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A rapid method for determining tRNA charging levels *in vivo*: analysis of yeast mutants defective in the general control of amino acid biosynthesis

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ABSTRACT

We describe a simple method to quantitate the intracellular levels of charged tRNA species representing all 20 amino acids. Small RNA species are isolated from yeast cells under conditions where amino acids remain bound to their cognate tRNAs. After chromatographic removal of free amino acids, the tRNAs are decharged, and the amounts of the released amino acids are then quantitated. This method was applied to yeast cells from a wild type strain and from three mutant strains that are defective both in the general control of amino acid biosynthesis and in protein synthesis. Two of these mutant strains, previously shown to be defective in the methionine or isoleucine tRNA synthetases, respectively contain undetectable amounts of charged methionine or isoleucine although their levels of the remaining 19 amino acids are similar to a wild type strain. In contrast, a *gcd1* mutant strain has normal levels of all 20 amino-acyl tRNA species. Thus, *gcd1* strains are defective in general control of amino acid biosynthesis for reasons other than artifactual starvation of an amino acid due to a failure in tRNA charging.

INTRODUCTION

When yeast cells are starved for any one of a variety of amino acids, they respond by coordinately inducing the transcription of many genes encoding amino acid biosynthetic enzymes (reviewed in 1). This induction is mediated by the binding of the GCN4 activator protein to specific upstream regulatory sequences, consensus ATGACTCATT (2), in the promoters of the coregulated genes (3). The intracellular level of GCN4 protein is regulated by the efficiency of translation of GCN4 mRNA (4,5). Under normal growth conditions, GCN4 mRNA is not translated due to the presence of upstream AUG codons; hence the amino acid biosynthetic genes are transcribed at basal levels. However, under conditions of amino acid starvation, the normal rules of translational initiation are broken, thus leading to the synthesis of GCN4 protein and the subsequent induction of the coregulated genes.

Experimentally, starvation conditions are achieved by the exogenous addition of a metabolic poison that blocks the synthesis or charging of a single amino acid. By analogy with the stringent-relaxed response of *E.coli* (6,7), it is presumed that the intracellular signal of amino acid starvation is produced during protein synthesis as a consequence of a low level of charged tRNAs. However, little is known about the molecular signal of amino acid starvation, or how this signal stimulates the synthesis of GCN4 protein, the ultimate effector.

The general control of amino acid biosynthesis requires the product of the GCD1 gene (8).

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The *gcd1*-1 allele results in induced mRNA levels of the coregulated genes even under normal growth conditions (9-11). In addition, at elevated temperatures, *gcd1*-1 strains become arrested at the start of the cell cycle and are unable to grow further (8). The inability to grow at high temperature is due at least in part to a general defect in protein synthesis (our unpublished results).

Although these observations suggest an interesting role for the GCD1 gene product, one trivial possibility must be ruled out. Specifically, the *gcd1*-1 phenotype could be explained by artifactual amino acid starvation due to a defect in tRNA charging. Indeed, mutations in the isoleucine or methionine tRNA synthestase genes (*ILS1*, *MES1*) prevent growth at high temperatures (12,13), and they cause constitutive induction of the amino acid biosynthetic enzymes at lower temperatures (14). For this reason, we wished to determine the levels *in vivo* of charged tRNA species in *gcd1*-1 strains. However, artifactual starvation and concommitant induction could in principle be due to a failure to charge any one of the 20 amino acids. Conventional tRNA charging assays are applicable only for individual amino acids and are rather cumbersome (12,13,15). In this paper, we describe a simple and quantitative method for simultaneously assaying all 20 amino acids for their presence on amino-acyl tRNA complexes. With this method, we analyze the levels of charged tRNA in wild type, *gcd1*-1, *ils1*-1, *mes*--1, and *cdc16*-1 strains.

