Volume 1 number 5 May 1974 Nucleic Acids Research

Transfer RNA methylating activity of yeast mitochondria

Nina Smolar and Ingvar Svensson

Institute of Biochemistry, University of Uppsala, Box 531,
S-751 21 Uppsala, Sweden

Received 3 April 1974

ABSTRACT

Mitochondria isolated from Saccharomyces cerevisiae and purified in Urografin or sucrose gradient contain tRNA methylating activity with specificities different from those of the cytoplasm. The main reaction product, using E.coli tRNA as methyl group acceptor, is N^2_2-N^2-dimethylguanine. The corresponding mitochondrial methylase is coded by nuclear DNA. A DNA methylating activity is also associated with yeast mitochondria.

INTRODUCTION

Mitochondria isolated from yeast as well as from other organism have been shown to contain their own machinery for the synthesis of nucleic acids and protein (1-3). There is also convincing evidence that several constituents of this machinery differ in their structures and properties from those of the cytoplasm, e.g. DNA polymerase (4), RNA polymerase (5), ribosomes (6), peptide chain elongation factors (7,8), tRNA nucleotidyltransferase (9) and at least some aminoacyl-tRNA synthetases (10-12). Mitochondria also contain unique species of tRNA (13-18), some of which have been shown to hybridize specifically with mitochondrial DNA (13,14).

Only a few reports concerning the methylation of mitochondrial tRNA have appeared so far. In hamster mitochondria, the degree of tRNA methylation has been estimated by labelling *in vivo* to be less than half of that in cytoplasmic tRNA (19,20). In agreement with these results, Klagsbrun (21) has found that in HeLa cell mitochondria there is much less methylating activity than in the cytoplasm and that they have different specificities.

In this paper, we present evidence that in yeast there is a difference between the tRNA methylating capacities of the mitochondria and the cytoplasm. We also show that yeast mitochondria

methylating activity is coded in the nuclear DNA and probably identical with the cytoplasmic enzyme.

MATERIAL AND METHODS

Strains and growth conditions

Saccharomyces cerevisiae, strains D84 and D38, were grown as previously described (22). Strain D38 is a derivative of D84 lacking an active (m_2^2G)methylase and thus producing a tRNA devoid of m_2^2G but with full complement of other methylated nucleosides (23).

Escherichia coli, strains B and K12 58-161 (known as W6), were grown as described earlier (24). Strain W6 is met^- and RC^{rel}, and it produces a generally submethylated tRNA during methionine starvation (25).

Chemicals

DNase I, RNase A from bovine pancreas, bovine albumin fraction V and protective Lacquer Spray were obtained from Sigma Chemical Co. Urografin was bought from Schering AG, Berlin, and spleen phosphodiesterase from Worthington Biochemical Corp. Sheets No 6064 without fluorescence indicator used for thin layer chromatography were purchased from Eastman Kodak Co. S-adenosyl--L-(methyl-^{14}C)-methionine(54 mC/mmole) and L-arginine(U) (336 mC/mmole) were bought from the Radiochemical Centre, Amersham. Bacterial proteinase fixed to agarose was a most generous gift from Dr. D. Gabel, Uppsala University, Sweden.

Preparation of mitochondria

Yeast mitochondria were isolated according to Guarnieri et al. (26) and further purified by centrifugation in a Urografin gradient (4) or in sucrose gradient from 0.98 to 1.66 M sucrose in 0.02 M Tris-HCl, pH 7.5, with 1 mM EDTA. The yield was approximately 0.4 mg of mitochondrial protein per 1 g of yeast (wet weight). This is within the range of variation, 0.25-2.5 mg, reported from different laboratories (27,28).

The integrity of mitochondria was routinely tested by measuring the ATPase activity (29). Only preparations with ATPase activities within the range of common literature values were used. The preparation was also examined under the electron microscope with magnification of 18,000x. No visible degradation and

no contaminating material could be observed. We thank Dr. B. Gerolhadfon, the Agricultural College, Sweden, for his kind help at the electron microscope work.

