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Isopentenyladenosine deficient tRNA from an antisuppressor mutant of Saccharomyces cerevisiae

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ABSTRACT

We have isolated a mutant of *Saccharomyces cerevisiae* that contains 1.5% of the normal tRNA complement of isopentenyladenosine (i^6A). The mutant was characterized by the reduction in efficiency of a tyrosine inserting UAA nonsense suppressor. The chromatographic profiles of $tRNA^{Tyr}$ and $tRNA^{Ser}$ on benzoylated DEAE-cellulose are consistent with the loss of i^6A by these species. Transfer RNA from the mutant exhibits 6.5% of the cytokinin biological activity expected for yeast tRNA. Transfer RNAs from the mutant that normally contain i^6A accept the same levels of amino acids *in vitro* as the fully modified species. With the exception of i^6A, the level of modified bases in unfractionated tRNA from the mutant appears to be normal. The loss of i^6A apparently affects tRNA's role in protein synthesis at a step subsequent to aminoacylation.

INTRODUCTION

Isopentenyladenosine (i^6A) is one of several hypermodified derivatives of adenosine that have been found in the tRNA of almost all organisms investigated[1]. Its location is restricted to the position adjacent to the 3' end of the anticodons of most tRNA species that recognize codons beginning with uridine[2].

A number of investigations have been undertaken to determine the role of this modified nucleoside in tRNA function. Fittler and Hall[3] demonstrated that chemical modification of the i^6A moiety in yeast $tRNA^{Ser}$ with iodine reduced the level of ribosome binding of the tRNA in response to a synthetic message. The level of *in vitro* aminoacylation of the modified tRNA was not altered. An analogous study involving bisulfite treatment of yeast $tRNA^{Tyr}$ resulted in similar findings[4]. Gefter and Russell[5] isolated $tRNA^{Tyr}$ deficient in 2-methylthio and 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A) from *E. coli* in-

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fected with a transducing bacteriophage carrying the tRNA gene. They demonstrated that i^6A is required for the efficient binding of $tRNA^{Tyr}$ to ribosomes in the presence of the appropriate codons. They also found that the kinetics of *in vitro* amino-acylation is not affected by the absence of i^6A. In a study involving the formation and dissociation of complexes between tRNAs with complementary anticodons, Grosjean *et al.*[6] examined the relationship between modification of the purine adjacent to the 3' end of the anticodon of $tRNA^{Phe}$ and the stability of the complex. They found that $tRNA^{Phe}$ from *E. coli* (with ms^2i^6A) complexed with $tRNA^{Glu}$ from *E. coli* as well as did $tRNA^{Phe}$ from yeast (with base Y). Complexes between $tRNA^{Phe}$ from *Mycoplasma* (Kid), which lacks a hypermodified constituent, and *E. coli* $tRNA^{Glu}$, and between yeast $tRNA^{Phe}$ with base Y removed and *E. coli* $tRNA^{Glu}$ were less stable. Thus, hypermodification of the base adjacent to the 3' end of the anticodon was correlated with an enhancement in the stability of anticodon-anticodon interaction. Litwack and Peterkofsky[7] isolated tRNA with reduced levels (50%) of i^6A from a mevalonate-requiring mutant of *Lactobacillus acidophilus*. They were unable, however, to separate the i^6A deficient tRNAs from the fully modified species. Contrary to the other reports, they found no difference in the level of polynucleotide directed amino acid incorporation from specific tRNA species into protein when partially modified and fully modified tRNAs were compared. In addition, Kimball and Söll[8] demonstrated that $tRNA^{Phe}$ from *Mycoplasma* (Kid) was fully active in promoting phenylalanine incorporation in a cell free, tRNA dependent, polyuridylic acid directed amino acid incorporating system,even though this tRNA species lacks i^6A or any related hypermodified base. These last two studies suggest that there is no absolute requirement for i^6A and related modifications *in vitro*.