MATERIALS AND METHODS

The yeast strains (including their relevant genotypes) used in these experiments were KY114 (wild type)(18), KY403 (*gcd1*-1)(2), 341a (*ils1*-1)(12), 296α (*mes1*-1)(13), and *cdc16*-1 (obtained from Francine Messenguy). 500 ml. cultures of each strain were grown at 23°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) until an $A_{600} = 1$. At this point, the cultures were shifted to 37°C and incubated for an additional 4 hours. The cells were killed by addition to 2.5% trichloroacetic acid, chilled quickly by immersion of the flask in a dry ice/ethanol bath, and subjected to centrifugation. The cells were washed with 0.1M sodium acetate pH 4.6, and resuspended in 10 ml. of the same buffer. An equal volume of phenol (equilibrated with 0.1M sodium acetate pH 4.6) was added, and the mixture was shaken vigorously for several minutes. Although this procedure does not result in cell lysis, it quantitatively liberates 5S and tRNAs (12). After phenol extraction and ethanol precipitation, the RNA was resuspended in 1-2 ml. of sodium acetate pH 4.6 containing a small amount of ^{35}S-methionine and loaded on a Sephadex G25 column equilibrated with the same buffer. In this way, the RNA (monitored by A_{260}) was completely separated from free amino acids (monitored by ^{35}S) and other small molecules. The purified RNA was ethanol precipitated and then resuspended at a concentration of 50 mg/ml in 0.2M sodium carbonate pH 10 (the final pH should be at least 9), conditions that quantitatively decharge amino acids from their tRNAs (15). After 30 minutes at 37°C, the mixture was then adjusted to pH 2.0 by the addition of HCl. It

should be noted that a 50 mg/ml RNA solution is equivalent to a concentration of 150mM, and hence acts as a significant buffer. Some of the RNA precipitates if the pH is lowered too much, but this does not affect the subsequent analysis. Using a Beckman 121MB amino acid analyzer, the 20 amino acids were selectively eluted on a AA10 anion-exchange column (2.8 X 30 cm) with a gradient of increasing pH and then quantitated by the ninhydrin reaction (17). Specifically, after loading the sample, the successive elution conditions were 27 minutes at 40°C in 0.2N lithium citrate pH 2.83; 35 minutes at 70°C in the same buffer; 60 minutes at 70°C in 0.2N lithium citrate pH 3.75, 1% isopropanol; and 90 minutes at 70°C in 1N lithium citrate pH 3.75, 6% isopropanol. The vast excess of RNA in the samples does not interfere with the amino acid analysis. Although not specifically tested, it is likely that other procedures for amino acid analysis would be equally suitable.

RESULTS

The assay for tRNA charging levels *in vivo*

The principle of the method is as follows. First, RNA is isolated at pH 4, conditions that preserve the amino-acyl tRNA complexes. Second, charged tRNA species are separated from free amino acids by virtue of their large difference in molecular weight. Third, the amino acids are decharged from their cognate tRNAs by mild alkali treatment (pH 9). Fourth, the products are subjected to amino acid analysis by conventional procedures. The mild chemical treatments involved in this procedure make it possible to measure directly the amount of each of the 20 amino acids that was originally bound to tRNA. This is in contrast to amino acid analysis of proteins, where glutamine, asparagine, and tryptophan are destroyed by the strong acids necessary to cleave the peptide bond.

tRNA charging levels in wild type and mutant strains

The principal reason for developing this method was to analyze tRNA charging levels in a *gcd1*-1 strain. As controls, we also analyzed the charged tRNA levels in a wild type strain, in strains containing mutations in the isoleucine or methionine tRNA synthetases (respectively *ils1*-1 and *mes1*-1), and in a *cdc16*-1 strain that blocks the normal cell division cycle but does not interfere directly with protein synthesis. The analysis was performed on cells incubated at 37°C because this temperature causes the most severe mutant phenotypes. Due to the temperature sensitive nature of the *gcd1*, *ils1*, *mes1*, and *cdc16* mutations (8,12,13), the strains were pregrown at 23°C and then shifted to 37°C for 4 hours. At this time, the mutant cells have stopped growing, and in the case of *gcd1*, *ils1*, and *mes1*, the levels of protein synthesis are extremely low.

