JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1156-1160
Vol. 113, No.3

Relationship Between Methionyl Transfer Ribonucleic Acid Cellular Content and Synthesis of Methionine Enzymes in *Saccharomyces cerevisiae*

Y. SURDIN-KERJAN, H. CHEREST, AND H. DE ROBICHON-SZULMAJSTER
Laboratoire d'Enzymologie, C.N.R.S., 91190 Gif-sur-Yvette, France

Received for publication 7 December 1972

Derepression of some methionine biosynthetic enzymes (methionine group I enzymes) obtained in methionine limitation has been found to be accompanied by a significant lack of in vivo charging of bulk methionine transfer ribonucleic acid ($tRNA^{Met}$) and in addition by a decreased rate of synthesis of all tRNAs. Under the same conditions, methionyl-tRNA synthetase (MTS) was derepressed rather than repressed. These results are in agreement with those previously published based on studies of a mutant with an impaired MTS (5) and reinforce the idea that the rate of synthesis of methionine group I enzymes can be related to the total content of methionyl (Met)-$tRNA^{Met}$ per cell. They also render unlikely that MTS could be a constituent of the regulatory signal.

Participation of charged transfer ribonucleic acids (tRNAs) rather than free amino acids in the regulation of synthesis of enzymes which belong to amino acid biosynthetic pathways in different microorganisms is receiving increasing support. The best documented system in this area is certainly the histidine biosynthetic pathway in *Salmonella typhimurium* in which the repression of synthesis of histidine enzymes (2) correlates with in vivo charging of $tRNA^{His}$ as well as with the amount of $tRNA^{His}$ synthesized (3, 13). More recently, evidence has been adduced that the presence of nucleosides substituted at different positions such as pseudouridine, dispensable for the correct participation of His-$tRNA^{His}$ in protein synthesis, are indispensable for the participation of this molecule in the repression phenomenon (19). Thus mutations in structural genes coding for enzymes which insure "maturation" of precursor tRNAs appear to behave like regulatory mutations in histidine biosynthesis and most probably in other amino acid pathways (20).

We have shown in a previous work that in *Saccharomyces cerevisiae*, repression of synthesis of enzymes belonging to the methionine biosynthetic pathway involves methionyl-tRNA as a regulatory signal (5). This conclusion was based on studies of a thermosensitive mutant (ts^-296) with an impaired methionyl-tRNA synthetase (L-methionine:sRNA ligase [AMP] EC 6.1.1.10) in which at least four of the methionine-specific enzymes (Met group I enzymes) remained unrepressed under conditions which led to repression in the parental strain. This absence of repression was accompanied by a marked decrease in in vivo charging of bulk $tRNA^{Met}$. However, since methionyl-tRNA synthetase (MTS) activity was impaired in the mutant strain, participation of this enzyme in the regulatory system could not be excluded. To answer this question, experiments have been carried out by using a methionine auxotroph during methionine limitation. In such experiments, the level of MTS has been estimated together with those of Met group I enzymes. Since methionine deprivation in methionine auxotrophs has been shown to lead to cessation of tRNA synthesis in bacteria (14) well as in *S. cerevisiae* (11), it was conceivable that even in methionine limitation as the total $tRNA^{Met}$ content of yeast cells would be affected. Consequently the total tRNAs, charged and uncharged $tRNA^{Met}$, and $tRNA^{Ile}$ have been determined.

Results presented in this paper mainly show that derepression in the chemostat as in the previous experiments with the ts^-296 mutant is accompanied by a significant lack of in vivo charging of bulk $tRNA^{Met}$ and also by a

decreased rate of synthesis of all tRNAs. Under the same conditions, MTS synthesis is derepressed rather than repressed.

MATERIALS AND METHODS

Strains. The haploid strain of *S. cerevisiae* used for this investigation was strain D6 from M. Grenson's collection. Its genotype is α, met2,ura (gene number unknown).

