Selective ^{32}P -labelling of individual species in a total tRNA population

Cinzia Traboni[†], Riccardo Cortese[†] and Francesco Salvatore[†][†]Istituto di Chimica Biologica, II Facoltà di Medicina, University of Naples, Naples, Italy, and[†]European Molecular Biology Laboratory, Heidelberg, GFR

Received 16 September 1980

ABSTRACT

A simple procedure to label individual tRNA species in a total tRNA preparation has been developed. The principle of the method is as follows: total crude tRNA (from *E.coli*) is incubated in the presence of a crude aminoacyl-tRNA synthetase preparation, containing most aminoacyl-tRNA synthetases and only one specific amino acid corresponding to the tRNA species which is intended to be labelled. This achieves the purpose of charging the desired tRNA species thereby protecting its 3'OH-terminus; obviously all the other tRNA species will have a free 3'OH group. Periodate oxidation, followed by β -elimination, destroys any free 3'OH. After deacylation of the specific aminoacylated tRNA at pH 8.8 the only free 3'OH group will be the one of the desired tRNA species. High specific activity (^{32}P)-pCp is ligated to this 3'OH by means of T4-RNA ligase. Two-dimensional polyacrylamide gel electrophoresis (2D-PGE) and sequence analysis of the isolated tRNA show that the method is very specific. Individually labelled tRNA species can be used as probes for cloning tRNA genes.

INTRODUCTION

Recombinant DNA research applied to the purification of tRNA genes has been very useful for the clarification of several aspects of gene organization (1-3), transcription and precursor processing (4-9) in a variety of organisms.

In order to purify specific tRNA genes, it is essential to have a radioactive probe for the routine procedures of colony and plaque hybridization or DNA blotting.

In many cases it has been possible to prepare high specific activity ^{32}P -labelled tRNA simply by growing cells in the presence of ^{32}P -orthophosphate; alternatively, especially for eukaryote organisms, in vitro labelled tRNA has been used as probe. In most cases in vitro labelling is more convenient, especially since a very efficient procedure has been developed, involving T4-RNA ligase and (^{32}P)-pCp (10). Furthermore, in vitro methods for labelling are particularly suitable for the recently developed fast RNA sequencing techniques (11,12), which can be used when terminally labelled

molecules are available.

At the present time both the in vivo and the in vitro methods of labelling tRNA species are not specific for any individual tRNA species. For many purposes, however, it could be extremely useful to be able to label individual tRNA with high specific activity. Obviously, this can be achieved by using a radioactive amino acid and the aminoacyl-tRNA synthetase. In this case, however, the isotope that can be used (^3H , ^{14}C or ^{35}S) is not suitable for use in colony or plaque hybridization experiments, nor for RNA sequencing.

In this paper we present a method which exploits the specificity of the aminoacyl-tRNA synthetases to label individual tRNA species with ^{32}P at high specific activity.

MATERIALS AND METHODS

Chemicals. All radioactive amino acids and cytidine 3',5'-(5'- ^{32}P)-bisphosphate (2000-3000 Ci/mmol) (pCp) were from Amersham, England. T4-RNA ligase was purchased from PL Biochemicals, all other chemicals were of the highest degree of purity commercially available.

Bacterial strains. An E.coli K12 clone harbouring a plasmid carrying the tRNA^{Ile} gene was a gift of Dr. H.Delius.

Aminoacyl-tRNA synthetases and tRNA. Purified aminoacyl-tRNA synthetases from E.coli were a gift of C.Dingwell. An E.coli extract containing all aminoacyl-tRNA synthetases was prepared according to Kelmers et al. (13). E.coli tRNA (type XXI from Sigma) was deacylated by incubation at 37°C for 1 hour in 1 M Tris, pH 8.8, and extensively dialyzed before use.

Polyacrylamide gel electrophoresis. First dimension: 10% polyacrylamide gel was in 7M urea, 90 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.3; second dimension: 20% gel was in the same solution. tRNA was eluted from the gel by crushing the gel slices in 0.5 M ammonium acetate, 10 mM MgCl_2 , 0.1% SDS. After incubation at 37°C for a few hours, acrylamide was removed by centrifugation through glass wool and tRNA was precipitated with ethanol.

