THE EFFECT OF A CONSTITUTIVE MUTATION IN PSEUDOMONAS AERUGINOSA ON RAPIDLY-LABELLED RNA

J. G. EDWARDS * and A. P. MATHIAS
Department of Biochemistry, University College London, Great Britain

Received 14 March 1969

1. Introduction

Pseudomonas aeruginosa 8602 possesses an inducible aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) [1,2]. We set out to detect in this organism a specific RNA, the amidase messenger, which should be present only in cells synthesising the enzyme. No phage or episome carrying the amidase structural gene was available for its detection by hybridisation. We therefore attempted to make use of the double-labelling technique of Hayashi et al. [3]. In this technique, a ^{14}C precursor is used to label (for example) the RNA of cells expected to be synthesising the messenger, and a ^{3}H precursor to label RNA of cells from which the messenger is missing. The two RNA populations are mixed and analysed by chromatography or density gradient centrifugation. A separation of amidase-specific messenger RNA from other species, would be revealed as a discrepancy between the ^{14}C and ^{3}H profiles. To ensure a high differential rate of amidase synthesis in exponentially growing cells, use was made of one of the constitutive mutants isolated by Brammar, Clarke and Skinner [4].

Reproducible differences in sedimentation and chromatographic behaviour of the RNA of the parent wild strain and the constitutive mutant were found. The discrepancies observed suggest that the technique may be revealing a difference between the two strains in their relative rates of synthesis of ribosomal and messenger RNA, rather than the presence of amidase specific messenger RNA in the constitutive mutant.

* Present address: Department of Cell Biology, University of Glasgow, Great Britain.

2. Materials and methods

2.1. *Strains*

The parent strain, *Pseudomonas aeruginosa* 8602 and constitutive mutant Cl were kindly provided by Mrs. P.H.Clarke and Dr. W.J.Brammar.

2.2. *Growth medium*

Both strains were grown in a minimal medium "C medium" of Roberts et al. [5] in which 1% (w/v) sodium pyruvate replaced glucose as the carbon source. Cultures were grown on a reciprocal shaker at 37°C. The mean generation time for both strains was about 70 min.

2.3. *Labelling*

60 ml cultures were labelled by addition of 20 μC (0.5 μmole) ^{14}C-uracil or 200 μC (0.5 μmole) ^{3}H-uracil (Radiochemical Centre, Amersham) at a cell density of 0.4 mg dry weight cells per ml. (The ^{3}H-uracil was diluted to the appropriate specific activity with unlabelled uracil to eliminate possible effects of uracil concentration on incorporation.) After 3 min at 37° cultures were poured onto 0.3 volumes of crushed, frozen growth-medium, containing 1 mM unlabelled uracil and 0.1 mM sodium azide. The chilled suspensions were mixed in isotopic pairs before harvesting by centrifugation.

2.4. *Isolation of RNA*

RNA was isolated by phenol extraction of French Press lysates, as described by Monier et al. [6] at 2–5°. The cells were washed in 25 mM tris-acetate, containing 1 mM magnesium acetate, pH 7.0, and

resuspended in 5 ml of this medium to which 0.1% w/v bentonite had been added. The suspension was extruded from the French Press at about 10,000 psi into an equal volume of 90% (w/v) redistilled AnalaR phenol. The aqueous phases from two consecutive phenol extractions were combined, and the RNA precipitated by addition of two vol of absolute ethanol. The RNA was redissolved in 10 mM Tris-acetate pH 7.0 containing 0.1 mM magnesium acetate, and 0.1% (w/v) bentonite, for reprecipitation by ethanol. For analysis by chromatography and density gradient centrifugation the RNA was finally re-dissolved in 5 mM Tris-HCl – 0.1 mM EDTA (pH 7.0) and 0.01% (w/v) bentonite.

2.5. *Chromatography on methylated serum albumin-Kieselguhr (MAK)*

The procedure described by Monier et al. [6] was followed using Bovine Plasma Fraction V (Armour Ltd.) and Hyflo Supercell (Hopkin & Williams Ltd., Chadwell Heath, Essex). Columns (20 × 1 cm) were always freshly prepared on the day of use, and washed with 200 ml of 0.75 M NaCl in 25 mM Tris-HCl, pH 7.0. RNA was eluted by linear gradients of NaCl in 500 ml of buffer, at 30 ml per hour.