The amounts of mitochondria are expressed as protein values, determined with the Lowry method.

Preparation of mitochondrial extract

The purified mitochondria were suspended in 0.05 M Tris-HCl, pH 8.0 and sonicated in a sonicator MSE, Kistnerlab, at 20 KHz for 1 min eight times with cooling.

Preparation of cytoplasmic tRNA methylases

An extract from yeast D84, containing the cytoplasmic tRNA methylases was prepared according to Phillips and Kjellin-Stråby (23).

Preparation of cytoplasmic aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases were prepared similarly to Svensson et al. (30).

Preparation of tRNA

Transfer RNA from yeast and E.coli was prepared as previously described (31), but with omission of the methyl cellulose extraction.

RNA from yeast D38 mitochondria was prepared according to Leon and Mahler (32), but mitochondria were treated with spleen phosphodiesterase for 45 min. After phenol extraction and ethanol precipitation, the RNA pellet was dissolved in 0.05 M triethylamine, pH 5.5, and chromatographed on a Sephadex G-200 column (1.8 x 90 cm) which had been previously calibrated with purified rRNA and tRNA from E.coli B. The fractions corresponding to the tRNA peak were collected and incubated for 1 h at 37° at pH 9.0 to hydrolyse off bound amino acids. The solution was freeze-dried and the tRNA further purified on a column of Sephadex G-100 (Stahl and Accoceberry, - personal communication). The recovered tRNA was dissolved in water. The ratio A_{260}/A_{280} was 1.85. The yield was 2.3 μg of tRNA per mg of mitochondrial protein.

Assay for methylase activity

The reaction mixture contained, in a total volume of 0.2 ml, 0.1 M Tris-HCl, pH 8.0, 0.1 mM dithiothreitol, 0.1 mM EDTA, 10 mM $MgSO_4$, 20 mM NH_4 Cl, 3.6 nmoles of ^{14}C-labelled S-adenosyl-

methionine and different amounts of tRNA and protein. Unless otherwise stated, the mitochondrial extracts were preincubated with 20 μg of DNase per 0.1 ml at 37° for 30 min.

Incubation of the reaction mixture was for 90 min at 37° with mitochondrial enzymes and for 90 min at 30° with cytoplasmic methylases. The reaction was stopped and measurements performed as already described (30).

Assay for amino acid acceptance

The reaction mixture contained, in a volume of 0.1 ml, 0.1 M Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 1 mM dithiothreitol, 2 mM ATP, 2 mM CTP, 0.3 nmoles of ^{14}C-labelled arginine and different amounts of tRNA and protein. Incubation was for 20 min at 30°, which was sufficient for complete charging. The aminoacylated tRNA was precipitated, washed and measured as previously described (30).

Characterisation of methylation products

Methylated tRNA was hydrolysed by acid, and the products were analysed by two-dimensional chromatography on paper or thin-layer cellulose sheets as already described (33,34).

The thin-layer spots were sprayed with protective lacquer, cut out and measured in a liquid scintillation counter (35).

RESULTS

Endogenous methylating activity of mitochondria

In our initial experiments we observed that incubation of both intact yeast D84 mitochondria and mitochondrial extracts with ^{14}C-labelled S-adenosylmethionine resulted in incorporation of significantly high amounts of radioactivity into acid precipitable material. Preincubation of a mitochondrial extract with DNase decreased the level of methyl group incorporation by 70-80 % (Fig. 1). This indicates that most of the self-methylating activity of mitochondria is due to DNA methylation.