A mutant of *S. cerevisiae* with a greatly reduced level of i^6A has been isolated, and affords the opportunity to compare *in vitro* results with *in vivo* observations. The mutation, which has been designated *mod5-1*[9], reduces the suppression by a dominant UAA suppressor, *Sup7-1*[10], so that only the more easily suppressed of several UAA mutations are suppressed. *SUP7-1* is

one of several efficient tyrosine-inserting UAA suppressors, and most probably codes for an altered $tRNA^{Tyr}$ [11-16]. Genes corresponding to these suppressors have been genetically mapped at eight different loci in the yeast genome [10,17], and yeast $tRNA^{Tyr}$ hybridizes to eight distinct yeast DNA fragments generated by *Eco*RI digestion[15]. This tRNA normally contains i^6A adjacent to the 3' end of the anticodon, as do yeast $tRNA_1^{Ser}$, $tRNA_2^{Ser}$, and $tRNA^{Cys}$ [18].

EXPERIMENTAL PROCEDURES

Mutant Screening

The strategy for selection of mutants characterized by a reduction in efficiency of Class I ochre-specific suppressors in S. cerevisiae was analogous to that described by McCready and Cox[19]. Strain 700:1: *a SUP7-1 can1-100 ade2-1 his5-2 lys1-1 trp5-48* was mutagenized with either ethyl methanesulfonate or ultraviolet radiation. Strain 700:1 is canavanine sensitive and adenine, histidine, lysine and tryptophan independent by virtue of the suppression of the UAA nonsense mutations *can1-100, ade2-1, his5-2, lys1-1, and trp5-48*. Mutants with partial loss of suppression were selected either as canavanine-resistant or adenine-requiring revertants. Most antisuppressor mutants, including *mod5-1*, remained histidine, lysine and tryptophan independent. The genetic characterization of the mutations is being published elsewhere[9].

All strains were constructed by utilizing standard techniques of mating, sporulation and ascus dissection. Nutritional requirements were determined by growth on selective media[20].

Transfer RNA Preparation

Transfer RNA was isolated from log phase S. cerevisiae cells by phenol extraction and DEAE-cellulose (Whatman DE23) chromatography.

Base Analysis of tRNA by Chemical Tritium Labelling

The procedure for nucleoside composition analysis by two-dimensional thin layer chromatography of tritium labelled derivatives was essentially that described by Randerath et al.[21,22]. Transfer RNA was enzymatically digested to nucleosides, and the products were tritiated by treatment with $NaIO_4$ (Matheson,

Coleman, and Bell) and $[^3H]KBH_4$ (Amersham). The mixture of tritiated nucleoside trialcohols was chromatographed on microcrystalline cellulose thin layers (J.T. Baker) and quantitated by liquid scintillation counting.

Analysis of Isopentenyladenosine

Isolation of i^6A by Sephadex LH20 (Pharmacia) chromatography was done essentially by the procedure of Armstrong et al.[23]. Transfer RNA was converted to nucleosides with snake venom phosphodiesterase (Worthington), pancreatic ribonuclease (Sigma), and bacterial alkaline phosphatase (Sigma). The identification of i^6A was made on the basis of elution position on Sephadex LH20 and by ultra-violet spectral analysis. Fractions containing i^6A were pooled, and the nucleoside was quantitated spectrophotometrically. Absorbance was converted to moles using the molar extinction coefficient of 20.0×10^{-3} at 269 nm and pH 7.

Amino Acid Acceptance

Amino acid acceptance assays were performed as previously described[24,25]. For each amino acid, the reaction mixture contained 400 µl of aminoacylation reagent, 0.1 mg of crude S. cerevisiae enzyme, and approximately 10 A_{260} units[26] of tRNA. Reactions were incubated for 30 min at 37°C.

