NONSENSE MUTATION IN THE REGULATORY GENE *ETH2* INVOLVED IN METHIONINE BIOSYNTHESIS IN *SACCHAROMYCES CEREVISIAE**

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ABSTRACT

Ethionine-resistant mutants, mapping at the locus *eth2*—the product of which is involved in pleiotropic regulation of methionine biosynthesis—have been isolated in a strain carrying five ochre nonsense mutations. Selection for nonsense suppressors in such a strain led to characterization of several allele-specific but gene non-specific suppressors which are active on the recessive heteroallele *eth2-2* (resulting in partial recovery of sensitivity toward ethionine) as well as on the five other suppressible alleles. Two of these suppressors are unlinked to the *eth2* gene and either dominant or semi-dominant. It is concluded that the mutation *eth2-2* resulted in a nonsense codon. Enzyme studies indicate that this mutation results in a complete absence of an active product of gene *eth2*, in contrast with the effect of a former mutation *eth2-1* which was interpreted as leading to a modified product of this gene (CHEREST, SURDIN-KERJAN and DE ROBICHON-SZULMAJSTER 1971). This conclusion is based on the absence of repressibility of methionine group I enzymes and the observation that in a heteroallelic diploid, *eth2-1* expression is not masked by *eth2-2*. The nonsense suppressors studied lead to at least partial recovery of repressibility of methionine group I enzymes. All these results support the idea that the product of gene *ETH2* is an aporepressor protein.

IN *Saccharomyces cerevisiae*, synthesis of the methionine biosynthetic enzymes is subject to a pleiotropic regulatory system. The mutation *eth2-1*† in the regulatory gene *ETH2* previously studied was found recessive to the wild-type allele and resulted in lowered repressibility of four methionine enzymes. Structural genes *met2* and *met8* corresponding to two of these enzymes (de ROBICHON-SZULMAJSTER and CHEREST 1967; CHEREST, EICHLER and DE ROBICHON-SZULMAJSTER, 1969) map independently (MORTIMER and HAWTHORNE, 1969) and are unlinked to the *ETH2* gene (CHEREST *et al.* 1969). This situation can be compared to regulation of arginine biosynthetic enzymes in *E. coli*, where five clusters respond to the regulatory gene *argR* (GORINI, GUNDERSON and BURGER 1961).

In a thermosensitive mutant with an impaired methionyl-tRNA synthetase (HARTWELL and McLAUGHLIN 1968), the genetic alteration was found unlinked with either *ETH2*, *met2* or *met8* (CHEREST *et al.* 1971). Biochemical studies with

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† The designation of alleles will be justified later.

this mutant suggested that met-tRNA might be involved as a corepressor in this pleiotropic regulatory system (CHEREST *et al.* 1971).

One could envisage that the *ETH2* gene product is either an aporepressor protein or the, yet hypothetical, "tRNA^{met} regulatory species." If suppressible nonsense mutations could be isolated in the *ETH2* gene, this would demonstrate the protein nature of its product. This kind of evidence has already been obtained for other regulatory genes such as the *trpR* gene in *Salmonella typhimurium* (MORSE and YANOFSKY 1969) and the *malT* gene in *Escherichia coli* (HATFIELD, HOFNUNG and SCHWARTZ, 1969).

Here we report the isolation of a nonsense mutation in the *ETH2* gene, which leads to complete non-repressibility of some methionine biosynthetic enzymes. Under the effect of nonsense suppressors, partial repressibility is recovered.

MATERIALS AND METHODS

Strains: The haploid strains of *Sacch.cerevisiae* used are the following: PM100:*a*; PM101:*a*; and 199M1:*α*, *trp1*‡, *arg4*‡, *his5*‡, *lys1*‡, *ade2*‡, *leu1*, from P. MEURIS's collection; CH71-1D:*a*, *ura1*; CH82-10D:*a*, *ura1*, *eth2*-1, from our own collection.

Mutagenesis: Mutants resistant to ethionine were obtained from the strain 199M1 by nitrous acid mutagenesis as follows. After 2 days growth (to stationary phase) on YPGA plates, cells were collected, suspended in sterile water and the cell number calculated from the optical density of the suspension (650nm) according to a standard curve. An aliquot of 5 ml, corresponding to $0.5-1 \times 10^7$ cells, was diluted with an equal volume of acetate buffer (0.2 M, pH 4.5) and 0.1 ml of freshly prepared 0.2 M NaNO₂ added. This suspension was incubated at 37°C for 28 min with gentle shaking. The mutagenic treatment was stopped by diluting 1 ml of the suspension into 49 ml of potassium phosphate buffer 0.1 M, pH 7.3. Cells were then centrifuged and 1×10^6 cells/plate spread on complete synthetic medium containing 1 mM DL-ethionine and the auxotrophic requirements of the strain. Survival under these conditions was 10% and mutation rate towards ethionine resistance was stimulated 40 fold.

Screening for specific resistance to ethionine: Mutations leading to ethionine resistance can affect the generalized amino acid uptake system AAP/aap. However, it was shown previously (SURDIN *et al.*, 1965) that mutations of this type lead to resistance to several non-homologous amino acid analogs. Since we were searching for nonsense regulatory mutations affecting methionine biosynthesis, it was expected that such mutations would confer resistance to high levels of ethionine. Consequently, the ethionine resistant clones were replica-plated on DL-parafluorophenylalanine 5 mM and on DL-ethionine 10 mM. Only the clones which remained resistant to this high ethionine concentration and sensitive to parafluorophenylalanine were studied further.