RNA sequencing. ^{32}P -labelled tRNA (about 0.01 μg) was dissolved in 3 μl of deionized formamide, containing 10 μg of cold tRNA as carrier, heated in a sealed capillary in a boiling water bath for 2.5 minutes, applied on a cellulose acetate strip (Schleicher and Schüll, Dassel, Germany) and electrophoresed at 5,000 volts for 30 min in 5% acetic acid, 7 M urea, pH 3.5. The cellulose acetate strip was blotted on a 20 x 40 cm/DEAE-cellulose thin layer plate (Polygram Cel 300 DEAE/HR-2/15, Macherey-Nagel, Duren, West

Germany) and subjected to homochromatography(14).

Colony hybridization. Bacterial colonies were transferred to membrane filters (Schleicher and Schüll, Dassel, Germany). The filters were treated three times with 1 mg/ml lysozyme, 25% sucrose, 50 mM Tris, pH 7.5, one time with 0.5 M NaOH, 0.2% Triton, one time with 0.5 M NaOH, twice with 1 M Tris, pH 7.6, one time with 0.1 M Tris, 1.5 M NaCl, washed with 0.3 M NaCl, baked at 80°C under vacuum for 2 hours and incubated with ³²P-labelled tRNA in 10% Denhardt buffer (15), 0.6 M NaCl, 0.12 M Tris, pH 8, 8 mM EDTA, 0.1% SDS at 65°C for 40 hours. After extensive washing with 0.6 M NaCl, 0.12 M Tris pH 8, 8 mM EDTA, 0.1% SDS, they were autoradiographed. It may be useful to specify that RNasetreatment, which is often used to reduce background, cannot be used in our case. The labelled 3'-terminus, which is often not encoded in the primary structure of tRNA, would be digested, thereby eliminating the radioactive signal.

RESULTS AND DISCUSSION

The rationale of the method is as follows: incubation of total crude tRNA in the presence of aminoacyl-tRNA synthetases (either as a crude extract or as a purified enzyme) and a specific amino acid will result in the aminoacylation of a tRNA species corresponding to the amino acid used. Subsequent treatment with sodium metaperiodate will oxidize all tRNA species which had not been charged. The charged species, after deacylation is the only substrate which can be specifically ligated at the free 3'-OH terminus to (5'-³²P)-pCp by the action of T4-RNA ligase. The overall result of these stepwise reactions will be the specific ³²P-labelling of the tRNA species of choice.

Aminoacylation reaction. Deacylated *E.coli* tRNA (150 µg) was incubated at 37°C for 30 min in 100 mM potassium cacodylate, pH 7.0, 10 mM MgCl₂, 2 mM ATP, 10 mM KCl, 2 mM β-mercaptoethanol, 120 µM of a single specific amino acid, as specified in figure legends, with various amounts of enzyme (0.16-0.6 µl purified synthetases; in the case of crude preparations we have used enough enzyme to reach the saturation of aminoacylation reaction in 20 min) in a final volume of 500 µl. After phenol extraction (pH 4.5) the tRNA was precipitated with ethanol, centrifuged and dried under vacuum.

Periodate oxidation and β-elimination. The tRNA was incubated with 50 µl of 0.1 M Na metaperiodate (in water, pH 4.5) for 30 min at room temperature in the dark (16). The reaction was stopped by addition of 5 µl of 1 M ramnose and incubated for 30 min at room temperature in the dark. After

ethanol precipitation tRNA was dried, resuspended in 100 μ l of 0.5 M lysine, pH 8.8, incubated at 37°C for 30 min, ethanol precipitated and dried.

pCp labelling by T4-RNA ligase. tRNA (10 μ g) was incubated overnight at 4°C with 10 μ Ci of (32 P)-pCp, 50 mM Hepes, pH 7.5, 15 mM MgCl₂, 3.3 mM dithiothreitol, 0.5 mM ATP, 10% DMSO, 1 unit of T4-RNA ligase, in a final volume of 10 μ l. The products of this reaction were analyzed on gel electrophoresis. In Fig.1 are shown the autoradiographies of the gels obtained by specifically labelling tRNA^{Gln} (panel 1), tRNA^{His} (panel 2), tRNA^{Tyr} (panels

Figure 1. Labelling of individual tRNA species using a crude preparation of aminoacyl-tRNA synthetase. Autoradiograms of 10% polyacrylamide gel electrophoresis. Panel 1, labelling of tRNA^{Gln}; panel 2, tRNA^{His}; panel 3, tRNA^{Tyr}; panel 4, tRNA^{Tyr} (slot 2) and tRNA^{Leu} (slot 2'). For experimental details see text.