Cultures. The chemostats were operated as described by Novik and Szilard (17). The storage vessel was filled with minimal medium G0 (7) containing 20 mg of uracil per liter, 0.08 mM DL-methionine, and 2% glucose as a carbon source. From this vessel, a steady stream of medium (150 ± 10% ml per h) was pumped into the growth vessel. The growth vessel was inoculated with 3.5×10^7 to 4.0×10^7 cells per ml previously grown in minimal medium containing 20 mg of uracil per liter and 0.5 mM *O*-acetyl-DL-homoserine. A side arm set the level of the medium in the growth vessel (2 liters), and the cell suspension flowing through the side arm was collected in an ice-cooled recipient to stop growth. Fractions of about 2 liters were collected; tRNAs were extracted from two-thirds of each fraction, and the remaining third was used for cell-free extracts.

Cell-free extracts. The cells were collected by centrifugation, washed with 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0), and extracted as described previously (5).

The crude extract was submitted to centrifugation for 90 min at 40,000 rpm in rotor 40 of a preparative Spinco ("Spinco extract") which lowers the blanks considerably for the adenosine 5'-triphosphate (ATP) sulfurylase (ATP:sulfate adenylyl transferase, EC 2.7.7.4).

Enzyme assays. ATP sulfurylase activity was measured by the method of Wilson and Bandurski (24) as described by de Vito and Dreyfuss (21). Homocysteine synthetase activity was assayed at 30 C by the method of Wiebers and Garner (22), and the homocysteine formed was estimated as described by Kredich and Tomkins (12). These two enzymes were assayed on the "Spinco extract." Methionyl-tRNA synthetase was assayed on the crude extract. The assay mixture contained in a volume of 0.1 ml:10 μmol of potassium phosphate buffer (pH 7.4), 1 μmol of KCl, 1 μmol of $MgCl_2$, 0.2 μmol of ATP (Na), 0.2 μmol of dithiothreitol, 0.01 μmol of ^{3}H-L-methionine (2×10^6 counts/min), 0.5 mg of commercial baker's yeast tRNA and crude extract containing 150 μg of protein. After incubation at 30 C for 10 min, the reaction was stopped with 1 ml of a 0.1% trichloroacetic acid solution followed by an addition of 2 ml of a cold 10% trichloroacetic acid solution containing 0.5% DL-methionine. The resulting precipitates were filtered through Whatman glass fiber circles GF/C and washed with a cold 5% trichloroacetic acid solution containing 0.5% DL-methionine. The radioactivity of the filters was counted in a scintillation counter (Intertechnique SL30).

Protein determination. The protein estimation was carried out by the biuret method (8) with bovine serum albumin as reference.

Specific activities. Specific activities are expressed as nanomoles per minute per milligram of protein, i.e., in international units $\times 10^{-3}$.

Determination of the fraction of tRNA present in the acylated form. The method used for determination of the degree of tRNA charged in vivo is the one described previously (5) with some modifications introduced after Lewis and Ames (13). The cell suspension from the chemostat was centrifuged and the cells were suspended in 25 ml of distilled water; 0.25 ml of a 3% solution of sodium dodecyl sulfate was added, followed immediately by 25 ml of an 80% solution of phenol in water (wt/wt). The mixture was shaken vigorously for 1 h at room temperature, and two phases were then separated by centrifugation at 10,000 $\times$ *g* for 10 min. The aqueous layer was washed twice with 50 ml of cold ether containing 5 ml of a 2 M solution of potassium acetate buffer (pH 5.0). A 50-ml amount of ethyl alcohol (−15 C) was then added, and the RNA was allowed to precipitate for at least 30 min at −15 C.

The precipitate was collected by centrifugation at 27,000 $\times$ *g* for 10 min and dissolved in 6 ml of 100 mM potassium acetate buffer (pH 4.6). The exact volume and the absorption at 260 nm of the solution were measured, and these figures were used for the determination of the total amount of tRNA extracted from each chemostat fraction.