Crosses: When auxotrophic requirements carried by the two parental strains were complementary, diploids were isolated on minimal medium and where this method could not be used, zygotes were obtained on YPGA plates by mixing the two parental strains, previously grown for 4 hr on the same medium. After 4 hr contact between the two haploids, zygotes were isolated by classical micromanipulation. In all cases the diploid clones obtained were checked for sporulating ability.

Asci dissection: A loopful of diploid cells was suspended and incubated for three days with shaking at 28°C in sporulation medium. Tetrads were dissected after mild digestion with snail juice (Industrie Biologique Française) according to JOHNSTON and MORTIMER (1959).

Random spore isolation: After incubation in sporulation medium as described, one volume of the suspension was incubated for 24 hr at 28°C with two volumes of sterile water and one volume of snail juice activated with cysteine HCl, and then violently shaken on a Vortex mixer. One volume of the treated suspension was then concentrated by shaking with one volume of sterile

‡ These alleles are suppressible by ochre suppressors; the *leu1* allele is not.

paraffin oil. The two phases were separated by a 5 min centrifugation at $1,500 \times g$. Appropriate dilutions of the upper phase were plated on YPGA medium.

Determination of spore phenotype: After two days growth on YPGA plates, spore clones were transferred to YPGA matrix plates (A) and later replicated to screening media, according to the auxotrophic requirements involved in the cross and to the presence of ethionine resistance characters.

Two techniques were used for mating type determination. In one, the YPGA plates (A) were used again in conjunction with another YPGA plate (B) on which two lines of cells were placed at regular intervals, one for each of the opposite mating-type tester strains. These were chosen carrying auxotrophic requirements which complement the haploid clones tested. The two types of plates (A and B) were then replicated at right angles to each other on the same plate (C). After two days growth these YPGA plates (C) were again replica-plated to minimal medium. The presence of prototrophs was recorded after two days. In the other technique, when only a few sporal clones were considered, the method for obtaining zygotes described above was used, and the presence of zygotes was determined under the microscope.

Selection of revertants carrying a nonsense suppressor: From a washed suspension of each ethionine resistant strain obtained from 199M1, 1×10^7 cells were plated on single omission media, and the revertants detected were scored after seven days. Among these, multiple revertants were further characterized by replica-plating on each of the omission media, according to GILMORE (1967). Any clone revertant for at least two of the nonsense alleles present in the strain and still auxotrophic for leucine was further checked for a return towards ethionine sensitivity by replica-plating on complete synthetic medium supplemented with DL-ethionine 5 mM, 8 mM and 10 mM, and scored after two days.

Media: Minimal synthetic medium (YNB): Yeast nitrogen Base without amino-acids (Difco): 7 g; D-glucose 20 g/l.

Supplemented synthetic media: The following compounds were added, as required, to the previous minimal synthetic medium in order to satisfy the auxotrophic requirements of each strain: DL-leucine, 100 mg/l; L-arginine, 10 mg/l; L-histidine HCl, 100 mg/l; L-lysine, 40 mg/l; L-tryptophan, 20 mg/l; adenine, 40 mg/l; uracil, 20 mg/l.

Natural complete medium (YPGA): This medium contains in 1 liter: 5 g Yeast extract (Difco); 5 g Bacto peptone (Difco); 30 g D-glucose and 40 mg adenine.

When necessary, all these media were solidified by the addition of Bacto agar (Difco), 30 g/l.

Sporulation medium: contains per liter: 2.5 g Yeast extract (Difco); 1 g D-glucose; 9.8 g anhydrous K acetate; 10 mM L-lysine. Supplements corresponding to the auxotrophic requirements present in the parental strains were added at concentrations identical to those used for supplemented synthetic media.

Growth curves: Cells from YPGA liquid cultures were inoculated at 5×10^5 cells/ml into 10 ml of synthetic media in special flasks equipped with a side arm for optical determination. Optical densities were recorded at regular intervals (650 nm) until the stationary phase was reached.

Enzyme assays: Growth of organisms and preparation of cell-free extracts were as previously described (CHEREST *et al.*, 1971). A single extract made in Tris buffer 0.1 M pH 8.0 was used for all enzyme determinations.

Homoserine dehydrogenase (L-homoserine:NAD oxidoreductase E.C.1.1.1.3) was assayed as described by KARASEVITCH and DE ROBICHON-SZULMAJSTER (1963), homocysteine synthetase (acetyl homoserine sulfhydrylase) was assayed according to CHEREST, TALBOT and DE ROBICHON-SZULMAJSTER (1968) and ATP sulfurylase (ATP:sulfate adenynyl transferase E.C.2.7.7.4) according to DE VITO and DREYFUSS (1964). For determination of the last enzyme, extracts were dialyzed against 5 l of 10 mM Tris buffer pH 8.0 containing 1 mM EDTA.

Protein determination was carried out according to GORNALL, BARDAWILL and DAVID (1949) by the biuret method with bovine serum albumin as reference. Specific activities are expressed as nanomoles of product formed per min per mg protein (i.e. in 10^3 international units).