3 & 4), tRNA^{Leu} (panel 4). In slot 3 there is bulk tRNA, where the tRNA species do migrate in series of bands, in the 4S region. Slots 2 and 2' are the results obtained by using a crude aminoacyl-tRNA synthetase preparation, in the presence of either glutamine, histidine, tyrosine or leucine (see legend to Fig.1). In slot 1 is the corresponding background (we define as background the result obtained by subjecting the tRNA preparation to the same procedure, but omitting the amino acid). An inspection of Fig.1 shows that uniquely different bands appear whenever the tRNA has been charged with a specific amino acid. It is clear, however, that there is a certain amount of non specific labelling. This is eliminated by using purified, rather than crude synthetases, as it is shown in Fig.2. It is

Figure 2. Labelling of individual tRNA species using purified aminoacyl-tRNA synthetases. Panel 1, tRNA^{Met}; panel 2, tRNA^{Val}; panel 3, tRNA^{Ile}.

Figure 3. Comparison of the use of crude versus purified aminoacyl-tRNA synthetase. Two-dimensional 10% to 20% polyacrylamide gel electrophoresis. Panel 1, use of crude synthetase preparation: (a) "background" with no amino acid, (b) with isoleucine; Panel 2, use of pure isoleucyl-tRNA synthetase with isoleucine.

obvious, then, that the background is due to the use of crude synthetase preparation, probably depending on trace amounts of amino acids produced by proteolysis. This also explains the different background patterns (slots

Figure 4. Selective labeling and 2D-PAGE of isoaccepting $tRNA^{Met_f}$ and $tRNA^{Met_m}$. In this experiment a purified methionyl-tRNA synthetase from *E. coli* was used. Panel 1: "background" without methionine; Panel 2: labeling after aminoacylation with methionine.

1) obtained by using different crude synthetase preparations at different times. In every case, however, two-dimensional gel analysis did allow satisfactory resolution and easy identification of the uniquely labelled tRNA species, as it is clearly shown in Fig.3.

Whenever it is possible to separate on two-dimensional gel isoaccepting species, one expects to see more than one spot specifically labelled. One example is given in Fig.4 when tRNA was charged with methionine. Spot

Figure 5. Partial sequence of tRNA^{His}. Panel 1: Two-dimensional fractionation of partially hydrolyzed ³²P-labeled tRNA^{His}; panel 2, schematic representation of the "wandering spot".

Figure 6. Partial sequence of spot A (Panel 1) and spot B (Panel 2) of Fig. 4.

A and spot B were identified as $\text{tRNA}^{\text{Metf}}$ and $\text{tRNA}^{\text{Metm}}$ respectively (see Fig. 6).

In a typical experiment we started with 40 picomoles of total tRNA from *E. coli* which was aminoacylated with histidine. The measured amount of charged tRNA was about 1% of the total. We found about 0.4 picomoles of (5'- ^{32}P)-pCp incorporated into tRNA^{His} , indicating that we achieved about

100% labelling of tRNA^{His}.

Partial sequence of specifically ³²P-labelled tRNA. tRNA^{His} was eluted from the gel and its partial sequence was determined by the "wandering spot" method (Fig.5). A stretch of 12 nucleotides could be easily "read" from the autoradiography. The obtained sequence corresponds exactly to the sequence at the 3'-end of tRNA^{His} (17). In the case of tRNA^{Met} the sequence of spot A (see Fig.4) is shown in Fig.6 panel i and corresponds to the first 12 nucleotides of tRNA^{Metf} from *E.coli* (18). Both spot B and B' have the same sequence at the 3'-end (panel 2) and they are probably two isoaccepting tRNA^{Metm} (19).

