*MUTANTS OF YEAST WITH TEMPERATURE-SENSITIVE ISOLEUCYL-TRNA SYNTHETASES**

By Leland H. Hartwell and Calvin S. McLaughlin

DEPARTMENT OF MOLECULAR AND CELL BIOLOGY, UNIVERSITY OF CALIFORNIA, IRVINE

Communicated by Renato Dulbecco, December 1, 1967

In order to investigate the mechanisms of macromolecule synthesis and their regulation in a eucaryotic organism, we have undertaken an investigation of temperature-sensitive mutants in yeast. The isolation of temperature-sensitive mutants of *Saccharomyces cerevisiae* and their characterization with respect to macromolecule synthesis, morphology, and cell division at the nonpermissive temperature has been described previously.[1] Such mutants display a variety of phenotypes, including those consistent with a primary lesion, in one of the following processes: protein synthesis, RNA synthesis, DNA synthesis, cell-wall formation, and cell division. We wish to report biochemical and genetic experiments which indicate that two of the mutants from the protein synthesis class, $ts^{-}341$ and $ts^{-}443$, have single, allelic temperature-sensitive (ts^{-}) mutations which result in a thermolabile isoleucyl-tRNA synthetase enzyme.

Materials and Methods.—Yeast strains: The following heterothallic, haploid strains of *Saccharomyces cerevisiae* were used in this investigation: A346A (α ga_1 hi_7 tr_1 ur_1 ad_1 ad_2 ly_2 ty_1), X1069-2D (α ad_1 hi_4 le_2 thr_4 me_2 tr_5 ur_1), 1505-3B (α ga_3 ga_7 hi_5 ur_3 me_{10} ly_2 ly_{11}), and 2262-2A (α ga_1 hi_5 ur_1 ad_1 le_2 ly_{11}). All strains were obtained from the collection of Drs. Roman and Hawthorne at the University of Washington, Seattle, except for strain X1069-2D which was obtained from Dr. Mortimer at the University of California, Berkeley. The mutants $ts^{-}341$ and $ts^{-}443$ were derived from strain A364A by mutagenesis with nitrosoguanidine.[1]

Media: The enriched liquid medium, YM-1, was described previously.[1] To score marker segregation, synthetic medium was used containing 6.7 gm/liter yeast nitrogen base without amino acids (Difco), 10 gm/liter glucose, 20 gm/liter agar, and whatever biochemical compounds were needed to supplement auxotrophic requirements.

Chemicals: Adenosine triphosphate (ATP) and cytidine 5′-triphosphate (CTP) were purchased from Sigma Chemical Company. Yeast tRNA was obtained from General Biochemicals Co. Radioactive amino acids were purchased from Schwarz BioResearch, Inc. C^{14} amino acids had a specific activity of 50 mc/mmole and a concentration of 1 mM. H^3 methionine from Schwarz was adjusted to 125 mc/mmole, 1 mM concentration.

Preparation of aminoacyl-tRNA synthetases: Yeast were grown in medium YM-1 at 23° to a density of 100 Klett units. The cells were harvested by centrifugation, and spheroplast preparations[2] were lysed in a 0.01 *M* Tris-maleate pH 7.6, 0.0005 *M* magnesium acetate, 0.02 *M* KCl buffer by homogenization. The extract was centrifuged at 27,000 *g* for 20 min, and the supernatant was centrifuged at 229,000 *g* for 70 min. The ribosomal pellet was discarded and the supernatant was dialyzed overnight against a 0.01 *M* tris-acetate pH 7.6, 0.0005 *M* magnesium acetate, 0.02 *M* KCl buffer.

Assay of aminoacyl-tRNA synthetase activity: The formation of aminoacyl-tRNA was assayed on DEAE paper (Whatman DE81).[3] Each 100 μliter incubation contained in a 0.05 *M* tris-acetate pH 7.6, 0.05 *M* magnesium acetate, 0.02 *M* KCl buffer (buffer A), 0.5 μmole of ATP, 0.05 μmole of CTP, 5 mμmoles of C^{14} or H^3 amino acid, 0.2 mg of yeast tRNA, 0.1 mg of gelatin, and 0.1 mg of enzyme protein. The incubation was carried out for 30 min at the specified temperature. The amount of isoleucyl-tRNA formed is proportional to the enzyme concentration up to concentrations of 0.2 mg of protein per incubation. Protein was determined by the Lowry procedure.[4]

Genetic techniques: The techniques used for the mating of haploid strains, the isolation and sporulation of the resulting diploids, and the analysis of tetrads by dissection were those described by Hawthorne and Mortimer.[5] Since all the ts$^-$ mutants are *a* in mating type, complementation studies between mutant ts$^-$341 and the other ts$^-$ mutants were carried out by mating all ts$^-$ mutants with a haploid strain carrying the ts$^-$341 mutation, the α allele for mating type, and the proper nutritional markers for prototrophic selection. This ts$^-$341 α strain was prepared by sporulation of a diploid resulting from the mating, ts$^-$341 *a* $\times$ X1069-2D α. Only complete tetrads are reported in the tables.