Methionine uptake: Measurements were made according to SURDIN *et al.* (1965).

Chemicals: o-acetyl-DL-homoserine was synthesized for us by M. CHEREST using the method

of SAKAMI and TOENNIS (1942). L-aspartate- β -semialdehyde was prepared according to BLACK and WRIGHT (1955). ^{14}C (methyl)-L-methionine was purchased from C.E.A., France. Amino acids, purines, pyrimidines and coenzymes used were from the best commercial sources available. Inorganic pyrophosphatase was obtained from Worthington Biochemical Corp.

RESULTS

Characterization of ethionine resistant mutants at the eth2 locus

Isolation: As described under MATERIALS AND METHODS, the strain 199M1 was mutagenized and clones resistant to 1 mM DL-ethionine were isolated. In such an experiment, 141 clones were isolated which did not show concomitant resistance to parafluorophenylalanine. Among those, 6 were found resistant also to the higher dose of DL-ethionine tested, i.e. 10 mM.

Recessivity of the ethionine resistance character: The six clones were crossed with the haploid sensitive strain CH71-1D and all the resulting diploids were found sensitive to 1 mM DL-ethionine. These six diploids were sporulated and monogenic segregation of the ethionine resistance character(s) was observed. It was then concluded that the character borne by each of the six resistant haploids was due to a single recessive mutation.

Phenotype of diploids between strains carrying the eth2-1 allele and the other ethionine resistant character: The six ethionine resistant strains were crossed with CH82-10D (strain carrying the *eth2-1* allele previously studied (CHEREST *et al.* 1969, 1971), resistant to 1 mM DL-ethionine) and the resulting diploids tested on 1 mM DL-ethionine. Among those, two were found sensitive, two had an intermediate phenotype and two were found resistant. The six diploids were sporulated. Segregation of ethionine resistance was digenic for 5 of these diploids. No ethionine-sensitive segregants were recovered from the sixth one, involving 199M1-102 (see further).

Methionine uptake: No difference was observed between a wild-type haploid strain and 199M1-102.

Segregation of diploids involving the resistant mutant 199M1-102: Table 1 shows the segregation pattern of the seven auxotrophic characters observed for two diploids which involved the ethionine resistant strain 199M1-102 and two sensitive strains CH71-1D and PM100 (*ETH2*). It can be seen that all the auxotrophic characters as well as the ethionine resistance character present in the cross, segregate monogenically.

The allelism between the ethionine resistance characters of strains CH82-10D and 199M1-102, is shown in Table 2. It can be seen that all the segregants from such crosses are resistant to 1 mM DL-ethionine. It was concluded that the two mutations affect the same gene. Consequently, the mutations borne by the two strains were further designated as *eth2-1* (CH82-10D) and *eth2-2* (199M1-102).

It was of interest to check, by classical tetrad analysis, if any linkage could be detected between the gene *eth2* and the characters present in the diploid CM53. Table 3 shows that the *eth2* gene segregates independently of the genes *trp5*, *arg4*, *his5*, *lys1*, *ade2*, *leu1*, and *ura1*. In addition, since *leu1* is closely linked to its centromere, and greater than 65% tetratype asci were observed, it seems so far unlikely that *eth2* could be centromere linked.

TABLE 1

Segregation of the characters carried by the strain 199M1-102 from two different diploids

Diploid	CM53	CM83	Percent average
Number of spores	112	176	
Selected allele			
<i>URA1</i>	55	—	49.0
<i>TRY5</i>	58	87	50.4
<i>HIS5</i>	56	88	50.0
<i>LYS1</i>	57	86	49.7
<i>ADE2</i>	55	87	49.4
<i>LEU1</i>	56	88	50.0
<i>ARG4</i>	55	88	49.6
Ethionine resistant spores	56	88	50.0

Diploid strains: CM53 = 199M1-102 (*eth r*) × CH71-1D (*eth s*): α *trp5 arg4 his5 lys1 ade2 leu1 URA* $\bar{a}$ *TRP ARG HIS LYS ADE LEU ura1*CM83 = 199M1-102 (*eth r*) × PM100 (*eth s*): α *trp5 arg4 his5 lys1 ade2 leu1* $\bar{a}$ *TRP ARG HIS LYS ADE LEU*

Tests were carried out on solid media as described under MATERIALS AND METHODS. The DL-ethionine concentration was 1 mM.

Excretion of methionine by the mutant strain 199M1-102: Since the *eth2-1*-bearing strains previously studied had all the criteria of a regulatory mutation and the mutant 199M1-102 bearing the *eth2-2* allele was resistant to high concentrations of ethionine, overproduction of methionine could be expected. Cross feeding experiments were then undertaken, using different methionine-requiring strains as testers. It can be seen in Table 4 that the strain 199M1-102 excretes a compound able to promote growth of all the strains used even the one (MH5) which is able to grow only on either methionine or s-methylcysteine. It

TABLE 2

Segregation of the characters carried by a diploid involving two ethionine resistant haploids