2.6. *Density gradient centrifugation*

RNA solutions were layered on 25 ml 5–20% (w/v) linear sucrose gradients, prepared in 5 mM Tris-HCl – 0.1 mM EDTA (pH 7.0), bentonite 0.01% (w/v) and centrifuged in a Beckman SW25 rotor as described in the legends. The gradients were fractionated and monitored for absorption at 260 mμ using an enlarged version of the apparatus of Edwards and Mathias [7]. Since accurate registration of ultraviolet absorption and radioactivity profiles proved important, individual fractions were also measured for absorption at 260 mμ.

2.7. *Estimation of radioactivity*

For liquid scintillation counting, RNA was precipitated in 5% trichloracetic acid, and collected, in absence of carrier, on Millipore filters (type HA 0.45 μ, 25 mm diameter). The filters were dried and immersed in liquid scintillation vials containing 10 ml of toluene-based scintillation fluid (0.04 g 1,4-bis-(5-phenyloxazolyl-2)-benzene and 0.4 g 2,5-diphenyloxazole per l toluene) and counted in a Nuclear Chicago Scintillation Counter. The ^{14}C channel counted at an efficiency of 27% with less than 0.02% of the ^{3}H counts. The other channel counted ^{3}H at 8% efficiency with an overlap of 9% of the total ^{14}C counts. At least 10,000 counts were registered for each fraction. There was no detectable sensitivity of ^{3}H efficiency to load, provided the RNA was kept below 20 μg per filter. Double isotope data were normalised by a procedure devised by James [8], using a specially written computer program.

3. Results

3.1. *MAK chromatography*

Fig. 1 shows the elution pattern of RNA from an MAK column. The two ribosomal species elute at about 0.95 and 1.0 M NaCl. In fig. 2 the elution of radioactivity from a double-labelled sample is shown. Very little radioactivity elutes in the first peak of unbound material and its low specific activity precludes ^{3}H counting by the filter technique. The peaks of radioactivity coincide with the ribosomal components, but the second peak of radioactivity is much more broadly spread than the 23S ribosomal RNA. In this RNA pair the ^{3}H-uracil was used to label the constitutive mutant and the difference profile between the two isotopes indicates an excess of RNA originating from this strain, eluting in correspondence with each

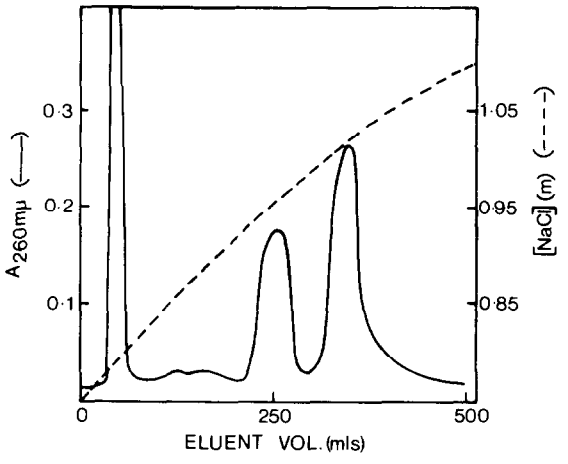

Fig. 1. Chromatography on methylated serum to albumin-Kieselguhr (MAK) of RNA extracted with phenol from *Ps. aeruginosa* 8602.

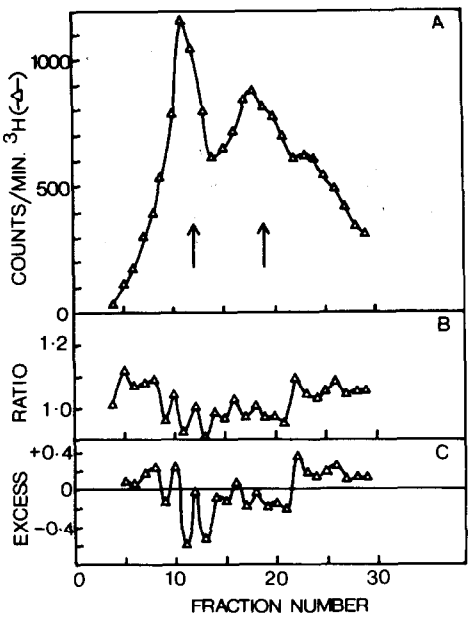

Fig. 2. Profile of elution of radioactivity from an MAK column. The wild type was labelled with ^{14}C-uracil and the constitutive mutant (Cl) with ^{3}H-uracil. The cultures were combined and the RNA extracted according to the description in the text. (A) ^{3}H counts in each fraction. The arrows mark the positions of the peaks of the 16S and 23S ribosomal RNA. (b) Ratio of percentage of total ^{3}H counts to percentage of total ^{14}C counts in each fraction (^{3}H/Σ^{3}H) – (^{14}C/Σ^{14}C). (C) Difference profile of the pair of isotopes in each fraction. A negative value indicates an excess of ^{3}H.