Since mitochondria might be contaminated with nuclear DNA, we have compared preparations of different purity in DNase-sensitive methylation. We found no difference between unpurified, sucrose-gradient and Urografin-gradient purified mitochondria. The DNase-sensitive methyl group incorporation in mitochondrial extracts may therefore reflect a retarded, homologous methylation

of mitochondrial DNA. Under this assumption, the level of incorporation can be estimated to be 1 methyl group per 100 nucleotides. The full methyl group complement of yeast mitochondrial DNA therefore seems to be much higher than that reported for mouse fibroblast mitochondrial DNA (36). Preincubation of mitochondrial extracts with RNase (10 μg per 0.06 mg of mitochondrial protein) for 30 min at 37° had no significant effect on the level of endogenous methylation, while a postincubation with 0.5 mg of subtilisin for 2 h at 37° decreased the level by 12 %. From these results it can be inferred that some protein but no RNA is being methylated in the mitochondrial extracts.

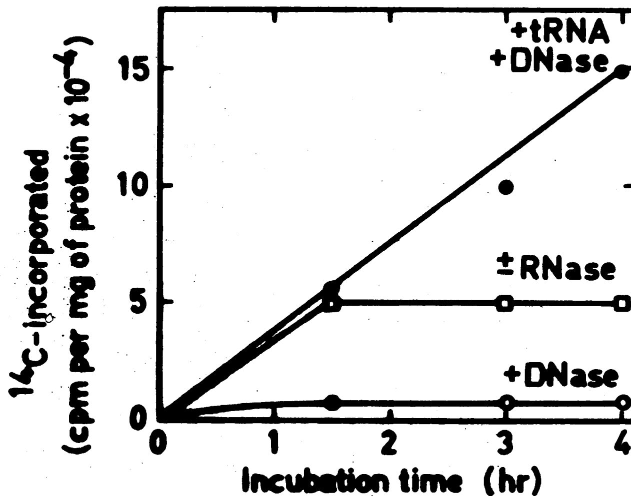


Fig. 1 DNA and tRNA methylating activity of yeast mitochondria. The reaction mixtures were preincubated for 30 min at 37° with 20 μg of DNase or 10 μg of RNase as indicated. The amount of mitochondrial protein varied between 0.02--0.06 mg. The incorporation values are calculated per mg of protein.

To diminish the apparent blank values when measuring tRNA methylase activity of mitochondrial extracts, preincubation of the samples with DNase was routinely performed.

Mitochondrial tRNA-methylase activity

DNase-treated mitochondrial extracts from S. cerevisiae D84 catalyze incorporation of methyl groups into submethylated tRNA from methionine-starved E.coli W6. Fig. 1 shows that the reaction is completely dependent on exogenously supplied tRNA and that it proceeds at a linear rate for at least 4 h at 37°. The reaction rate, however, varies greatly between extracts.

Yeast tRNA from strain D38, lacking m_2^2G, was found to be a much better, although more specific, substrate for the mitochondrial methylase. While in an experiment submethylated tRNA from E.coli W6 accepted 0.002 nmoles of methyl groups per min per mg of protein, tRNA from yeast D38 accepted 0.006.

The tRNA-methylating activity associated with the mitochondrial preparation might be due to contamination with cytoplasmic enzymes. We examined this possibility by treating intact mitochondria from strain D84 for different times at 37° with an agarose-bound proteinase. The mitochondria were reisolated, and the remaining methylase activity was measured against submethylated tRNA from E.coli W6. About 50 % of the tRNA methylase activity was lost within 15 min of incubation with proteinase, while more than 40 % remained even after prelonged treatment up to 45 min. The result indicates that part of the tRNA methylase activity associated with our mitochondrial fraction may be cytoplasmic contamination and that part of the activity emanates from within the mitochondria. Another possible interpretation is that the loss of activity during proteinase treatment is due to partial damage to the mitochondrial membrane.

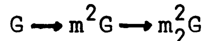
Pattern of tRNA methylation

To characterize the mitochondrial tRNA methylase activity further, the mitochondrial extract and a cytoplasmic methylase preparation were compared in their abilities to methylate tRNA from different sources. The maximum methyl group incorporation obtained with enzyme in excess, limiting amounts of tRNA and prolonged incubation times is shown in Table I. It is evident that the mitochondrial methylase preparation is inferior to the cytoplasmic enzymes. The reason for this is obscure at present.