The results of the experiment are shown in Table 1. For the wild type strain, the 20 amino-acyl tRNA complexes are easily detectable. Although we have not directly quantitated the amount of tRNA, it is known from previous work (12-14) that tRNA species in growing yeast cells are essentially 100% charged with their cognate amino acids. Thus, the values in Table 1,

Table 1: Levels of amino-acyl tRNA complexes

Amino acid	Wild type	*gcd1*	*ils1*	*mes1*	*cdc16*	Ave.
Alanine	36.0	44.5	43.3	37.5	42.7	37.0
Arginine	43.6	33.8	33.1	27.6	25.1	22.0
Asparagine	11.9	15.6	17.8	17.8	10.0	25.5
Aspartic acid	39.6	31.6	37.6	32.7	35.9	29.5
Cysteine	+	+	+	+	+	5.5
Glutamate	16.1	23.9	23.4	27.0	25.2	32.5
Glutamine	21.7	17.8	17.8	24.2	24.2	18.5
Glycine	39.2	48.0	44.5	43.2	52.6	34.5
Histidine	23.0	19.6	18.3	19.6	9.6	10.5
Isoleucine	43.1	35.8	0	35.5	46.2	31.5
Leucine	70.9	41.6	29.7	40.5	48.7	42.5
Lysine	71.4	32.2	40.5	28.6	20.5	37.5
Methionine	24.5	13.8	12.2	0	20.2	9.0
Phenylalanine	34.5	24.4	19.7	30.6	29.0	19.5
Proline	18.8	20.6	12.1	15.8	19.3	20.5
Serine	25.6	43.1	47.1	40.4	32.3	36.0
Threonine	38.8	44.4	37.8	41.2	53.2	27.5
Tryptophan	23.0	8.2	22.9	12.9	4.0	5.0
Tyrosine	37.9	15.7	8.9	6.1	23.3	17.0
Valine	21.6	33.5	23.1	25.5	38.0	36.5
TOTAL	641.2	548.1	499.8	506.7	559.9	(500)

Entries for each amino acid represent the number of pmoles released from tRNA per mg of total RNA. The number of pg of total RNA per cell (calculated from the A_{600}) was 6.1 (wt), 5.9 (*gcd1*-1), 5.6 (*ils1*-1), 4.9 (*mes1*-1), 5.5 (*cdc16*-1). The + indicates that cysteine was detectable, but its amount was not easily quantitated. The last column represents an average amino acid composition for yeast proteins derived from the presumptive sequences of 78 proteins (normalized to 500 for comparison).

which are normalized to the amount of total RNA, should represent fully charged levels. As expected, the relative amounts of these charged amino acids are in good accord with the average amino acid composition of 78 yeast proteins. Similar values were obtained with the *cdc16* mutant strain, suggesting that the tRNAs remain completely charged even when the cells have stopped growing.

Analysis of the *ils1* and *mes1* mutants revealed normal levels of only 19 amino-acyl tRNA complexes. As expected, the *ils1* mutant did not contain detectable levels of charged isoleucine, and the *mes1* mutant lacked charged methionine. These results confirm those obtained with more conventional charging assays, and they indicate that the method described here can easily detect defects in tRNA charging.

In contrast to the results obtained with the synthetase mutants, the *gcd1* strain had normal levels of all 20 amino-acyl tRNA complexes even when the cells were not growing due to the absence of protein synthesis. This strongly suggests that phenotypes caused by the *gcd1*-1 mutation are not due to defects in tRNA charging, and that the *GCD1* gene does not encode a tRNA synthetase.