Results.—The two mutants on which we are reporting in this paper—mutants ts$^-$341 and ts$^-$443—were suspected of having lesions in the protein synthetic machinery due to their rapid cessation of protein synthesis after a shift from 23°, the permissive temperature, to 36°, the nonpermissive temperature. Both mutants synthesize less than 10 per cent as much protein during a three-hour period at 36° as does the ts$^+$ parent strain, A364A. Complementation studies demonstrated that these two mutants have lesions in the same gene. The haploid mutant ts$^-$341 was mated with 367 other ts$^-$ mutants and the resulting diploids were tested for temperature-sensitivity. Only one recessive mutant ts$^-$443 was found not to complement with mutant ts$^-$341. Since these mutants were isolated from different mutagen-treated cultures, they are independent in origin.

Crosses were performed and tetrads dissected in order to test for allelism between mutants ts$^-$341 and ts$^-$443 (Table 1). In crosses between the two

TABLE 1. *Tetrad analysis of crosses with mutants ts$^-$341 and ts$^-$443.*

Cross	Number of Tetrads with a ts$^-$:ts$^+$ Segregation Pattern of: 4:0	3:1	2:2	1:3	0:4
ts-341 $\times$ 2262-2A	0	7	87	2	0
ts-443 $\times$ 2262-2A	0	3	16	2	0
ts-341 $\times$ ts-443	27	0	0	0	0

mutants and strain 2262-2A (ts$^+$), temperature-sensitivity segregated in a 2:2 fashion in the great majority of the tetrads, indicating that the two mutants probably each carry a single ts$^-$ mutation. In the cross ts$^-$341 $\times$ ts$^-$443, a 4:0 segregation for ts$^-$:ts$^+$ was observed for all 27 tetrads, indicating that the two mutations are closely linked. The two mutations are not identical, however, since they recombine to give ts$^+$ progeny. In a random spore preparation from the cross ts$^-$341 $\times$ ts$^-$443, approximately 2×10^{-5} of the progeny were ts$^+$.

Aminoacyl-tRNA synthetase activity: Cell-free extracts from mutant ts$^-$341 and the parent strain, A364A (ts$^+$), were assayed for their ability to catalyze the transfer of C^{14} amino acids onto tRNA. Sixteen different amino acids were tested. The extracts were incubated for 30 minutes at 35° to allow for the inactivation of a temperature-sensitive enzyme and then assayed at 25°. The activities of the various aminoacyl-tRNA synthetases are recorded in Table 2. Only the isoleucyl-tRNA synthetase activity is seen to be strikingly low in the extract from mutant ts$^-$341 as compared to the activity in the extract from A364A. If the preincubation at 35° is omitted, the level of isoleucyl-tRNA synthetase activity is nearly the same in extracts of A364A and ts$^-$341 (see Fig. 1).

A study was undertaken of the isoleucyl-tRNA synthetase activity in extracts of the mutants ts^{-}341 and ts^{-}443 and of the parent strain A364A (ts^{+}). The activity of the enzyme was examined at a variety of temperatures (Fig. 1). The initial rate of formation of isoleucyl-tRNA by extracts of A364A increased with increasing temperature, as might be expected. However, the ultimate amount of isoleucyl-tRNA formed at 40°, 45°, and 50° is less than that at 35°,

TABLE 2. *Survey of aminoacyl-tRNA synthetase activities in extracts of mutant ts^{-}341 and strain A364A.*

	Mμmoles of Aminoacyl-tRNA Formed/Mg of Protein/Hr at 25°		
Amino acid	A364A	ts $^{-}$341	Ratio ts^{-}341/A364A
Alanine	2.70	2.78	1.03
Arginine	4.05	3.69	0.92
Aspartic acid	3.67	3.96	1.08
Glutamic acid	2.14	1.83	0.85
Glycine	0.59	0.40	0.68
Histidine	1.48	1.75	1.18
Isoleucine	3.35	0.45	0.13
Leucine	1.40	1.18	0.85
Lysine	4.51	3.54	0.79
Methionine	0.91	0.42	0.46
Phenylalanine	3.06	2.50	0.82
Proline	1.54	1.35	0.88
Serine	1.72	1.08	0.63
Threonine	2.04	1.91	0.94
Tyrosine	2.49	2.11	0.85
Valine	6.00	3.99	0.67