Transfer RNA Fractionation and Benzoylated DEAE-Cellulose

Equal weights of benzoylated DEAE (BD)-cellulose (prepared by M. Miyazaki by the procedure of Gillam et al.[27]) were used to pour two columns (1.3 x 11.0 cm each). The columns were pre-equilibrated with 0.05 M sodium acetate, pH 4.75, 0.45 M NaCl, 0.01 M $MgCl_2$, 0.1 mM EDTA. One was loaded with 36.25 mg of *mod5-1* tRNA and the other with 36.25 mg of tRNA from a nonmutant control. The columns were eluted with a single linear NaCl gradient that was divided and simultaneously pumped through both columns at a flow rate of 7.2 ml/hr. The NaCl concentration ranged from 0.45 to 1.0 M in a total volume of 400 ml. The columns were purged of bound material with 0.05 M sodium acetate, pH 4.75, 1.2 M NaCl, 0.01 M $MgCl_2$ in 15% ethanol. Thirty minute fractions were collected and assayed for amino acid acceptance activities by the procedure referred to above, with the following modifications. Each reaction contained 90 µl column effluent, 100 µl aminoacylation reagent, and 0.05 mg enzyme. With the ex-

ception of fractions 72 and 74, in which tRNA was precipitated and redissolved in aminoacylation reagent, no steps were taken to standardize salt conditions or tRNA concentration.

Tobacco Tissue Culture Bioassay

The bioassay used to detect cytokinin activity has been described [28,29]. For each determination, bases derived from approximately 35 mg of tRNA were incorporated into the tissue culture medium, RM-1965[28], and tested in fivefold serial concentrations of tRNA from approximately 0.1 mg/l to 350 mg/l. Four replicate 50 ml flasks, each containing 20 ml of medium, were prepared for each tRNA concentration. Three pieces of tobacco callus (*ca.* 25 mg each) were planted per flask. Controls containing kinetin (6-furfurylaminopurine) at concentrations of 0,1,2,3,5,7,10,15, and 20 μg/l (eight replicate flasks per concentration) were also assayed. Tissue yields (fresh weight) were determined after five weeks growth. A standard curve based on the yields obtained from the kinetin control flasks was used to determine the cytokinin activities recovered from the tRNA samples, expressed as kinetin equivalents (KE); i.e., micrograms of kinetin required to give the same growth stimulation as the test sample.

RESULTS

Base Composition Analyses

The genetic selection scheme which generated several distinct suppressor inactivating, or "anti-suppressor" mutations, was designed to screen for lesions in enzymes responsible for tRNA modification. The nucleoside composition of unfractionated tRNA extracted from each mutant was first characterized by chromatography of tritium labelled nucleoside derivatives. There were no significant differences in nucleoside composition with respect to those nucleosides detectable by this procedure (Table 1). There were also no significant differences in tRNA nucleoside composition for six other distinct antisuppressor mutants (*mod1-1, mod2-1, mod3-1, mod4-1, mod6-1 and moda-1*) with phenotypes similar to the *mod5-1* mutant[30].

Quantitation of i^6A is not amenable to the thin layer pro-

TABLE 1. Base Composition of Unfractionated tRNA from *mod5-1* and Nonmutant Strains[a]

Nucleoside	Percent Total	
	mod5-1[b]	Nonmutant[c]
Adenosine (A)	19.1	18.9
Cytidine (C)	24.9	25.4
Guanosine (G)	27.2	26.5
Uridine (U)	17.0	17.0
Inosine	0.49	0.46
1-MethylA	0.52	0.66
N^6-(*N*-Threonylcarbonyl)A	0.35	0.33
5-MethylC	0.91	1.17
1-MethylG	0.63	0.70
N^2-MethylG	0.89	0.80
N^2,N^2-DimethylG	0.57	0.60
N^7-MethylG	0.57	0.48
Ribothymidine	0.81	0.74
Pseudouridine	3.19	2.94
Dihydrouridine	2.84	3.21

[a]Values for the four major nucleosides are $\leq \pm 4\%$; values for all others are $< \pm 10\%$.

[b]Data from two analyses.