Diploid	CM52	
Number of spores	96	Percent
Selected allele		
<i>URA1</i>	48	50
<i>TRY5</i>	49	51
<i>HIS5</i>	48	50
<i>LYS1</i>	49	51
<i>ADE2</i>	47	49
<i>LEU1</i>	48	50
<i>ARG4</i>	49	51
Ethionine resistant spores	96	100

Diploid CM52 = 199M1-102 (*eth r*) × CH82-10D (*eth r*): α *trp5 arg4 his5 lys1 ade2 leu1 URA* $\bar{a}$ *TRP ARG HIS LYS ADE LEU ura1*

TABLE 3

Tetrad analysis for the different characters presents in the diploid CM53

Parental association	Ascus type (percent)			N*
	PD	NPD	TT	
<i>trp5, eth2-2</i> × <i>TRP, ETH2</i>	20.0	4.0	76.0	25
<i>arg4, eth2-2</i> × <i>ARG, ETH2</i>	16.0	12.0	72.0	25
<i>his5, eth2-2</i> × <i>HIS, ETH2</i>	15.3	7.7	77.0	26
<i>lys1, eth2-2</i> × <i>LYS, ETH2</i>	13.0	21.7	65.3	23
<i>ade2, eth2-2</i> × <i>ADE, ETH2</i>	7.7	15.3	77.0	26
<i>leu1, eth2-2</i> × <i>LEU, ETH2</i>	23.0	15.5	65.5	26
<i>ura1, ETH2</i> × <i>URA, eth2-2</i>	8.2	20.8	70.8	24
<i>α, eth2-2</i> × <i>α, ETH2</i>	12.0	32.0	56.0	25

* N: total number of tetrads observed.

According to MORTIMER and HAWTHORNE (1969) the genetic localization of the known characters involved is as follows: *trp5*, chromosome VII (left arm); *arg4*, chromosome VIII (right arm); *his5*, fragment 4; *lys1*, chromosome IX (right arm); *ade2*, fragment 1; *leu1*, chromosome VII (centromere linked); *ura1*, fragment 5; and mating type, chromosome III (right arm).

PD: parental ditype; NPD: non parental ditype; TT: tetratype.

seems then that methionine at least is one of the compounds excreted by 199M1-102.

Growth of eth2-2-bearing strains in the presence of ethionine: The haploid 199M1-102 and two diploids, CM53 (*ETH2/eth2-2*) and CM52 (*eth2-1/eth2-2*) were grown on synthetic medium supplemented with various amounts of ethionine. Figure 1 summarizes the observed growth behaviour. It can be seen that, in the presence of the *eth2-2* allele, the generation time of a haploid strain remains unaffected up to a concentration of 10 mM DL-ethionine. In this strain, the generation time is prolonged, even in the absence of ethionine. This rather slow growth might be due partly to the presence of six auxotrophic requirements in 199M1-

TABLE 4

Cross feeding of different methionine auxotrophs caused by an eth2-2-bearing strain

	Methionine requiring tester strains		
	MH5	MH7	MH15
Growth on supplements (mM)			
DL-methionine	+	+	+
S-methyl-L-cysteine*	+	+	+
DL-homocysteine	—	+	+
L-cysteine	—	+	+
Growth in the presence of other strains			
—	—	—	—
4094-B	—	—	—
199M1-102	+	+	+

* Not directly involved in methionine biosynthesis.

Strains: 199M1-102: *α, trp5, arg4, his4, lys1, ade2, leu1, eth2-2*. 4094-B: *α, ade2, ura1*.MH5-MH7-MH15 methionine auxotrophs isolated through mutagenic treatment of a haploid strain *α, ade2, ura1* (H. CHEREST). When cross feeding occurs, it is evident after two to three days.

FIGURE 1.—Influence of exogenous ethionine concentrations upon growth of strains carrying the *eth2-2* allele. Strains: 199M1-102: *α*, *trp5*, *arg4*, *his5*, *lys1*, *ade2*, *leu1*, *eth2-2*.

CM53 = 199M1-102 × CH71-1D:

$$\frac{\alpha}{a} \frac{trp5}{TRP} \frac{arg4}{ARG} \frac{his5}{HIS} \frac{lys1}{LYS} \frac{ade2}{ADE} \frac{leu1}{LEU} \frac{URA}{ura1} \frac{eth2-2}{ETH2}$$

CM52 = 199M1-102 × CH82-10D:

$$\frac{\alpha}{a} \frac{aug4}{ARG} \frac{his5}{HIS} \frac{lys1}{LYS} \frac{ade2}{ADE} \frac{leu1}{LEU} \frac{URA}{ura1} \frac{eth2-2}{eth2-1}$$

Growth curves were established as described under MATERIALS AND METHODS. Mean generation time (MGT) expressed in hours was calculated from the exponential part of each growth curve. DL-ethionine (at the concentrations indicated), was present from the beginning of growth.

102. In order to overcome this difficulty, a segregant having only two auxotrophic requirements was used for biochemical studies.

The recessivity of the *eth2-2* allele over the wild-type allele, already noticed on plates, was confirmed by the complete inability of diploid CM53 to grow in the presence of DL-ethionine 0.5mM. It was previously shown that the *eth2-1* allele was entirely recessive to the wild-type allele (CHEREST *et al.* 1968, 1969, 1971). Yet the heteroallelic diploid *eth2-1/eth2-2* shows a resistance to ethionine comparable to the resistance observed with a haploid strain bearing *eth2-1*. It seems then that in this heteroallelic combination, *eth2-1* can still express its particular phenotype; in other words that *eth2-1* is dominant over *eth2-2* (see further).