The conditions of the assay are as described under *Materials and Methods* except that the gelatin is omitted and the enzyme preparations were preincubated in buffer A for 30 min at 35°.

indicating that some inactivation of the enzyme occurs at these higher temperatures. Nevertheless, the final extent of charging at 50° is as much as 50 per cent of that at 35°. The mutant extracts display a different pattern when compared with the parent strain and with each other. The initial rate of formation of isoleucyl-tRNA by extracts of ts^{-}443 is almost the same at 25° and at 35°, while the rate at 40° is lower. Extracts from both ts^{-}443 and ts^{-}341 show hardly any charging at 45° and 50°. Mutant ts^{-}341 shows a progressive decrease in the initial rate of charging as the temperature is increased from 25° to 35° to 40° in contrast to the pattern with ts^{-}443 and A364A. Thus, the two mutants have a thermolabile isoleucyl-tRNA synthetase. The activities of the three extracts are very similar at 25° near the permissive temperature (23°), while the pattern of thermolability begins to express itself in the mutants near the nonpermissive temperature (36°). The fact that the temperature profile of activity is different for the two mutants suggests that the sites of the two lesions are different, a conclusion which is in agreement with the recombination data.

The low activity of the isoleucyl-tRNA synthetase in extracts of the mutants at high temperature could be due to an instability of the enzyme, the inability of the enzyme to charge tRNA, or to the presence of an inhibitor. Although the