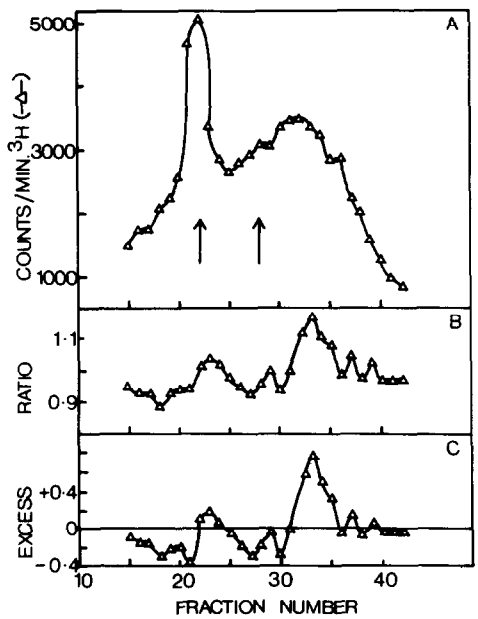

Fig. 3. Profile of elution of radioactivity from an MAK column. The isotopes are reversed from the experiment in fig. 2 and the wild type is labelled with ^{3}H-uracil. (A) ^{3}H counts in each fraction. (B) Ratio of percentage of total ^{3}H counts to percentage of total ^{14}C counts. (C) Difference profile.

of the ribosomal RNA species. This observation is the opposite of what would arise if the discrepancy were an artifact caused by the absorption on the Millipore filters of low energy β-particles by the ribosomal RNA. However, to eliminate any other possible effects stemming from lack of equivalence of the two isotopes, the experiment was repeated but with the isotopes reversed. Fig. 3 shows that with ^{14}C used to label the constitutive mutant, ^{14}C rich regions replace the former ^{3}H excesses. The agreement between the two experiments is qualitative only, in the sense that the relative size of the two discrepancies is altered.

3.2. *Sucrose density gradient centrifugation*

The high salt concentrations needed to elute RNA from these columns are known to cause strong interactions between pre-existing ribosomal RNA and newly synthesised RNA [9]. Because of this there was an uncertainty as to whether the isotope discrepancies were to be attributed to newly synthesised ribosomal RNA. Similar RNA pairs were analysed by density gradient centrifugation in a medium of low ionic strength, to minimise RNA-RNA interactions. Control experiments in which both ^{14}C- and ^{3}H-uracil were added to either wild type or constitutive mutant showed negligible discrepancies in the profiles of radioactivity recovered from the gradients. Fig. 4 shows the sedimentation analyses of an RNA pair. The discrepancies are reproducible and reverse on reversal of the isotopes. In agreement with the chromatographic result, there are two distinct regions of excess material of constitutive origin, but each sediments slightly faster than the two ribosomal RNA species. Furthermore, they can be seen to occur at

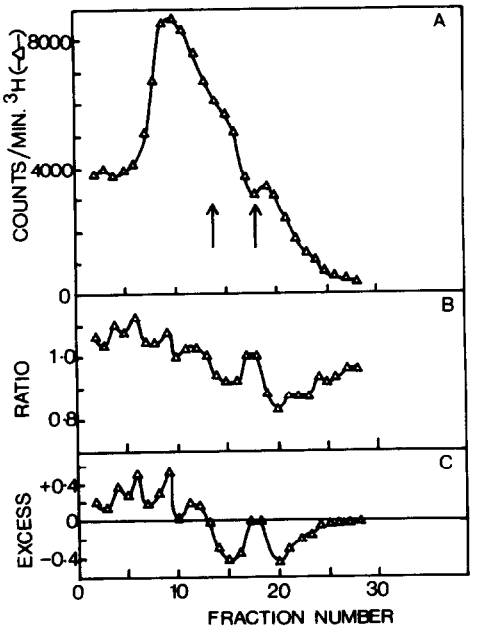

Fig. 4. Sedimentation in a sucrose gradient of labelled RNA. Centrifugation was performed in SW 25-1 rotor for 20 hr at 24,000 rev/min at 5°. The load (0.3 ml) was equivalent to the RNA extracted from 2 ml of each culture. In this experiment ^{3}H-uracil was used to label the constitutive mutant and ^{14}C the wild type. (A) Profile of ^{3}H labelling. The absorption at 260 mμ (not shown) was used to determine the positions of the 16 + 23 S ribosomal RNA which are marked with arrows. Sedimentation is from left to right. (B) Ratio of percentage of ^{3}H counts to percentage of total ^{14}C counts. (C) Difference profile.

the expense of label sedimenting more slowly than 16S RNA.