Table I. Extent of methylation of tRNA by yeast cytoplasmic and mitochondrial extracts. Incubations were done with standard mixtures for up to 8 h. Maximum methyl group incorporation obtained is expressed in nmoles per 100 O.D. units of tRNA. 1 mg of tRNA corresponds to 24 O.D. units at 260 nm and 1 cm pathway.

tRNA source	Enzyme extract from		
	D84 mitochondria	D84 cytoplasm	D38 mitochondria
E.coli W6, methionine-starved	9	470	3
S. cer. D38 cytoplasm	108	200	0.0
S. cer. D38 mitochondria	-	24	-
S. cer. D84 cytoplasm	0.5	0.6	-
S. cer. D84 mitochondria	0.6	-	-

The pattern of methylated products was also analyzed. Table II shows that the yeast D84 mitochondrial extract preferentially methylates guanine in tRNA from both E.coli B and methionine--starved E.coli W6. The main products are m^2G and m_2^2G. Since the maximum methyl group incorporation in this case was not obtained (Table I), most of the m^2G found probably represents the intermediate product in the sequence



$$G \longrightarrow m^2G \longrightarrow m_2^2G$$

at the site of m_2^2G synthesis in tRNA. The greatest part of the mitochondrial tRNA-methylating activity would therefore be tRNA (m_2^2G) methylase. This is in sharp contrast to the distribution of tRNA-methylating activities in the cytoplasm (37).

The presence of tRNA (m_2^2G) methylase within the mitochondria in yeast D84 would mean that a preparation of mitochondrial tRNA from that strain is already saturated with m_2^2G under normal conditions. Table I shows that this is indeed so, since an extract from D84 mitochondria, containing active tRNA (m_2^2G) methylase, cannot methylate D84 mitochondrial tRNA further *in vitro*. This finding also supports the view that at least part of the methylating activity associated with the mitochondria belongs to the interior.

Table II. Pattern of tRNA methylation by cytoplasmic and mitochondrial extracts from yeast. Reactions were carried out with standard mixtures for 8 h. Transfer RNA was extracted with phenol, precipitated with ethanol, hydrolyzed and chromatographed as described (34,35). No radioactivity was detected in m^7G, m^2A, m^6A and m^6_2A. The unidentified radioactivity is probably associated with ribose-methylated compounds.

Enzyme source, S. cer.	tRNA source	Radioactivity recovered, cpm	Recovery %	Recovered radioactivity in % of total recovery						
				m^1G	m^2G	m^2_2G	m^1A	mCp	mUp	unknown
D84 mitochondria	E.coli W6, methionine-starved	1760	98	1	31	63	0	1	0	4
"	E.coli B	1780	102	1	28	64	1	1	0	6
D84 cytoplasm	D38 mitochondria	670	104	11	10	69	1	2	1	6
D38 mitochondria	E.coli W6, methionine-starved	460	72	9	4	0	0	7	55	25

Origin of mitochondrial tRNA (m_2^2G) methylase

The access to the yeast mutant D38, which lacks an active tRNA (m_2^2G) methylase and, consequently, m_2^2G in its tRNA, gave us the means to test the origin of the mitochondrial tRNA (m_2^2G) methylase of strain D84. If this enzyme were coded by mitochondrial DNA, a mitochondrial extract from strain D38 should contain the enzyme, while a cytoplasmic extract does not. We have, however, not been able to demonstrate any tRNA (m_2^2G) methylase activity in extracts from D38 mitochondria, neither with D38 cytoplasmic tRNA (Table I) nor with methionine-starved E.coli W6 tRNA (Table II). We therefore conclude that the tRNA (m_2^2G) methylase in D84 mitochondria may be coded by the same gene as the cytoplasmic enzyme on the nuclear DNA. The mutation in this gene in strain D38 thus results in loss of this enzyme in both cytoplasm and mitochondria.