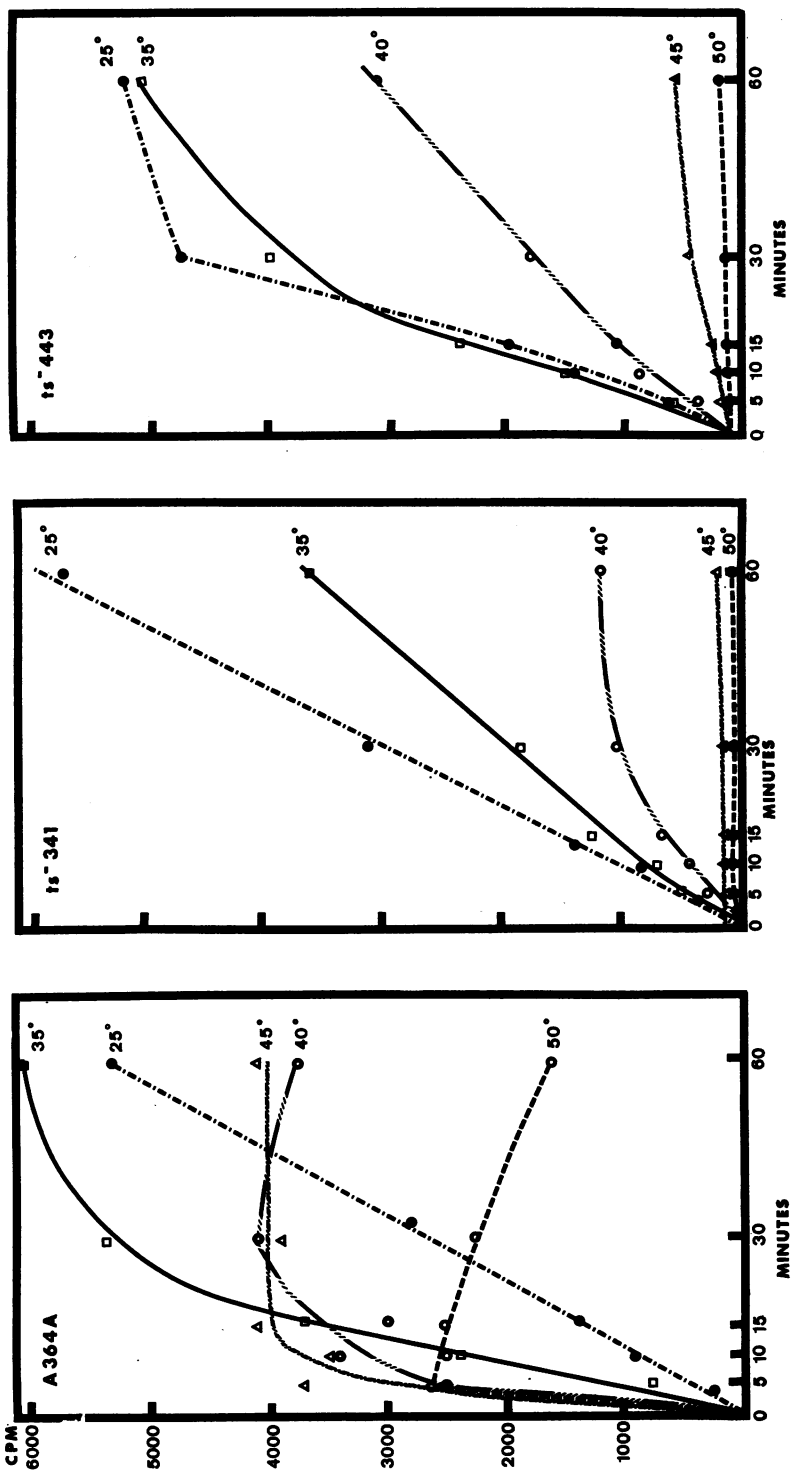

FIG. 1.—Effect of temperature on the kinetics of isoleucyl-tRNA formation. Counts per min of C^{14}-isoleucine bound to tRNA as a function of time by extracts of A364A, ts⁻341, and ts⁻443 incubated at various temperatures.

latter possibility is rather unlikely since both mutations are recessive, further evidence on this point was sought by assaying mixed extracts at high and low temperature (Table 3). At all temperatures investigated a mixed extract containing enzyme from both the parent strain, A364A (ts^+), and the mutant, ts^-341, displays at least the activity expected from the sum of the separate activities. We conclude that an inhibitor is not responsible for the observed inactivity of the mutant extracts at high temperature. Other experiments[6]

TABLE 3. *In vitro test for a temperature-induced inhibitor.*

Extract source	Isoleucyl-tRNA Formation in 30 Minutes (cpm) 25°	40°	45°	50°
1. ts-341 (50 μg)	900	43	31	22
2. A364A (50 μg)	2700	1772	997	288
3. A364A (50 μg) ts-341 (50 μg)	3700	2441	1361	431
4. Sum of 1 and 2.	3600	1815	1028	300

The assays were carried out as described under *Materials and Methods* except that each incubation mixture contained only 50 μg of the designated crude aminoacyl-tRNA synthetase preparation.

TABLE 4. *Segregation of isoleucyl-tRNA synthetase activity in dissected asci of the cross $ts^-341 \times 1505\text{-}3B$ (ts^+).*

Tetrad	Spore	Me	Le	Ty	ts	Isoleucyl-tRNA Synthetase Activity (cpm of ileu-tRNA formed/30-min incubation) 25°	50°
I	1	+	−	−	+	1300	390
	2	−	+	−	−	2300	60
	3	−	+	+	−	2400	40
	4	+	−	+	+	1950	620
II	1	−	+	+	−	850	20
	2	−	+	−	+	2300	700
	3	+	−	−	−	2100	20
	4	+	−	+	+	2400	510
III	1	+	−	+	+	3200	800
	2	−	+	−	−	1400	20
	3	−	+	−	−	1900	20
	4	+	−	+	+	2400	750
IV	1	+	−	−	+	2500	450
	2	+	−	−	−	3200	20
	3	−	+	+	+	1000	400
	4	−	+	+	−	1750	20

involving a preincubation of the enzyme at 45° followed by assay at 25° indicate that the irreversible thermal inactivation of the enzyme at 45° is not sufficiently rapid to account for the inability of the enzyme to charge tRNA at 45°. It is therefore apparent that the mutant enzyme undergoes a rapid reversible inhibition of activity at 45° as well as a slow, irreversible thermal inactivation.

Segregation of enzyme activity at meiosis: The segregation of the gene for isoleucyl-tRNA synthetase activity at meiosis was correlated with the segregation of ts^- in order to see if the observed alteration in enzyme activity is closely linked to the mutation which affects growth at 36°. The mutant, ts^-341, was