Effect of the allele eth2-2 on repressibility of different methionine enzymes: It was shown previously that the presence of the allele *eth2-1* lowers the repressibility of at least four enzymes specifically involved in methionine biosynthesis

TABLE 5

Synthesis of different methionine enzymes under various growth conditions in an eth2-2-bearing haploid strain

Enzymes	Media				
	Minimal	Minimal + DL-methionine (mM)			
		0.4	0.6	2	20
Group I					
Homocysteine synthetase	142	136	98	177	155
	174	193	—	189	161
ATP sulfurylase	142	136	166	210	121
	248	259	—	250	240
Group II					
Homoserine dehydrogenase	290	254	360	285	253
	227	252	—	238	172

Strain CM53-6A: *α*, *lys1*, *ura1*, *eth2-2*.

Minimal medium: YNB supplemented by uracil and lysine.

Results are expressed in specific activities (see MATERIALS AND METHODS). When two numbers are recorded they correspond to quite separate experiments.

(methionine group I enzymes (CHEREST *et al.* 1971)). However, two other enzymes involved in homoserine biosynthesis (methionine group II enzymes (CHEREST *et al.* 1969, 1971)) although susceptible to repression by methionine seem unaffected by *eth2-1*. In the present study, two group I enzymes (homocysteine synthetase, ATP sulfurylase) and one group II enzyme (homoserine dehydrogenase) were assayed. Table 5 summarizes the results obtained when a strain carrying the *eth2-2* allele was grown in the presence of various methionine concentrations. The repressibility of the two group I enzymes has been compared for strains carrying the wild type and the mutant *eth2-2* alleles (Table 6). It can be seen that, whatever the exogenous concentration of methionine used, no significant repression of the two methionine group I enzymes tested was observed.

Note: As far as homoserine dehydrogenase is concerned, it should be pointed out that, although this enzyme generally exhibits a weaker response to methionine-mediated repression than methio-

TABLE 6

Comparative repressibility of homocysteine synthetase and ATP sulfurylase in ETH2 and eth2-2-bearing strains

DL-methionine mM	Homocysteine synthetase		ATP sulfurylase	
	ETH2	<i>eth2-2</i>	ETH2	<i>eth2-2</i>
0.4	67	0	81	0
0.6	76	28	90	15
2	80	0	92	0
20	80	0	92	8

Values for the wild-type (ETH2) strain are mean values from previous experiments (CHEREST *et al.* 1971); values for the mutant (*eth2-2*) are calculated from Table 5.

Values are expressed in percent of repression.

nine group I enzymes, its exclusion from this group was based mainly upon its non-coordinate synthesis with group I enzymes (CHEREST *et al.* 1971). In the *eth2-2*-bearing strain studied here, some repression of homoserine dehydrogenase synthesis could be observed in one of the two experiments only, at the higher (20 mM) methionine concentration. If further substantiated, the failure to repress homoserine dehydrogenase synthesis in the other cultures, could, of course, raise the question of classification of this enzyme in group I instead of group II. However, these results could also be explained by secondary effects of the *eth2-2* mutation.

In view of the demonstration that *eth2-2* is recessive with respect to ethionine resistance, it was of interest to show that recessivity also extends to the biochemical expression of the *eth2* gene, i.e. repressibility of the methionine group I enzymes. Results in Table 7 show that this is the case since the enzymes tested recovered complete repressibility in a diploid *ETH2/eth2-2*.

In the heteroallelic diploid *eth2-1/eth2-2*, resistance to ethionine was found which was similar to that occurring in an haploid *eth2-1* strain. In a preliminary experiment, with such a diploid only one concentration of exogenous methionine was chosen in order to provide some information on repressibility of several methionine enzymes (DL-methionine 2mM). It was found that synthesis of both ATP sulfurylase and homoserine dehydrogenase were repressed to a similar extent to that in the haploid *eth2-1*. In this experiment homocysteine synthetase remained unrepressed, although a 50% repression was expected (CHEREST *et al.* 1971). However, it can be recalled that the synthesis of homocysteine synthetase is slightly less sensitive to repression at intermediate concentrations of methionine than ATP sulfurylase. These fragmentary results therefore seem to reinforce the afore-mentioned physiological observation and show that biochemical expression of *eth2-1*, as well as its phenotypic expression, can occur in the presence of *eth2-2*.

Characterization of nonsense suppressors in strains carrying eth2-2

Isolation: As described under MATERIALS AND METHODS, spontaneous multiple

TABLE 7

Comparative repressibility of methionine enzymes in strains carrying the wild-type ETH2 and the mutant allele eth2-2

Enzyme	Alleles involved		
	<i>eth2-2</i>	<i>ETH2</i> *	<i>eth2-2</i>
	(1)	(2)	<i>ETH2</i>
Percent repression			
Group I			
Homocysteine synthetase	0	81.0	81.0
ATP sulfurylase	0	94.3	81.0

* H. CHEREST, unpublished results.

