NONSENSE MUTATIONS IN THE *ADE3* LOCUS OF *SACCHAROMYCES CEREVISIAE*¹

ELIZABETH W. JONES

Department of Biology, Case Western Reserve University, Cleveland, Ohio

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ABSTRACT

Fifty seven mutations at the *ade3* locus have been crossed to ochre, amber and ochre-amber suppressors. 70% (39/56) of the mutations at this locus are nonsense mutations; 61% (34/56) are ochre mutations and 9% (5/56) are amber mutations. The frequency of nonsense mutations among *ade3* alleles recovered is very high and raises the interesting possibility that only polar mutations at this locus are recovered. An hypothesis to explain these genetical findings as well as physiological properties of these mutations is proposed.

STRAINS of *Saccharomyces cerevisiae* carrying point mutations in the adenine locus, *ade3*, have been shown to be 90% deficient in formyltetrahydrofolate synthetase (EC 6.3.4.3) and methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) and to be lacking one of two isoenzymes of methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) (JONES and MAGASANIK 1967a; LAZOWSKA and LUZZATTI 1970a, 1970b). The pathway for synthesis and interconversion of selected coenzyme forms of tetrahydrofolate and the relationship of the above mentioned enzymes to this metabolic system can be seen in Figure 1. As a consequence of the *ade3* lesion, mutant strains have decreased levels of 10-formyltetrahydrofolate and 5, 10-methenyltetrahydrofolate and are hence unable to convert glycineamide ribonucleotide to α -N-formylglycineamide ribonucleotide and 5-aminoimidazole-4-carboxamide ribonucleotide to 5-formamidoimidazole-4-carboxamide ribonucleotide (JONES and MAGASANIK 1967b). Failure to carry out these two latter reactions results in a requirement for adenine or hypoxanthine. The strains carrying the *ade3* mutation also have a histidine requirement which is related to the insufficiency of tetrahydrofolate coenzymes.

This paper reports results of genetic studies directed toward characterization of the mutational lesions present in 57 *ade3* mutant strains which lead to simultaneous loss of three enzymatic activities. Fifty seven *ade3* mutations have been examined for suppressibility by ochre and amber suppressors (HAWTHORNE and MORTIMER 1968). Mapping data are presented for selected alleles.

A minimum estimate for the frequency of nonsense mutations at the *ade3* locus, deriving from these studies, is 70%—a very high frequency. These data, as well as the biochemical characteristics of the mutants and of tetrahydrofolate

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FIGURE 1.—Pathway and enzymes involved in interconversion of selected coenzymes of tetrahydrofolic acid.

metabolism, are consistent with a model in which the *ade3* locus is the structural gene for one of two isoenzymes of methylenetetrahydrofolate dehydrogenase and is the proximal of three contiguous cistrons (the other two cistrons being structural genes for formyltetrahydrofolate synthetase and methenyltetrahydrofolate cyclohydrolase) which are included in one unit of transcription. Further characteristics of this model are presented in the discussion.

MATERIALS AND METHODS

Yeast strains: Starting strains of *Saccharomyces cerevisiae* were obtained from H. L. ROMAN and D. C. HAWTHORNE. The *ade3* mutants were obtained as white *ade2 ade3* derivatives of red *ade2* strains (ROMAN 1956). Genotypes of the parental strains employed and the *ade3* alleles isolated from them are given in Table 1. Alleles 46–54 were isolated from cultures treated with nitrous acid. The cells were treated in 0.1 M acetate buffer pH 4.5 with 0.024 M NaNO₂ for various times before plating on SC. Whites were isolated as sectors in red colonies. Alleles 73 and 75–84 were isolated from colonies which had been grown on a modified SC medium (2mg/l adenine sulfate, 12mg/l leucine) containing 1 mg/ml of 2-aminopurine. All other alleles were of spontaneous origin. X1687-16C-R1, a strain carrying the ochre suppressor *SUP2* (GILMORE, STEWART and SHERMAN 1971) first isolated by GILMORE (1967), was obtained from T. R. MANNEY. C200-10B was obtained as a segregant from a cross of X1687-16C-R1 to a strain bearing the ochre alleles present in X1687-16C-R1. D609-28C and 6190D were obtained from G. R. FINK. Genotypes of these four strains are given in Table 1.