The tRNA solution was split into two 3 ml-portions. One was treated with 1 ml of a freshly prepared 10 mM sodium periodate solution (in 100 mM potassium buffer, pH 4.6) and the other was treated with 1 ml of potassium acetate buffer alone. After 30 min at 37 C in the dark, 1.3 ml of potassium acetate (1 M, pH 4.6) and 11 ml of cold (−15 C) ethyl alcohol were added, and the precipitated RNA was collected by centrifugation (27,000 $\times$ *g*, 10 min). The excess of periodate was destroyed by dissolving the precipitate in 4 ml of ethylene glycol (100 mM) in potassium acetate buffer (100 mM, pH 4.6) and allowing it to stand in the dark at room temperature for 10 min. In the two fractions, the RNA was precipitated again with 1.3 ml of potassium acetate (1 M, pH 4.6) and 11 ml of cold (−15 C) ethyl alcohol and collected by centrifugation at 27,000 $\times$ *g* for 10 min. Each precipitate was dissolved in 1 ml of distilled water, and the stripping was performed by adding to each RNA solution 1 ml of 3.6 M Tris-acetate buffer (pH 8.2), and allowing it to stand for 2 h at 37 C. The RNA of each fraction was then precipitated by addition of 11 ml of cold (−15 C) ethyl alcohol, collected by centrifugation (27,000 $\times$ *g*, 10 min), and dissolved in 0.5 ml of distilled water.

Acceptor activity of the periodate-treated and untreated tRNAs was determined as described previously, except that tritiated methionine was used in the incubation mixture instead of ^{14}C-methionine (5). The preparation of the amino acyl tRNA synthetases used for the determination of the tRNA acceptor activities for methionine and isoleucine has been described previously (5).

Results are expressed as the percent of the tRNA

charged in vivo, i.e., the ratio of the acceptor activity of periodate treated over the acceptor activity of untreated tRNA × 100. The acceptor activity of untreated tRNA was used for the determination of total $tRNA^{Met}$ synthesized in each fraction of the chemostat.

Chemicals. *O*-acetyl-DL-homoserine was synthesized by M. Cherest by the method of Sakami and Toennies (18). Sodium dodecyl sulfate was recrystallized in ethyl alcohol. Commercial yeast tRNA was purchased from Schwarz BioResearch. L-Methionine-*methyl*-^3H and DL-isoleucine-^{14}C were purchased from the C.E.A., France.

RESULTS

In a first series of experiments, the effluent of the chemostat was sampled after various times and in vivo $tRNA^{Met}$ charging was measured in the successive fractions together with the specific activity of homocysteine synthetase which had been chosen as a typical representative of methionine group I enzymes. It was previously shown that enzymes which belong to this group are derepressed under methionine limitation (5). Results of a typical experiment (Table 1) show that in vivo $tRNA^{Met}$ charging decreased sharply as soon as methionine limitation was established. Under these conditions, the $tRNA^{Ile}$, taken as a control, remained fully charged. As expected, the specific activity of homocysteine synthetase was increased three-fold.

TABLE 1. *Effect of methionine limitation on homocysteine synthesis and on the percentage of $tRNA^{Met}$ and $tRNA^{Ile}$ charged in vivo*

Fraction	HC synthetase[a]	$tRNA^{Met}$ charged in vivo (%)	$tRNA^{Ile}$ charged in vivo (%)
Control[b]	96	96	100
Fraction I	125	54	100
Fraction II	235	60	100
Fraction III	300	55	100

[a] Specific activities of homocysteine synthetase are expressed as nanomoles per minute per milligram of protein.

[b] The day before the chemostat was inoculated, 6 liters of G0 minimal medium containing 20 mg/liter of uracil and 0.5 mM *O*-acetyl-DL-homoserine were inoculated with strain D6 from a preculture (4). The next day, at the end of the exponential phase of growth (2.0 to 2.5 × 10^7 cells per ml) the cells were collected by sterile centrifugation. One part was resuspended in G0 medium containing 20 mg/liter of uracil and 0.08 mM DL-methionine and was used to inoculate the chemostat as described in Materials and Methods. The remaining culture was split into two parts, one for tRNA extraction, the other for cell-free extracts. The results are given in Table 1 as "control." Fractions of the chemostat were collected at time intervals corresponding to one doubling of cell mass. The percentage of tRNA charged in vivo was calculated as described in Materials and Methods.