If the mitochondria of strain D38 really lack tRNA (m_2^2G) methylase, then tRNA from D38 mitochondria should lack m_2^2G and consequently serve as methyl group acceptor when incubated with an active tRNA (m_2^2G) methylase. To test this assumption, we prepared tRNA from yeast D38 mitochondria.

The tRNA was first tested for acceptance activity with arginine to ascertain its biological integrity. In the presence of both ATP and, obligatory, CTP, the tRNA accepted 2.3 nmoles of arginine per mg of tRNA, which is in good agreement with published data (18).

The D38 mitochondrial tRNA was then incubated with an enzyme extract from D84 cytoplasm. Table I shows that the tRNA accepted a substantial amount of methyl groups, although the level of incorporation was much lower than that of the corresponding cytoplasmic tRNA. Product analysis revealed that most of the incorporated methyl groups concerned m_2^2G (Table II). These results show that yeast mitochondrial tRNA can be methylated by tRNA (m_2^2G) methylase and in normal yeast strains also is methylated.

DISCUSSION

The results presented in this paper are interpreted in terms of tRNA methylases present and functional within the mitochondria of yeast. The arguments in favour of this view are:

1. The tRNA methylase activity associated with our mitochondrial fraction could not be completely digested away by proteinase. However, it is a possibility that the remaining activity was cytoplasmic contamination strongly attached to the outside of mitochondria and resistent to proteinase degradation. On the other hand, it is equally possible that the loss of activity after proteinase treatment only mirrors a partial damage to the mitochondrial membrane, permitting the methylase to leak out and become degraded.

2. Klagsbrun (21) has reported that in HeLa cells a tRNA (m^2A) methylase is uniquely associated with the mitochondria. Although this result is based on marginal data from the experiment with already fully methylated E.coli tRNA, and without pertinent controls, it lends some support to the view that mitochondria really possess tRNA methylases.

3. Mitochondrial tRNA is methylated to some extent. Our work shows that yeast mitochondrial tRNA contains m_2^2G. Work by Dubin (19,20) shows that also in hamster mitochondria tRNA is methylated. Since many different tRNA species have been shown to be specific for either mitochondria or cytoplasm, tRNA seems to be unable to pass the mitochondrial membrane in any direction. If this turns out to be generally true, the presence of methylated tRNA in mitochondria thus presupposes the existence of inside tRNA methylases.

4. We have shown that the tRNA (m_2^2G) methylase associated with yeast mitochondria is probably coded by nuclear DNA. This interpretation is not quite conclusive, since there is a slight possibility that the strain D38 might carry a mutation in both the gene for cytoplasmic tRNA (m_2^2G) methylase and a hypothetical gene on mitochondrial DNA for the mitochondrial enzyme. However, if we assume a nuclear gene for the mitochondrial methylase, it may still be a gene distinct from the gene coding for the cytoplasmic methylase. This, in our view, is not very probable, and we have assumed as a working hypothesis that there is only one gene on the nuclear DNA for both cytoplasmic and mitochondrial tRNA (m_2^2G) methylase.

The question of how a nuclear gene product finds its way to the mitochondrion is very intriguing. There is a possibility

that mRNA unlike protein, rRNA and tRNA, can traverse the mitochondrial membrane and be specificly translated, if necessary, by the mitochondrial ribosome system.

ACKNOWLEDGEMENTS

The work has been supported by The Ollie and Elof Ericsson Fund, The Knut and Alice Wallenberg Fund, and The Hjerta-Retzius Fund.