Strains: (1) CM53-6A: α , *lys1*, *ura1*, *eth2-2*. (2) 4094-B: α , *ade2*, *ura1*, *ETH2*. (3) CM83 = 199M1-102 $\times$ PM100: $\frac{\alpha}{a}$ *trp5* *arg4* *his5* *lys1* *ade2* *leu1* *eth2-2*.
 $\frac{a}{a}$ *TRP* *ARG* *HIS* *LYS* *ADE* *LEU* *ETH2*.

Cultures were carried out in synthetic minimal medium supplemented or not with 1 mM DL-methionine.

TABLE 8

Classification of spontaneous suppressor-bearing revertants obtained from the strain 199M1-102

Suppressed auxotrophies*					Number of clones	Number of ethionine sensitive clones	Group number
[TRY]	[ARG]	[HIS]	[LYS]	[ADE]			
1	1	1	1	1	69	13	4
1	1	1	1	6	1	1	10
1	1	1	6	6	1	0	..
1	3	1	1	1	1	0	..
1	3	6	1	1	1	0	..
1	1	1	1	.	10	1	8
1	1	6	1	.	1	0	..
1	6	1	3	.	1	0	..
1	1	.	.	.	20	0	..
1	3	.	.	.	10	1	5
1	6	.	.	.	6	0	..
3	3	.	.	.	2	0	..
1	.	.	1	.	1	0	..
1	.	.	3	.	2	0	..

For experimental procedure, see MATERIALS AND METHODS.

* Numbers below indicate days of incubation prior to appearance of growth on the plates.

revertants were selected from 199M1-102. They were further classified according to their phenotypic expression. As shown in Table 8, the most numerous class (group 4—first line of the table) is the one in which suppressor expression is attained in one day for all the nonsense suppressible mutations introduced in the strain. Other classes differ either by the length of time necessary for expression or by the number of suppressed auxotrophic traits. In all cases, the internal control mutation, *leu1* (insensitive to ochre suppressors), remained unaffected.

All the suppressor-bearing revertants were tested for their sensitivity to ethionine. The highest proportion of suppressors active on ethionine resistance was in group 4. For practical reasons only two of these strains (R7 and R9) were subjected to extensive analysis.

Dominance studies of suppressors present in strains R7 and R9 over eth2-2 mutation: Growth studies were carried out on haploid and diploid strains carrying one or the other suppressor. Figure 2A shows the partial recovery of sensitivity towards ethionine caused by the suppressor present in R7 (199M1-102R₄7) and R9 (199M1-102R₄9) strains respectively. It will be recalled that the parental sensitive strain is completely inhibited at 0.5 mM DL-ethionine and that the *eth2-2* mutant isolated from it, is resistant to 10 mM DL-ethionine.

In part B of Figure 2, the growth response of the diploid CM109 (heterozygote for the suppressor gene present in strain R9 and homozygote for *eth2-2*) to ethionine shows that this suppressor is at least semi-dominant. An analogous diploid (CM122) bearing the suppressor present in R7 showed a more pronounced dominance since it remained sensitive to 0.5 mM DL-ethionine. In addition to a difference in suppressor efficiency, the difference in behaviour of the two diploids

FIGURE 2.—Influence of exogenous ethionine concentration upon growth of *eth2-2* and nonsense suppressor bearing strains. Strains: 199M1-102R₄7: α , *trp5*⁺, *arg4*⁺, *his5*⁺, *lys1*⁺, *ade2*⁺, *leu1*, *eth2-2*⁺, *SuR7*. 199M1-102R₄9: α , *trp5*⁺, *arg4*⁺, *his5*⁺, *lys1*⁺, *ade2*⁺, *leu1*, *eth2-2*⁺, *SuR9*. CM109 = 199M1-102R₄9 $\times$ CM53-18B: $\frac{\alpha}{a}$, $\frac{trp5}{trp5}$, $\frac{arg4}{ARG}$, $\frac{his5}{HIS}$, $\frac{lys1}{LYS}$, $\frac{ade2}{ADE}$, $\frac{leu1}{leu1}$, $\frac{eth2-2}{eth2-2}$, $\frac{SuR7}{SU}$. Growth was carried out in synthetic medium supplemented with leucine.

- (A) $\bullet$ 199M1-102R₄7 } no ethionine added
 $\circ$ 199M1-102R₄9 }
 $\blacktriangle$ 199M1-102R₄7 } DL-ethionine 0.5 mM
 $\triangle$ 199M1-102R₄9 }
 $\blacksquare$ 199M1-102R₄7 } DL-ethionine 5 mM
 $\square$ 199M1-102R₄9 }
 (B) Diploid CM109
 $\bullet$ no ethionine
 $\triangle$ DL-ethionine 0.5 mM
 $\circ$ DL-ethionine 1 mM
 $\square$ DL-ethionine 5 mM

might also be due to the fact that CM109 was heterozygous for four out of five of the suppressed auxotrophies and was homozygous only for *trp5* (suppressed) and *leu1* (unsuppressed) while CM122 was homozygous for four of the suppressible characters, *arg4*, *his5*, *lys1*, *ade2*.