[c]Averages from duplicate analyses of five $MOD5^+$ strains related to *mod5-1*.

cedure and was facilitated by Sephadex LH20 chromatography. The elution profile in Figure 1A is representative of those recorded for tRNA digests from several nonmutant controls (with and without *SUP7-1*) and five of six mutants. The concentration of i^6A in unfractionated tRNA from these strains ranged from 60 mmoles per mole of tRNA to 120 mmoles per mole of tRNA. The elution profile in Figure 1B is representative of those recorded for tRNA digests from several different strains, each carrying the antisuppressor mutation, *mod5-1*. Unfractionated tRNA from each of these strains yielded approximately 1.2 mmoles of i^6A per mole of tRNA, or about 1.5% of the above concentrations.

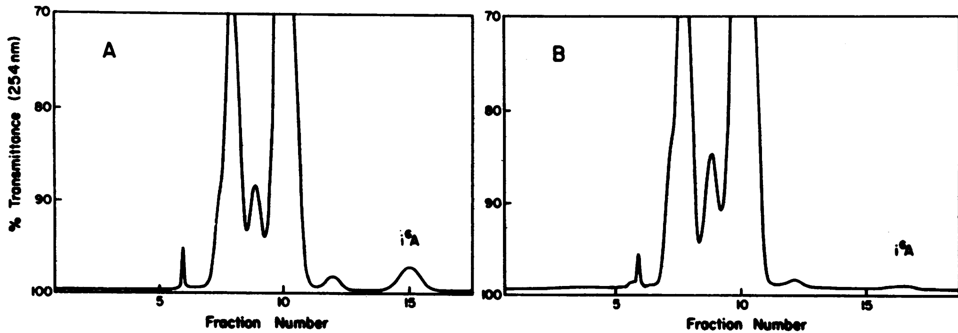
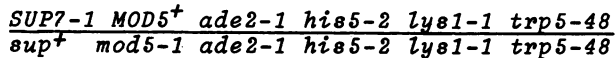


Figure 1. Separation of isopentenyladenosine on Sephadex LH20. (A) Transfer RNA digest from nonmutant control; (B) Transfer RNA digest from *mod5-1*.

Correlation Between Antisuppressor Phenotype and i^6A Reduction

To confirm the correlation of antisuppressor phenotype with i^6A deficiency, tRNA was isolated from 14 different yeast segregants identified genetically as *mod5-1* and from five nonmutant segregants. All 19 segregants were derived from the same cross by dissection of individual yeast asci. Segregants resulted from the diploid:



$$\frac{SUP7\text{-}1\ MOD5^{+}\ ade2\text{-}1\ his5\text{-}2\ lys1\text{-}1\ trp5\text{-}48}{sup^{+}\ mod5\text{-}1\ ade2\text{-}1\ his5\text{-}2\ lys1\text{-}1\ trp5\text{-}48}$$

(Although a diploid homozygous for *SUP7-1* would have facilitated the identification of *mod5-1* segregants, such diploids sporulated poorly.) *SUP7-1 mod5-1* segregants required adenine and were canavanine resistant; *SUP7-1* $MOD5^+$ segregants were adenine independent and canavanine sensitive. In the absence of *SUP7-1*, the identification of *mod5-1* could not be directly made because mutant and nonmutant strains are indistinguishable in a genetic background lacking a Class I suppressor. Nonsuppressor segregants were therefore crossed to *SUP7-1 mod5-1* strains of opposite mating type, and the phenotypes of the resulting diploids were analyzed. In the case of sup^+ *mod5-1* segregants, diploids heterozygous for *SUP7-1* and homozygous for *mod5-1* proved to be adenine and histidine requiring; in the case of sup^+ $MOD5^+$ segregants, diploids heterozygous for both *SUP7-1* and *mod5-1* proved to be adenine requiring but histidine independent.

Transfer RNA from 13 of the *mod5-1* segregants was combined into three pools, such that two pools each contained tRNA from four different segregants, and the third pool contained tRNA from the remaining five segregants. If tRNA from any single mutant segregant contained the normal complement of i^6A, the concentration of i^6A in the pooled sample was expected to be at least 20% of that in the nonmutants. Nonmutant tRNA was not pooled. The concentrations of i^6A in the pooled samples were approximately the same as that in the unpooled tRNA isolated from a single *mod5-1* segregant (Table 2), approximately 1.5% of nonmutant levels. These results indicate that the mutant segregants are uniformly deficient in i^6A, and that the loss of suppressor function is correlated with the reduction of i^6A.