DISCUSSION

Generality of the method

When compared to conventional assays for tRNAs charging levels *in vivo* which involve purification and chemical modification of the tRNA as well as preparation of active tRNA synthetases (14), the method described is rapid, and it involves simple preparative and analytical procedures. More importantly, the method permits an internally controlled and simultaneous determination of charging levels for all 20 amino acids. To obtain equivalent information by the standard procedure, it would be necessary to perform 20 separate determinations each containing one labeled amino acid, an expensive and time consuming process.

When normalized either to the number of cells or to the amount of total RNA, the amount of each of the 20 amino acids present on tRNA complexes is quite consistent, although there are occasional 2-fold differences among the different strains (Table 1). We suspect that these differences reflect experimental error rather than actual charging levels. Levels for tryptophan and tyrosine are more variable, possibly due to artifacts associated with the method of amino acid analysis. Cysteine can be detected, but absolute levels are difficult to quantitate. The experimental error associated with our method is not markedly different to that associated with the conventional procedure.

Thus, although the method is quantitative, it is more suitable for situations in which charging defects are relatively severe. We estimate that for most amino acids, our method would easily detect circumstances up to 20% of full charging and possibly, with repeated determinations, detect situations involving 30-40% charging. Charging defects involving tryptophan, tyrosine, or cysteine would have to be relatively more severe in order to be detected.

The major value of the procedure described in this paper is that it is possible to screen mutant strains for defects in tRNA charging. For example, it is very likely that among the numerous yeast mutants that fail to grow at high temperature, some of them will have defects in tRNA charging. Such mutants could define the genes encoding various tRNA synthetases or tRNA modifying enzymes. Conversely, as demonstrated here, the method is extremely useful in excluding tRNA charging defects in strains that are known to be defective in protein synthesis. Although our method is best suited for strains with low tRNA charging levels, it is worth noting that all previously characterized mutants (*ils1*-1 and *mes1*-1) are completely defective in charging, and that situations of 30-50% charging (as seen in *ils1*-1 or *mes1*-1 strains grown at intermediate temperatures) do not significantly affect cell growth (12-14). Thus, the quantitative limitations of our method are unlikely to interfere with the analysis of mutants that have been identified by their growth phenotypes. Finally, although the method is applied here exclusively to yeast cells, it should be possible to adapt the procedure to essentially any other organism.

Implications for the general control of amino acid biosynthesis

The coordinate induction of amino acid biosynthetic genes is mediated by the binding of a common activator protein, GCN4, to common *cis*-acting promoter sequences (2,3). The

regulated expression of these genes can be accounted for simply by the level of GCN4 protein under the relevant environmental conditions (4,5). However, mutations in at least 10 other genes abolish the coordinate regulation of amino acid biosynthetic genes, yet the functions of their gene products are unknown at the molecular level (18,19). It is hoped that at least some of these gene products are directly involved in the production of or the response to the starvation signal and/or the mechanism of translational control of GCN4 mRNA. However, as there are usually many indirect ways to alter a cell's physiology, it is often difficult to determine whether a regulatory mutation defines an interesting mechanistic feature or a side issue.

The observation that *gcd1*-1 strains contain normal levels of charged tRNAs strongly suggests that their defect in general control is not due to artifactual starvation for an amino acid. Instead, it is likely that the protein synthesis defect caused by the *gcd1*-1 mutation is directly related to the mechanism of general control. Two specific models for how a gene product involved in normal protein synthesis can be important for general control are proposed. One possibility is that the *GCD1* gene product is part of the machinery that produces the starvation signal when the ribosomes stall because of their inability to add the next amino acid. By this model, the *gcd1*-1 mutation could produce the starvation signal even under normal conditions. Alternatively, the *GCD1* gene product might be involved in the mechanism(s) by which only the 5' proximal AUG codon is selected as the site of translational initiation and reinitiation at downstream AUG codons is prevented (20). A *gcd1*-1 mutation could alleviate the block to reinitiation, and thus permit initiation at the downstream AUG codon that begins the GCN4 protein coding sequence.

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