TABLE 9

Segregation of the characters involved in a diploid carrying eth2-2 and the suppressor from strain R7

Observed phenotype	Spores with the observed phenotype	Total spore tested	Percent
TRY	178	224	79.5
HIS	163	224	72.8
LYS	168	224	75.0
ADE	164	224	73.5
ARG	127	160	79.5
LEU	109	224	48.8
(1) <i>eth2-2</i>	73	216	32.5
(2) <i>eth2-2</i> , <i>SuR7</i> [<i>eth r</i>]	12	224	5.3
<i>eth2-2</i> , <i>Su</i> ⁺ ((1)-(2))	..	..	27.2

Strain: CM104 = 199M1-102R₄7 × PM100:

α *trp5* *arg4* *his5* *lys1* *ade2* *leu1* *eth2-2* *SuR7*

$\bar{\alpha}$ *TRP*, *ARG*, *HIS*, *LYS*, *ADE*, *LEU*, *ETH2*, *SU*.

For explanations see the text and MATERIALS AND METHODS.

Segregation of the suppressor present in strain R7 and its effect on suppressible alleles: A diploid (CM104) was constructed between 199M1-102R₄7 and a wild-type sensitive haploid PM100. Segregation of all the characters present in the cross was studied. In Table 9 it can be seen that the five known ochre suppressible mutations present in the cross segregate as expected for an unlinked ochre suppressor. In addition, it is obvious that the recovery of ethionine resistant segregants shows the presence of the *eth2-2* allele in the parental suppressed strain R7.

The recovery of resistant segregants is slightly higher than the 25% expected for independent segregation of *eth2-2* and the suppressor. If one considers those resistant segregants in the population which lack the suppressor, the *eth2-2* allele segregates correctly. It should be pointed out here that, in crosses between either heteroalleles *eth2-1* or *eth2-2* and wild-type strain, moderate differences between segregants are routinely observed in expression of resistant phenotype which do not appear, so far, to be related to defined genetic characters. These phenomena can be attributed to differences in the genetic background among the segregants. It seems reasonable therefore to count the resistant segregants bearing the suppressor among the sensitive population. If this is correct, the class (*eth2-2*; *SU*⁺) would represent only 27% of the total population, which is close to the expected proportion.

Another diploid (CM103) was constructed between 199M1-102R₄9 and the wild-type sensitive haploid strain PM100, and sporulated. One suppressor-bearing sensitive segregant was then crossed with another wild-type sensitive haploid strain PM101 (diploid CM121). Since recovery of four spore asci was rare, random spore isolation was used. As for the previous diploid, CM104, ethionine resistant segregants were recovered in a proportion compatible with the expected suppression pattern of *eth2-2*.

TABLE 10

Comparative levels of two methionine enzymes in haploid strains carrying the wild-type allele ETH2 or the mutated allele eth2-2 in the presence or absence of suppressor R7, after growth in complex repressive medium

Enzymes	<i>eth2-2</i> (a)	<i>eth2-2 SuR7</i> (b)	<i>ETH2</i> (c)
Group I			
Homocysteine synthetase	88 (2)	34 (2)	47 (1)
ATP sulfurylase	40 (2)	15 (2)	4 (1)

Strains: (a) CF53-6A: α , *lys1*, *ura1*, *eth2-2*. (b) 199M1-102R₁7: α , *trp5*, *arg4*, *his5*, *lys1*, *ade2*, *leu1*, *eth2-2*, *SuR7*. (c) 199M1: α , *trp5*, *arg4*, *his5*, *lys1*, *ade2*, *leu1*.

Medium: YPGA.

Number in parenthesis correspond to the number of independent experiments. Mean values are expressed in specific activities (see MATERIALS AND METHODS).

Effect of the nonsense suppressor of eth2-2 on repressibility of methionine enzymes: It was mentioned in a previous section that strains carrying many suppressible auxotrophic characters showed reduced growth rates in synthetic media. This difficulty was also observed for suppressor-bearing revertants. Consequently, it was very difficult to obtain from cultures in supplemented synthetic media, the standard amount of cells necessary for the biochemical study of these strains. However, a basis for comparison can be still obtained from cultures in YPGA (see Table 10). In such conditions homocysteine synthetase and ATP sulfurylase are synthesized at repressed levels as compared to the *ETH2* sensitive strain. It should first be pointed out that growth in such complex media lowers the specific activity of the two enzymes considered in the *eth2-2*-bearing strain (compare with values in Table 5). This observation could be explained in different ways such as non specific or catabolite repression, instability, degradation of enzyme molecules, etc. No attempt has been made so far to test any of these hypotheses. Nevertheless, these preliminary results seem to indicate that the presence of *SuR7* in an *eth2-2* bearing strain leads at least to partial recovery of repressibility of these two methionine group I enzymes. Experiments are in progress in order to correlate more precisely the effect of suppressors upon methionine resistance and their effect upon repressibility.

DISCUSSION

The results presented here have shown the existence of a nonsense mutation at the locus *eth2*. This evidence has been obtained through selection of nonsense suppressors having the widest action spectra possible (see MORTIMER and HAWTHORNE 1969 for review of suppression in *Sacch. cerevisiae*). All the nonsense alleles introduced into the initial strain used for selection of methionine-resistant mutants, were of the ochre type. Thus suppressors active on them are expected to be either ochre-specific suppressors or ochre-amber suppressors. Consequently, it is not possible to say if the *eth2-2* mutation is of the ochre or amber type.