As stated above, it was of importance to determine the evolution of methionyl-tRNA synthetase and to establish whether the overall content of tRNA was modified under methionine limitation. In a second series of chemostats, methionyl-tRNA synthetase activity, total tRNA, $tRNA^{Met}$, and $tRNA^{Ile}$ contents were followed together with activities of two group I enzymes (homocysteine synthetase and ATP sulfurylase). To allow a better comparison of the rates of synthesis of these different cellular constituents, the results of a typical experiment are presented, stating as 100% the values obtained with the culture used to inoculate the chemostat (Fig. 1).

In this representation, one would expect that if the rate of synthesis of any constituent is unaffected by methionine limitation, its relative increase will strictly follow the relative increase in cellular mass ("growth curve"); any constituent which is synthesized at a faster rate will show a higher slope and, in turn, any constituent synthesized at a slower rate will show a lower slope than the one corresponding to cellular mass.

In Fig. 1, it can be seen that the two biosynthetic enzymes are both synthesized at the same increased rate as compared to the growth curve. This result agrees with the previous finding that these enzymes are coordinately derepressed (5). In this figure, methionyl-tRNA synthetase appears slightly derepressed, although not coordinately with the methionine group I enzymes. Such derepression was not always found but, in any case, a decreased rate of synthesis of methionyl-tRNA synthetase was never observed. On the other hand, Fig. 1 shows that, in this same experiment, the rate of total tRNA synthesis is slowed down by methionine limitation. This result indicates that methionine limitation is already strong enough to lead to a "stringent effect." Moreover, in all experiments, the rates of synthesis of $tRNA^{Met}$ and $tRNA^{Ile}$ were both decreased to the same extent as total tRNA, showing no preferential decrease of $tRNA^{Met}$ synthesis. These two sets of experiments indicate that not only the in vivo charging of $tRNA^{Met}$ but also its total amount per cell is decreased under conditions which lead to derepressed synthesis of Met group I enzymes.

DISCUSSION

Results presented here reinforce the conclusions made previously that methionyl-tRNA participates in the regulation of methionine biosynthesis in *S. cerevisiae*. Experiments using the thermosensitive mutant ts-296 have shown a correlation between a 50% decrease of in vivo charging of $tRNA^{Met}$ and a lack of repressibility of methionine group I enzymes (5). Present experiments show that when derepression occurs, a similar decrease in in vivo charging of $tRNA^{Met}$ is observed. However, it should be pointed out that this decrease is already apparent in the first fraction of the chemostat and remains constant in further fractions. This is not surprising, since the in vivo charging must reflect the methionine concentration which has been ascertained to be limiting and constant in chemostat conditions. However, concomitantly, the rate of synthesis of RNA including $tRNA^{Met}$ decreases by roughly a factor of two. This results in a gradual decrease in the amount of methionyl-tRNA per cell while gradual derepression of methionine group I enzymes occurs. It should be noted that in Met G mutants of *S. typhimurium*, an impaired methionyl-tRNA synthetase does not lead to derepressed synthesis of some methionine biosynthetic enzymes. On this basis, Gross and Rowbury did not consider methionyl-tRNA as a possible co-repressor of methionine biosynthesis in this organism (9, 10). However, it remains possible that the limitation of in vivo charging of $tRNA^{Met}$ resulting from the mutations studied is not definite enough to have influenced the rate of synthesis of the enzymes considered in this organism.

On the other hand, present results do not support participation of methionyl-tRNA synthetase per se in the regulatory process. Indeed, one would expect that if this enzyme were one of the elements involved in the repression mechanism, its concentration should decrease during derepression. In fact, results obtained do not agree with such an assumption. Not only does the rate of synthesis of this enzyme never decrease, but in some experiments, a slight derepression is observed (see Fig. 1). This derepression fits in with the numerous recent findings that aminoacyl tRNA synthetases are themselves under the repressive control exerted by their cognate amino acid (1, 5a, 15, 16, 23). In any case, the synthesis of methionyl-tRNA synthetase from *S. cerevisiae* does not appear to be coordinate with that of methionine biosynthetic enzymes. These findings agree with those reported by Gahr and Nass concerning the regulation of synthesis of isoleucyl-tRNA synthetase and threonine deaminase in *Escherichia coli* (6).