Chromatographic Behavior of i^6A Deficient tRNAs

Because of the strongly hydrophobic character of i^6A, the chromatographic behavior of those tRNA species normally containing this nucleoside should be greatly altered when the modification is absent. Transfer RNA species containing i^6A are significantly retarded on BD-cellulose subjected to a NaCl gradient[27]. Figure 2 illustrates that $tRNA^{Tyr}$ (Fig. 2A) and $tRNA^{Ser}$ (Fig. 2B)

TABLE 2. Levels of i^6A in tRNA from yeast segregants genetically defined with respect to *mod5-1*.

tRNA source	i^6A (mmoles/mole tRNA)
a (*mod5*)	1.1
b (*mod5*)	1.2
c (*mod5*)	1.4
1 (*mod5*)	1.2
2 (nonmutant)	82
3 (nonmutant)	118
4 (nonmutant)	81
5 (nonmutant)	99

For samples a and c, tRNA from each of four different *mod5-1* strains was combined; for sample b, tRNA from five different *mod5-1* strains was combined. In all other determinations, tRNA from different individual segregants derived from the same cross was used.

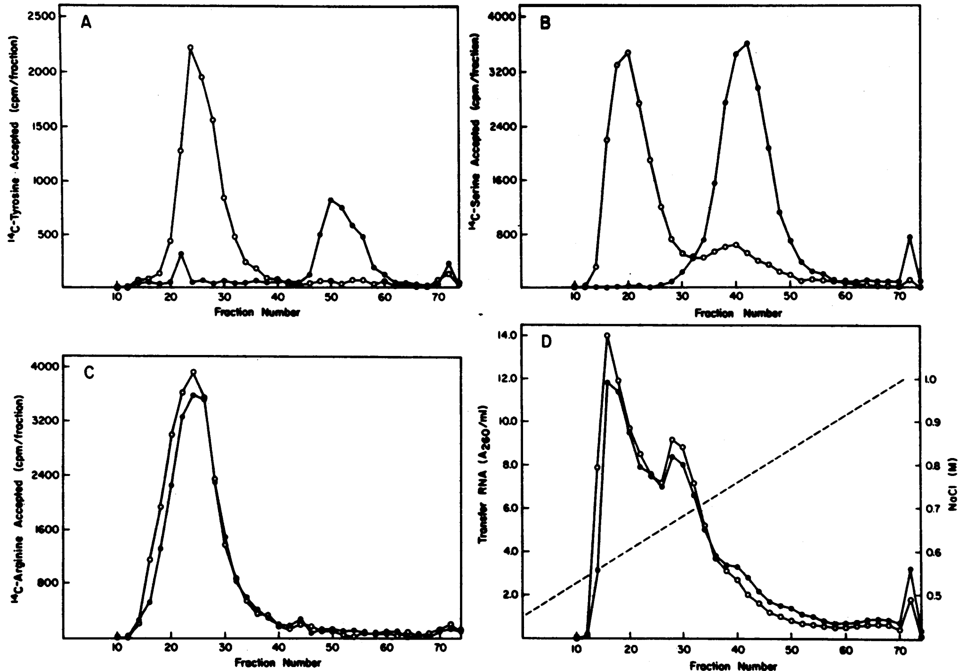


Figure 2. Comparison of tRNAs derived from nonmutant and *mod5-1* cells by BD-cellulose chromatography. (A) Tyrosine acceptance; (B) Serine acceptance; (C) Arginine acceptance; (D) Absorbance profile. (●——●) tRNA derived from the nonmutant; (o——o) tRNA derived from the *mod5-1* mutant; (---) NaCl concentration.