The phenotypic expression of the nonsense mutation *eth2-2* is interesting in

many respects. As expected, it promotes resistance to very high levels of ethionine (10 mM). This high resistance is easily explained by the finding that methionine is excreted by an *eth2-2*-bearing strain. It will be recalled that, in sensitive strains, inhibition by a given concentration of ethionine can be reversed by about a 10-fold lower concentration of methionine. The extent of methionine overproduction has not yet been quantitated. It can be remarked that the strain carrying the *eth2-2* mutation exhibits a much slower growth rate than the parental sensitive strain. One of the reasons might be a severe lack of energy (3 moles of ATP and one mole of acetyl CoA used for each mole of methionine formed) caused by methionine overproduction. In any case, since methionine excretion could be observed following mutation at the *eth2* locus, it seems to indicate that, of the two possible regulatory mechanisms, repression and feedback inhibition (for example inhibition of homocysteine synthetase activity by methionine (CHEREST *et al.* 1969), the former is of major importance for the control of methionine synthesis in *S. cerevisiae*.

Evidently the primary effect of the *eth2-2* mutation studied here resides in the complete absence of repressibility of methionine biosynthetic enzymes, the other effects such as methionine excretion and resistance to ethionine being secondary consequences of the first. This extreme phenotype brought by the *eth2-2* allele is exactly that expected from the introduction of a nonsense mutation, that is, the absence of a complete translation product of the *ETH2* gene.

Since the *eth2-1* allele previously studied led to roughly a 10 fold lowering of sensitivity toward repressive effects of exogenous methionine, it seems plausible that this mutation resulted in an impaired product of gene *ETH2*. Examination of a diploid *eth2-1/eth2-2* gave results which were in agreement with this interpretation, since it was found that the repressibility of methionine enzymes, in this heteroallelic diploid, follows the one characteristic of an *eth2-1* haploid strain.

If one now turns attention towards the *eth2-2* suppressed strains analyzed (especially the strain carrying the suppressor R7), it appears that the recovery of sensitivity to ethionine is only partial, as compared with that of the original sensitive strain. Although conclusive biochemical experiments have not yet been achieved, it is not unlikely that only a partial recovery of repressibility occurs in suppressed strains. If this point were to receive further experimental support, an attractive hypothesis could be that the R7 suppressor permits only a reduced amount of repressor to be synthesized. This situation would contrast with the situation in *eth2-1* strains, in which, it was concluded that the amount of repressor synthesized was not altered, but rather that its affinity for the co-repressor was modified.

The existence of a nonsense mutation in the *eth2* locus points to the protein nature of its product. The methods used here have been directed toward selecting ethionine-resistant mutants which would not complement with the former mutation *eth2-1*. This selection systematically eliminates heteroallelic mutations which could result in intragenic complementation with the *eth2-1* allele. Indeed, the existence of such complementing mutations is conceivable, and would imply

that the *ETH2* product is a polymeric protein. Other experimental procedures could be used to select such mutations.

It is noticeable that when the three *eth2* mutants so far studied (*eth2-1*, *eth2-2* and a third one not described here) are grown in minimal medium, the level of methionine biosynthetic enzymes formed is comparable to that observed for an *ETH2* strain, but that the maximally derepressed level (which is observed in methionine-limited growth of methionine auxotrophs) (CHEREST *et al.* 1971) is not attained. Thus, the effect of mutations at the *eth2* locus seems to modify the rate of synthesis of methionine enzymes between the "normal" and fully repressed levels, but does not seem to affect the rate of synthesis of the same enzymes between the normal and fully derepressed levels. This remark also applies to the *ts*⁻²⁹⁶ mutation which seems to modify the amount of corepressor made. It is tempting then to suggest that the aporepressor-corepressor system which we have described here and previously (CHEREST *et al.* 1971) acts at the translational level. If this is the case, it would then implicate a somewhat different regulatory system acting at the transcriptional level. The existence of regulatory mechanisms acting at the two levels has already received support from studies on regulation of arginine (LAVALLE 1970; McLELLAN and VOGEL 1970) and tryptophan (LAVALLE and DE HAUWER 1970) biosynthetic enzymes in *E. coli*.

Note added in proof: Since this paper was written, we succeeded to grow several strains bearing the *eth2-2* allele and various suppressors, in synthetic medium supplemented or not with various methionine concentrations. The following table summarizes the results so far obtained, expressed in percent of repression.

mL-Methionine (mM)	Homocysteine synthetase			ATP sulfurylase		
	R ₇	R ₁₀ 11	R ₉	R ₇	R ₁₀ 11	R ₉
1	66	44	0	94	96	51
5	—	—	0	—	—	53
10	80	48	—	96	92	—

It shows that recovery of repressibility can vary, depending upon the suppressor considered, R₇ being the most efficient, and R₉ the least efficient one. It can be noticed that ATP sulfurylase repressibility is more easily recovered than the one of homocysteine synthetase. In addition, when low efficiency of suppression is observed (homocysteine synthetase in R₁₀11 and ATP sulfurylase in R₉) it seems that increasing methionine concentration does not lead to an increased repressibility. This behaviour might reinforce the hypothesis (presented above, see DISCUSSION) of a limiting amount of active repressor being synthesized under the action of those suppressors.

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