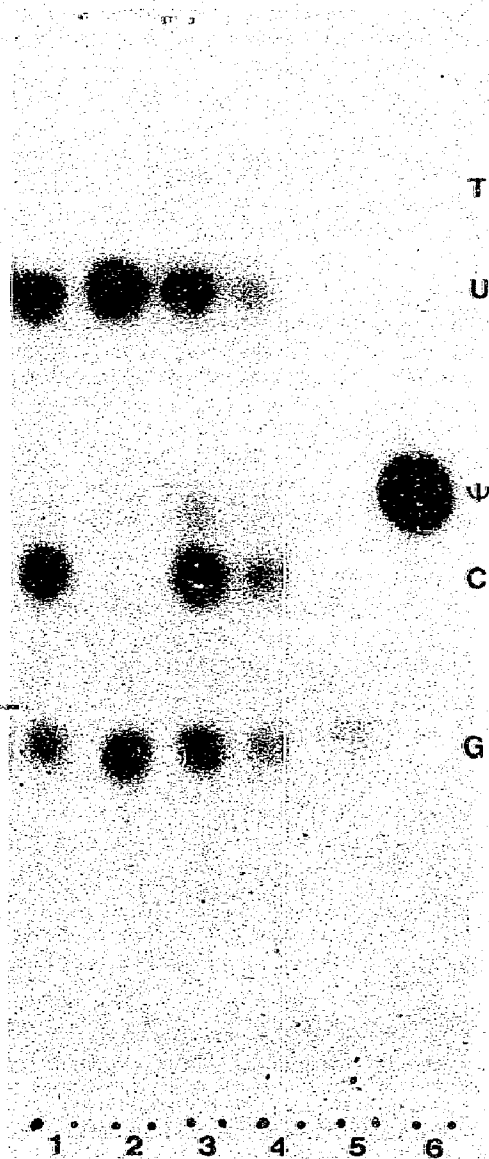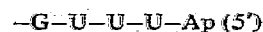
by pulse labelling a phage infected *E. coli* in the presence of chloramphenicol, contained extra nucleotides. The structure therefore proposed for the termini of precursor 5 S RNA is:

—G—U—U—U—Ap (5')
—C—A—U—A_{2-5}A—OH (3')

This would appear to be the first RNA species found in prokaryotic cells which contains sequences of adenosines at its 3'-end. The significance of this is not clear; certainly very little extra stability would be given to the molecule itself by the presence of these extra residues.

Studies on RNA's from bands C, D, and F' (fig. 1) suggested that these three species might be precursors of transfer RNA's. From their positions on the acrylamide gel, RNA from bands C and D should be similar in size to the tyrosine precursor tRNA described by Altman and Smith [17], whereas band F'RNA by analogy with 4.5 S RNA should contain approximately 110 nucleotides [18]. All three species were found to contain pppGp as a 5'-terminal nucleotide. Fingerprints of T_1-ribonuclease digests showed them to have an oligonucleotide in the position expected for U_2CG (TΨCG). The band C product contained only U, C and G, whereas the similarly migrating product from bands D and F' showed partial modification to thymineriboside and pseudouridine, as shown in fig. 5. Unfortunately, only small yields of the materials from bands C and D were obtained, so it was not possible to purify them to a further state of purity, or make any structural assignments. With the material from F', some information was obtained, however, as shown in fig. 4. Attempts to correlate this data with any of the known structures of *E. coli* tRNA have not yet been successful. Preliminary evidence suggested that the RNA's from bands C, D and F' were not related to each other. In a separate experiment (not previously described) where a 15 min pulse of [^{32}P]phosphate was used, several larger RNA species, migrating just slightly ahead of the origin on a 10% acrylamide gel, were observed. One of these contained all the T_1-ribonuclease products found in band C, in addition to many other oligomers. It is therefore possible to suggest that band C RNA might be a partial maturation product of an even larger species.

Fig. 5. The separation by thin layer chromatography of mononucleotides from spots with the expected composition U_2CG obtained from T_1-ribonuclease digest of RNA's from (1) band C (3) band D and (4) band F'. (2), (5) and (6) are reference materials.

Abbreviations:

T = thymineriboside; U = uridine; Ψ = pseudouridine; C = cytidine and G = guanosine.

and 6—7 A:1 U in another. These experiments suggest a short run of oligo-A at the 3'-end of precursor 5 S RNA. They confirm an earlier observation of Pieczenik [16] that the 3'end of 5 S RNA, obtained

We feel that the methods described in this paper should be useful for studying precursors of stable *E. coli* RNA molecules, and may lead to the discovery of yet more species of RNA. Moreover, we are optimistic that they may also allow a study of the maturation kinetics of these species.

Acknowledgements

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