REFERENCES

1. Borst, P. (1972) Annu.Rev.Biochem. 41, 333-376
2. Ashwell, M. and Work, T.S. (1970) Annu.Rev.Biochem. 39, 251-291
3. Rabinowitz, M. and Swift, H. (1970) Physiol.Revs. 50, 376-429
4. Wintersberger, U. and Wintersberger, E. (1970) Eur.J.Biochem. 13, 20-27
5. Tsai, M.Y., Michaelis, G. and Criddle, R.S. (1971) Proc.Nat. Acad.Sci.U.S. 68, 473-477
6. Morimoto, H. and Halvorson, H.O. (1971) Proc.Nat.Acad.Sci.U.S. 68, 324-328
7. Richter, D. and Lipmann, F. (1970) Biochemistry 9, 5065-5070
8. Scragg, A.H. (1971) FEBS Lett. 17, 111-114
9. Mukerji, S.K. and Deutscher, M.P. (1972) J.Biol.Chem. 247, 481-488
10. Buck, C.A. and Nass, M.M.K. (1968) Proc.Nat.Acad.Sci.U.S. 60, 1045-1052
11. Barnett, W.E., Brown, D.H. and Epler, J.L. (1967) Proc.Nat. Acad.Sci.U.S. 57, 1775-1781
12. Chiu, A.O.S. and Suyama, Y. (1973) Biochem.Biophys.Acta 299, 557-563
13. Halbreich, A. and Rabinowitz, M. (1971) Proc.Nat.Acad.Sci.U.S. 68, 294-298
14. Casey, J., Cohen, M., Rabinowitz, M., Fukuhara, H. and Getz, G.S. (1972) J.Mol.Biol. 63, 431-440
15. Accoceberry, B. and Stahl, A.J.C. (1971) Biochem.Biophys.Res. Commun. 42, 1235-1243
16. Barnett, W.E. and Brown, D.H. (1967) Proc.Nat.Acad.Sci.U.S. 57, 452-458
17. Guderian, R.H., Pulliam, R.L. and Gordon, M.P. (1972) Biochem. Biophys.Acta 262, 50-65
18. Accoceberry, B., Schneller, J.M. and Stahl, A.J.C. (1973) Biochimie 55, 291-296
19. Dubin, D.T. and Montenecourt, B.S. (1970) J.Mol.Biol. 48, 279-295
20. Dubin, D.T. and Friend, D.A. (1971) FEBS Lett. 18, 287-289
21. Klagsbrun, M. (1973) J.Biol.Chem. 248, 2606-2611
22. Björk, G.R. and Svensson, I. (1969) Eur.J.Biochem. 9, 207-215
23. Phillips, J.H. and Kjellin-Stråby, K. (1967) J.Mol.Biol. 26, 509-518
24. Svensson, I., Boman, H.G., Eriksson, K.G. and Kjellin, K. (1963) J.Mol.Biol. 7, 254-271
25. Mandel, L.R. and Borek, E. (1963) Biochemistry 2, 560-566

26. Guarnieri, M., Mattoon, J.R., Balcavage, W.X. and Payne, C. (1970) Anal.Biochem. 34, 39-45
27. Accoceberry, B. and Stahl, A.J.C. (1970) Bull.Soc.Chim.Biol. 52, 1113-1123
28. Mattoon, J.R. and Balcavage, W.X. (1967) in Methods in Enzymology, Vol. 10, pp. 135-142,(Estabrook, R.W. and Pullman, M.E. eds) Academic Press, New York/London
29. Kovač, L., Bednárowá, H. and Grešak, M. (1968) Biochem.Biophys.Acta 153, 32-42
30. Svensson, I., Isaksson, L. and Henningson, A. (1971) Biochem. Biophys.Acta 238, 331-337
31. Svensson, I. (1967) Biochem.Biophys.Acta 146, 239-252
32. Leon, S.A. and Mahler, H.R. (1968) Arch.Biochem.Biophys. 126, 305-319
33. Björk, G.R. and Svensson, I. (1967) Biochem.Biophys.Acta 138, 430-432
34. Isaksson, L. and Phillips, J.H. (1968) Biochem.Biophys.Acta 155, 63-71
35. Björk, G.R. and Neidhardt, F.C. (1973) Virology 52, 507-519
36. Nass, M.M.K. (1973) J.Mol.Biol. 80, 155-175
37. Svensson, I., Björk, G.R. and Lundahl, P. (1969) Eur.J.Biochem. 9, 216-221