Use of ³²P-labelled specific tRNA for gene cloning. We wanted to prove that individual tRNA specifically ³²P-labelled by this method could be used for the identification of bacterial colonies carrying plasmids containing a specific tRNA gene. Plasmid pBK8 is a pBR322 derivative containing a 6,000 bp insert including a *E.coli* tRNA^{Ile} gene (H.Delius, personal communication). Cells harbouring this plasmid contain between 10-30 copies of tRNA^{Ile} gene. Colony hybridization (20) using total ³²P-labelled tRNA fails to detect any difference in the autoradiographic signal between colonies harbouring the pBR322-tRNA^{Ile} plasmid and wild-type *E.coli* (R.Cortese, unpublished). It is probable that this is due to the high number of *E.coli* chromosomal tRNA genes, all of which hybridize with a total tRNA probe. The results of an experiment of colony hybridization, where wild-type *E.coli* and *E.coli* harbouring the pBK8 plasmid were hybridized to ³²P-labelled tRNA which had been precharged with isoleucine, are shown in Fig.7. This demonstrates that

Figure 7. Experiment of colony hybridization. Panel 1, row A: six identical colonies of *E. coli* harbouring the pBK8 plasmid, row B: six colonies of wild-type *E. coli*; panel 2, autoradiogram of the colony hybridization.

the signal was very specific for the colonies containing the tRNA^{Ile} genes.

ACKNOWLEDGEMENTS

Some preliminary experiments which led to this work were done in the laboratory of J.D. Smith (Cambridge, England), to whom we are indebted for hospitality and many stimulating discussions. We also thank Rodolfo Frunzio for helpful discussion. We acknowledge EMBO for a short-term fellowship to F.S. and FEBS for a short-term fellowship to C.T. This work was partially supported by grants from the CNR, Rome, Italy (International Cooperative project, and National Group for the study of Macromolecules) to the Istituto di Chimica Biologica, Naples, Italy.

REFERENCES

1. Clarkson, S.G., Birnstiel, M.L. and Serra, V. (1973) *J.Mol.Biol.* 79, 391-410.
2. Beckman, J. S., Johnson, P.F. and Abelson, J. (1977) *Science* 196, 205-208.
3. Horermann, B., Sharp, S., Yamada, H. and Söll, D. (1980) *Cell* 19, 889-895.
4. Cortese, R., Melton, D.A., Tranquilla, T. and Smith, J.D. (1978) *Nucl. Acid Res.* 5, 4593-4611.
5. Melton, D.A. and Cortese, R. (1979) *Cell* 18, 1165-1172.
6. Melton, D.A., De Robertis, E.M. and Cortese, R. (1980) *Nature* 284, 143-148.
7. Knapp, G., Beckman, J.S., Johnson, P.F., Fuhrman, S.A. and Abelson, J. (1978) *Cell* 14, 221-236.
8. Garber, R.L. and Gage, L.P. (1979) *Cell* 18, 817-828.
9. Hagenbuchle, O., Larson, D., Hall, G.I. and Sprague, K.U. (1979) *Cell* 18, 1217-1229.
10. England, T.E., Bruce, A.G. and Uhlenbeck, D.C. (1980) in *Methods in Enzymology*, Grossman, L. and Moldave, K. Eds.; Vol.65, pp.65-74. Academic Press, New York.
11. Simoncsits, A., Brownlee, G.G., Brown, R.S., Rubin, J.R. and Guilley, H. (1977) *Nature* 269, 833-836.
12. Peattie, D.A. (1979) *Proc.Natl.Acad.Sci. USA* 76, 1760-1764.
13. Kelmers, A.D., Novelli, G.D. and Stulberg, M.P. (1965) *J.Biol.Chem.* 240 3979-3983.
14. Ziff, E.B., Sedat, J.W. and Galibert, F. (1973) *Nature New Biology* 241, 34-37.
15. Denhardt, D.T. (1966) *Biochem.Biophys.Res.Comm.* 23, 641-648.
16. Folk, W.R. and Berg, P. (1970) *J.Bact.* 102, 204-211.
17. Singer, C.E. and Smith, G.R. (1972) *J.Biol.Chem.* 247, 2989-3000.
18. Dube, S.K., Marcker, K.A., Clark, B.F.C. and Cory, S. (1968) *Nature* 218, 232-233.
19. Cory, S., Marcker, K.A., Dube, S.K. and Clark, B.F.C. (1968) *Nature* 220, 1039-1040.
20. Grunstein, M. and Hogness, D.S. (1975) *Proc.Natl.Acad.Sci. USA* 72, 3961-3965.