species from the *mod5-1* mutant elute from the BD-cellulose column much earlier than the fully modified tRNA species from a nonmutant strain. The behavior of arginine accepting tRNA species, which do not contain i^6A, is not influenced by the presence of the *mod5-1* mutation (Fig. 2C). There was also no change in the chromatographic behavior of $tRNA^{Phe}$, which contains base Y adjacent to the 3' end of the anticodon (not shown). The major peak of tyrosine acceptor activity for the nonmutant in Figure 2A is considerably smaller than the peak for the mutant because tyrosine charging is inhibited by high salt concentrations present in assays of late column fractions[24]. The concentrations of the tyrosine acceptor activities in unfractionated tRNA from mutant and nonmutant strains are, in fact, comparable (see below). The trace of unmodified $tRNA^{Tyr}$ in the nonmutant

preparation most probably reflects incomplete isopentenylation in logarithmic phase cells. Although fully modified $tRNA^{Ser}$ elutes from BD-cellulose in a single peak, this peak contains several unresolved isoaccepting species. At least one of these that expected to translate AGU and AGC codons, should not normally contain i^6A. In Figure 2B, most but not all of the serine accepting species are shifted in the mutant preparation. The small amount of unshifted material is most likely due to this $tRNA^{Ser}$. The shift in the elution profiles of some species to earlier positions is also reflected in the absorbance profile (Fig. 2D). Twenty per cent of the tRNA from the mutant elutes from the column after fraction 34, while 27% of the tRNA from the nonmutant elutes after this fraction. This 7% difference is rougly equivalent to the sum of tyrosine, serine, and cysteine tRNAs found in unfractionated tRNA from yeast.

Cytokinin Activity in i^6A Deficient tRNA

Isopentenyladenosine and its related derivatives promote cell division or cytokinesis in plants[31,32]. Although these cytokinins, as they are called, are present in virtually all organisms[1], their hormonal activity has been conclusively demonstrated only in higher plants[31,32]. It has been suggested that tRNA is the primary, if not sole, source of naturally occurring cytokinins[29]. The only tRNA constituent in yeast known to have cytokinin activity is i^6A[33,34], and a reduction in cytokinin activity should parallel the reduction in i^6A.

To further corroborate the reduction of i^6A and to determine if the i^6A deficient tRNA exhibits significant cytokinin activity, mixtures of bases generated from unfractionated tRNA were assayed for their ability to stimulate plant tissue growth. The results of the tobacco tissue culture bioassay are presented in Table 3. Transfer RNA from the nonmutant strain contained 0.63 KE per mg tRNA. Because isopentenyladenine is ten times as potent as kinetin in this assay[35], this represents 62 nanograms of i^6A per mg tRNA or 9.2 mmoles of i^6A per mole of tRNA. Transfer RNA from the *mod5-1* strain contained 0.04 KE per mg tRNA or 0.6 mmoles of i^6A per mole of tRNA, 6.5% of that in the nonmutant. These concentrations are lower than those determined spectrophotometrically on Sephadex LH20 purified i^6A, but the

TABLE 3. Cytokinin activity in tRNA from nonmutant and *mod5-1* strains

Nonmutant strain									
tRNA conc. (mg/l)	350	70	14	2.8	0.56	0.11			
Ave. fresh weight (gm/flask)	5.88	7.39	5.75	1.10	0.43	NG[a]			
Cytokinin activity (KE/mg tRNA)	-	-	0.62	-	-	-			
***mod5-1* strain**									
tRNA conc. (mg/l)	375	75	15	3	0.60	0.12			
Ave. fresh weight (gm/flask)	8.00	2.45	0.41	NG[a]	NG[a]	NG[a]			
Cytokinin activity (KE/mg tRNA)	-	0.04	-	-	-	-			
Kinetin standards									
Kinetin conc. (μg/ml)	0	1	2	3	5	7	10	15	20
Ave. fresh weight (gm/flask)	0.6	1.1	1.9	2.6	4.1	4.2	6.3	7.4	6.4

[a]No growth

relationship between mutant and nonmutant i^6A levels is still maintained. The low values obtained from the bioassay are probably the result of chemical degradation during reactions required for sample preparation prior to the assay.