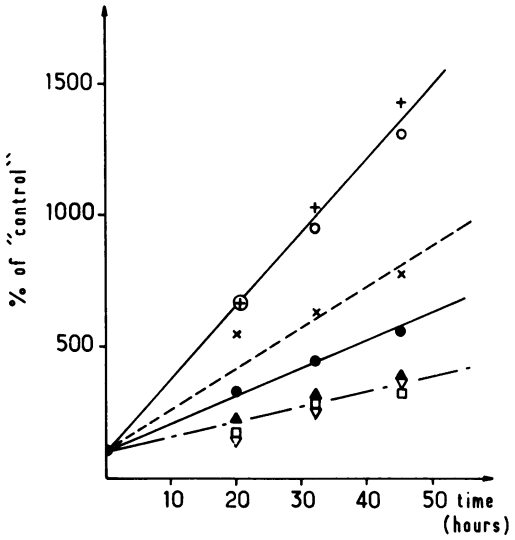

FIG. 1. *Effect of methionine limitation on the synthesis of different constituents of the cell.* ●, *Growth curve. The total number of cells collected at each fraction was calculated and expressed as a percentage of the number of cells used to inoculate the chemostat (this corresponds to the "control" in Table 1). Enzyme synthesis: homocysteine synthetase* (+), *ATP sulfurylase* (○), *and methionyl-tRNA synthetase* (×). *The quantity of each enzyme present in the inoculum of the chemostat (total units) was taken as 100%. Each point then represents the total increase in units of each enzyme from the beginning of the chemostat. tRNA synthesis: total tRNA* (□), *$tRNA^{Met}$* (▽), *and $tRNA^{He}$* (▲). *Calculations of total tRNA synthesized for each time were based on the absorbance at 260 nm determined after the first alcohol precipitation (see Materials and Methods). Calculations for $tRNA^{Met}$ and $tRNA^{Ile}$ were made on the same basis as for enzyme units.*

Also, a fourfold decrease in methionyl-$tRNA^{Met}$ content per cell results in a four- to fivefold increase in generation time (2 h in optimally growing cells versus 9.2 h in our chemostat conditions). This indicates that the amount of methionyl-$tRNA^{Met}$ per cell in optimal growth conditions is probably not in excess.

ACKNOWLEDGMENTS

This work was greatly assisted by grants from the D.G.R.S.T.-France, the F.R.M.F. and the C.E.A.-France.

We thank D. Henry and F. de la Torre for expert technical assistance.

LITERATURE CITED

1. Archibold, E. R., and L. S. Williams. 1972. Regulation of synthesis of methionyl-propyl-, and threonyl-transfer

ribonucleic acid synthetases of *Escherichia coli*. J. Bacteriol. **109**:1020–1026.
2. Brenner, M., and B. N. Ames. 1971. The histidine operon and its regulation, p. 349–387. *In* M. Vogel (ed.), Metabolic regulations, Vol. 5. Academic Press Inc., New York.
3. Brenner, M., and B. N. Ames. 1972. Histidine regulation in *Salmonella typhimurium*. IX Histidine transfer ribonucleic acid of the regulatory mutants. J. Biol. Chem. **247**:1080–1088.
4. Cherest, H., F. Eichler, and H. de Robichon-Szulmajster. 1969. Genetic and regulatory aspects of methionine biosynthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **97**:328–336.
5. Cherest, H., Y. Surdin-Kerjan, and H. de Robichon-Szulmajster. 1971. Methionine-mediated repression in *Saccharomyces cerevisiae:* a pleiotropic regulatory system involving methionyl transfer ribonucleic acid and product of gene *eth2*. J. Bacteriol. **106**:758–772.

5a. Ehresmann, B. F. Karst, and J. H. Weil. 1971. Regulation of the biosynthesis of valyl-tRNA synthetase in yeast. Biochem. Biophys. Acta **254**:226–236.