mated with a ts^+ strain, 1505-3B, and the resulting diploid was sporulated. The asci, each containing four haploid spores, were dissected by micromanipulation, and the clone which grew up from each spore was assayed for enzyme activity. In this case, the assays were carried out at 25° and 50° (Table 4). Extracts from all clones displayed significant isoleucyl-tRNA synthetase activity at 25°; however, at 50° the extracts prepared from the ts^+ clones are 10- to 20-fold higher in activity than the extracts prepared from the ts^- clones. Thus, in all four asci the altered isoleucyl-tRNA synthetase activity segregated in an identical pattern to the gene determining inability to grow at 36°. The chance of obtaining an identical segregation pattern in all four asci for two genes that are located on different chromosomes or even far apart on the same chromosome is 1 in 1296. The ts^- mutation and the mutation producing an altered isoleucyl-tRNA synthetase enzyme are, therefore, closely linked on the same chromosome. A haploid yeast cell contains at least 14 chromosomes.[7] Other nutritional markers segregating in this cross, me^-, le^-, ty^-, segregated independently of the ts^- marker. The conclusion appears warranted that mutants ts^-341 and ts^-443 carry a single temperature-sensitive mutation in a structural gene for the isoleucyl-tRNA synthetase enzyme.

Discussion.—Aminoacyl-tRNA synthetase mutants of *E. coli* have been described.[8, 9] In these mutants protein synthesis is rapidly inhibited after a shift to the nonpermissive temperature. This behavior provides direct evidence for the role of aminoacyl-tRNA synthetases in protein synthesis *in vivo*. Furthermore, RNA synthesis is also inhibited, indicating that the presence of a complete complement of amino acids is not sufficient to allow RNA synthesis but that these amino acids must at least be attached to their respective tRNA's. Furthermore, upon *in vivo* thermal inactivation of the valyl-tRNA synthetase of *E. coli*, derepression of the valine biosynthetic enzymes is observed.[10] Therefore, free valine is apparently not the repressor of this enzyme but the repressor is formed at or beyond the activation of valine onto tRNA.

The mutants of yeast which we have described here allow one to attack these same questions in a eucaryotic organism. The data presented indicate that protein synthesis is rapidly inhibited in this mutant after a shift to the nonpermissive temperature. Thus, the isoleucyl-tRNA synthetase is an obligatory component of the protein synthetic machinery under *in vivo* conditions in yeast. Furthermore, since a single mutation destroys greater than 99 per cent of the isoleucyl-tRNA synthetase activity (assayed *in vitro* at 50°), it is likely that the haploid yeast cell contains only a single species of this enzyme. The question of the role of amino acid activation in the control of RNA synthesis and in the repression of the isoleucine biosynthetic enzymes will be taken up in another publication. Finally, this mutant allows an approach to a question which is unique to eucaryotic organisms. Recent work by Barnett *et al.*[11] indicates that the mitochondria of *Neurospora crassa* have aminoacyl-tRNA synthetase enzymes which have different properties from those of the rest of the cytoplasm. The isoleucyl-tRNA synthetase mutants of yeast offer an obvious and unambiguous way of confirming these data in another eucaryotic organism.

Summary.—Two temperature-sensitive mutants of yeast are described which

have lesions in a structural gene for the isoleucyl-tRNA synthetase enzyme. These mutations result in a thermolabile isoleucyl-tRNA synthetase enzyme which is active *in vitro* at the permissive temperature. A haploid yeast cell probably contains a single species of isoleucyl-tRNA synthetase, and this enzyme is an obligatory component of the protein-synthetic system *in vivo*.

The authors wish to acknowledge the competent technical assistance of Pamela Michaels and Don Sanfilippo. One of us (L.H.) is indebted to Dr. Donald Hawthorne for his instruction in the art of tetrad dissection.

* This work was supported by research grant GM 13304 from the U.S. Public Health Service and grant GB 4828 from the National Science Foundation.

[1] Hartwell, L., *J. Bacteriol.*, **93**, 1662 (1967).
[2] Hutchison, H. T., and L. H. Hartwell, *J. Bacteriol.*, **94**, 1697 (1967).
[3] Ingram, V. M., and J. G. Pierce, *Biochemistry*, **1**, 580 (1962).
[4] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[5] Hawthorne, D. C., and R. K. Mortimer, *Genetics*, **45**, 1085 (1960).
[6] McLaughlin, C. S., and L. H. Hartwell, manuscript in preparation.
[7] Mortimer, R. K., and D. C. Hawthorne, *Genetics*, **53**, 165 (1966).
[8] Eidlic, L., and F. C. Neidhardt, *J. Bacteriol.*, **89**, 706 (1965).
[9] Yaniv, M., F. Jacob, and F. Gros, *Bull. Soc. Chim. Biol.*, **47**, 1609 (1965).
[10] Eidlic, E., and F. C. Neidhardt, these PROCEEDINGS, **53**, 539 (1965).
[11] Barnett, E. W., D. H. Brown, and J. L. Epler, these PROCEEDINGS, **57**, 1775 (1967).