4. Discussion

The differences in rapidly labelled RNA observed here between the two strains, involve an altered distribution of 2–5% of the total precursor incorporated in three minutes. The differential rate of amidase synthesis by Cl under the conditions of this work is about 6% of total protein. About a third of the RNA labelled in three minutes would be messenger, the remainder precursor to ribosomal RNA [10]. Thus if amidase messenger were translated at a rate equal to the average for all other messenger, it might represent about 2% of the rapidly labelled RNA. The observed differences are of the correct order of magnitude to be due to the presence, in the regions enriched with material from the constitutive mutant, of RNA derived from the amidase structural messenger.

There are, however, a number of grounds for believing that the difference patterns do not reveal the presence of amidase specific RNA, but rather slight increase in the relative flow of precursor into ribosomal RNA in the constitutive mutant. Firstly, very similar discrepancies in labelling have been obtained in this laboratory in comparisons of RNA from wild type strain of *E. coli* M.L. 30 with that from mutant producing β-galactosidase constitutively (M.L. 35). Secondly, the occurrence of two discrepancies in the normalised isotope profiles and the manner in which they track rather closely with the two ribosomal RNA species is not convincingly dismissed merely as a coincidence. They may reflect disturbance of the proportion of labelled precursor entering ribosomal RNA compared to that incorporated into messenger RNA. This argument is supported by reports that immature ribosomal RNA may sediment faster than the RNA of completed ribosomes [12]. An alternative could be that the slower sedimenting peak is a breakdown product of the faster. This explanation is the one favoured by Martin for the rather similar patterns he obtained in his comparison of histidine constitutive and deletion mutants of *Salmonella typhimurium* [9]. Thirdly, any tendency for the messenger RNA to be degraded preferentially during the isolation procedure by shearing in the French press [13] militates against separation of amidase-specific messenger from the rest of the messenger population. RNA already incorporated into ribosomal precursor particles may well be protected against such degradation. As a consequence its separation from fragmented messenger RNA would be improved. This would explain the finding that in density gradients the constitutive excess material is made up for by deficiency in the lower sizes of rapidly labelled RNA.

The postulated diversion of precursor into ribosomal RNA in Cl, compared with 8602 may be due to a coincidental, unrelated difference between the strains. However there is a distinct possibility that it is a direct consequence of the effect of derepression of the enzyme on the general economy of the cell. This possibility is supported by the finding of similar

RNA difference patterns using the lactose system of *E. coli* [11].

We conclude that a possible shift in rate between synthesis of ribosomal and messenger RNA should be taken into account in any experiment designed to detect qualitative changes in the messenger RNA population.

Acknowledgements

This work was carried out during the tenure by one of us (J.E.) of a scholarship from the Medical Research Council and of a Beit Memorial Fellowship. We thank D.James and M.J.Evans for many helpful conversations.

References

[1] M.Kelly and P.H.Clarke, J. Gen. Microbiol. 27 (1962) 316.
[2] W.J.Brammar and P.H.Clarke, J. Gen. Microbiol. 37 (1964) 307.
[3] M.S.Hayashi, S.Spiegelman, N.Franklin and S.E.Luria, Proc. Natl. Acad. Sci. U.S. 49 (1963) 729.
[4] W.J.Brammar, P.H.Clarke and A.J.Skinner, J. Gen. Microbiol. 47 (1966) 87.
[5] R.B.Roberts, P.Abelson, D.B.Cowie, E.T.Bolton and R.J.Britten, Studies of biosynthesis in *E. coli*, Carnegie Inst. Washington Publication No. 607 (1955).
[6] R.Monier, S.Naono, D.Hayes, F.Hayes and F.Gros, J. Mol. Biol. 5 (1962) 311.
[7] J.G.Edwards and A.P.Mathias, Nature 199 (1963) 603.
[8] D.James, personal communication.
[9] R.G.Martin, Cold Spring Harbor Symp. Quant. Biol. 28 (1963) 357.
[10] E.T.Bolton and B.J.McCarthy, Proc. Natl. Acad. Sci. U.S. 48 (1962) 1390.
[11] J.G.Edwards, D.R.James, A.P.Mathias and M.J.Evans, Biochem. J. 106 (1967) 32P and unpublished observations.
[12] P.S.Syperd and B.S.Fansler, J. Bact. 93 (1967) 920.
[13] M.Fry and M.Artman, Nature 217 (1968) 661.