Amino Acid Acceptance

Unfractionated tRNA from *mod5-1* and nonmutant cells was assayed for tyrosine and serine acceptor activities. Transfer RNA from nonmutant cells accepted 26 and 53 pmoles/A_{260} unit of tRNA for tyrosine and serine, respectively. From *mod5-1* cells, tRNA accepted 24 and 53 pmoles/A_{260} unit of tRNA for tyrosine and serine, respectively. Thus, the loss of i^6A has no apparent effect on the level of amino acid charging of two tRNAs that normally contain this modification.

DISCUSSION

The *mod5-1* mutation is recessive to the wild-type allele, segregates as a nuclear gene, and is unlinked to *SUP7-1*. Other than reducing suppressor efficiency, the mutation appears to have little effect on its host, although *mod5-1* homozygotes failed to sporulate. Mutant strains grow as well as nonmutants in both complex (peptone, yeast extract, and dextrose) and simple (vitamins, salts, dextrose, and nutritional supplements) media at both moderate (28°C) and elevated (37°C) temperatures.

Generation times and cell yields do not differ significantly (Laten and Gorman, unpublished).

The application of <u>in vitro</u> findings to the problems of <u>in vivo</u> functions is limited by the difficulty of duplicating a cellular environment in a reaction mixture. Unlike previous efforts to determine the function of the i^6A modification in cell-free systems[3-8], we have generated a mutant that affords the opportunity to study the effect of i^6A deficiency in growing cells. That *mod5-1* cells apparently grow as well as nonmutant cells under conditions where suppression is not required for growth suggests that the i^6A deficient tRNAs do not limit the rate of protein synthesis in the mutant. The efficiency of suppression by *SUP7-1*, however, may be a more sensitive indicator of the limitations imposed by i^6A deficient tRNA with respect to protein synthesis. The suppressor tRNA constitutes only a fraction of the total $tRNA^{Tyr}$ and must compete with termination factors responding to the nonsense mutation. Thus, suppressor tRNA concentration could be growth limiting, or nearly so, under conditions requiring suppressor function, and impairment of tRNA function would visibly affect growth under these conditions. The reduced suppressor efficiency in the mutant, as measured by growth on selective media, most probably results from the impaired functioning of the i^6A deficient suppressor tRNA in protein synthesis. It does not result from decreased levels of i^6A deficient tRNA. Unfractionated tRNA from the mutant contains as much $tRNA^{Tyr}$ and $tRNA^{Ser}$ as nonmutant tRNA. The i^6A deficiency therefore does not affect the relative rates of tRNA synthesis and degradation. In addition, *mod5-1* cells do not differ from nonmutant cells in accumulation of tRNA precursors (Park, unpublished). Our finding that the acceptor activities of the above two species are not reduced by the loss of i^6A suggests that the translational deficiency <u>in vivo</u> is related to tRNA interactions subsequent to aminoacylation (ribosomal binding and codon-anticodon or elongation factor interactions). This conclusion is in agreement with the findings of Fittler and Hall[3], Furuichi <u>et al</u>.[4], Gefter and Russell[5], and Grosjean <u>et al</u>.[6]. On the other hand, the negative findings of Litwack and Peterkofsky[7] and Kimball and Söll[8] are not unexpected

in view of the subtle in vivo consequences of the reduction in i^6A.

An i^6A deficient mutant has recently been found among a collection of antisuppressor mutants of Schizosaccharomyces pombe (G. Vögeli, personal communication). The mutation appears to be analogous to *mod5-1*.

There were no significant differences in tRNA nucleoside composition among the six other distinct antisuppressor mutants (and nonmutant controls) with respect to the nucleosides listed in Table 1. There were also no significant differences in the nucleoside composition of $tRNA^{Tyr}$ among these strains with respect to these same nucleosides[30]. This limits but does not preclude the possibility that these mutants are also defective in tRNA modifying enzymes. Undermodification of a tRNA species other than that responsible for suppression may effect antisuppression (J. Roth, personal communication), and analysis of global tRNA may not detect undermodification of a small number of tRNA species.

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