6. Gahr, M., and G. Nass. 1972. Regulation of the formation of isoleucyl-tRNA synthetase and the level of isoleucine biosynthetic enzymes in *E. coli* K12. Mol. Gen. Genet. **116**:348–359.
7. Galzy, P., and P. P. Slonimski. 1957. Evolution de la constitution enzymatique de la levure cultivée sur acide lactique ou sur glucose comme seule source de carbome. C. R. Acad. Sci. **245**:2556–2558.
8. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
9. Gross, T. S., and R. J. Rowbury. 1969. Methionyl tRNA synthetase mutants of *Salmonella typhimurium* which have normal control of their methionine biosynthetic enzymes. Biochim. Biophys. Acta **184**:233–236.
10. Gross, T. S., and R. J. Rowbury. 1971. Biochemical and physiological properties of methionyl sRNA synthetase mutants of *Salmonella typhimurium*. J. Gen. Microbiol. **65**:5–21.
11. Kjellin-Straby, K., and J. H. Phillips. 1969. Methyl deficient transfer ribonucleic acid and macromolecular synthesis in methionine-starved *Saccharomyces cerevisiae*. J. Bacteriol. **100**:679–686.
12. Kredich, N. M., and G. N. Tomkins. 1966. The enzyme synthesis of L-cysteine in *E. coli* and *S. typhimurium*. J. Biol. Chem. **241**:4955–4965.
13. Lewis, J. A., and B. N. Ames. 1972. Histidine regulation in *Salmonella typhimurium*. XI The percentage of transfer RNA^{his} charged *in vivo* and its relation to the repression of the histidine operon. J. Mol. Biol. **66**:131–142.
14. Maaløe, O., and N. O. Kjeldgaard. 1966. A study of DNA, RNA and protein synthesis in bacteria, p. 125–153. Control of macromolecular synthesis. W. A. Benjamin, Inc., New York.
15. McGinnis, E., and L. S. Williams. 1971. Regulation of synthesis of aminoacyl transfer ribonucleic acid synthetases for the branched chain amino acids of *Escherichia coli*. J. Bacteriol. **108**:254–262.
16. Nass, G., and F. C. Neidhardt. 1967. Regulation of formation of aminoacyl-ribonucleic acid synthetase in *Escherichia coli*. Biochim. Biophys. Acta **134**:347–359.
17. Novick, A., and L. Szilard. 1950. Experiments with the chemostat on spontaneous mutations of bacteria. Proc. Nat. Acad. Sci. U.S.A. **36**:708–719.
18. Sakami, W., and G. Toennies. 1942. The investigation of amino acid reactions by methods of non aqueous titrimetry. II. Differential acylation of hydroxy groups, and a method for the preparation of the acetyl derivatives of hydroxy amino acids. J. Biol. Chem. **144**:203–217.
19. Singer, C. E., G. R. Smith, R. Cortese, and B. N. Ames. 1972. Histidine regulation in *Salmonella typhimurium*. XII Mutant transfer RNA^{his} ineffective in repression and lacking two pseudo uridine modifications. Nature N. Biol. **238**:72–74.
20. Twardzik, D. R., E. H. Grell, and K. B. Jacobson. 1971. Mechanism of suppression in Drosophila: a change in tyrosine transfer RNA. J. Mol. Biol. **57**:231–245.
21. Vito, P. C. de, and J. Dreyfuss. 1964. Metabolic regulation of adenosine triphosphate sulfurylase in yeast. J. Bacteriol. **88**:1341–1348.
22. Wiebers, J. L., and H. R. Garner. 1967. Acyl derivatives of homoserine as substrates for homocysteine synthesis in *N. crassa* yeast and *E. coli*. J. Biol. Chem. **241**:5644–5649.
23. Williams, L. S., and F. C. Neidhardt. 1969. Synthesis and inactivation of aminoacyl tRNA synthetases during growth of *Escherichia coli*. J. Mol. Biol. **43**:529–550.
24. Wilson, L. G., and R. S. Bandurski. 1958. Enzymatic reactions involving sulfate, sulfite, selenate and molybdate. J. Biol. Chem. **233**:975–981.