TABLE 1
Genotypes of parental strains

Strain	Genotype	Derivative <i>ade3</i> alleles
C30-20B	α <i>ade2-1* his1 met1 ura1-1</i>	3, 4, 7, 8, 10, 11, 13, 17, 19, 20, 22, 26, 30, 31, 33, 34, 36, 37, 38
A1327A	<i>a ade2-1* leu1-1 Mal1 suc1</i>	39, 40, 41, 43, 45, 46, 47, 48, 50, 51, 54, 70, 73, 74, 75, 78, 79, 81, 83, 84
C19-17C	α <i>ade2-1* lys2 met2 trp5-2* ura1-1</i> <i>mal1 SUP1 cyh2</i>	58, 59, 63, 67
1721C	<i>ade2-1*</i>	55, 56
C62-30B	α <i>ade2-1* trp5-2* lys2* ura1-1 Suc1 mal1</i>	85, 86, 87, 88, 89, 90, 94, 95, 96, 97, 98, 99, 100, 101
X1687-16C-R1	<i>a trp5-48* arg4-17* his5-2* lys1-1*</i> <i>ade2-1* leu1-12 met1 SUP2</i>	
C200-10B	α <i>trp5-48* arg4-17* his5-2* lys1-1*</i> <i>ade2-1* met1 SUP2</i>	
D609-28C	<i>a arg4-17* his5-2* lys1-1* trp1-1† tyr7-1†</i>	
6190D	α <i>his5-1* lys1-1* trp1-1† tyr7-1†§</i>	

* Alleles suppressible by ochre suppressors.

† Alleles suppressible by amber suppressors.

§ The symbols *ade*, *arg*, *his*, *leu*, *lys*, *met*, *trp*, *tyr*, and *ura* signify requirement for adenine, arginine, histidine, leucine, lysine, methionine, tryptophan, tyrosine and uracil; *Mal* and *Suc* indicate the ability to ferment maltose and sucrose respectively; *cyh* indicates recessive resistance to cycloheximide and *SUP2* indicates an ochre suppressor (GILMORE 1967).

Media: One liter of medium contains the components given in the amounts indicated. *YEPD*—10 g Difco Yeast Extract, 20 g Difco Bacto-Peptone, 20 g dextrose; *Synthetic complete: (SC)*—20 g dextrose, 6.7 g Difco Yeast Nitrogen Base w/o amino acids, 20 mg adenine sulfate, 20 mg arginine, 20 mg histidine, 60 mg leucine, 50 mg lysine, 40 mg methionine, 20 mg phenylalanine, 20 mg tyrosine, 10 mg uracil, 20 mg tryptophan; *Omission medium:*—SC with one or more nutrients omitted, e.g. —Ad is SC without adenine; *Sporulation medium:* 15 g potassium acetate, 1 g dextrose, 2.5 g yeast extract. All media were solidified when necessary by 15 g Bacto-Difco Agar.

Genetic methods: The genetic methods used were as previously described. (JONES 1972).

RESULTS

Suppression of ade3 mutations by ochre suppressors: A characterization of the mutational lesions present in *ade3* mutant strains was begun by determining the frequency of nonsense mutations among *ade3* alleles. Various independently isolated *ade3* alleles were crossed to strains carrying nonsense suppressors. Segregation patterns for the diploids and the phenotypes of spore clones of genotype *ade3-x SUP-x* were determined.

In Table 2 are presented the results of crosses between strains carrying the ochre suppressor, *SUP2*, (GILMORE, STEWART and SHERMAN 1971) and 27 independently isolated *ade3* alleles. The relevant genotype of the diploids is given at the top of this table. The ochre allele *ade2-1* is homozygous in these crosses. It is suppressible by *SUP2*. If the *ade3* allele is suppressible by *SUP2* all spores

carrying the suppressor, whether of genotype *ade2-1 SUP2* or *ade2-1 ade3-x SUP2*, should be adenine independent (AD+). All tetrads should segregate 2+:2- for the adenine requirement. Only those spores not carrying *SUP2* should have a requirement for adenine (AD-). If the *ade 3* allele is not suppressible by *SUP2*, 2+:2-, 1+:3- and 0+:4- tetrads should be seen in the ratio of 1:4:1. These three classes are respectively the parental ditype, tetratype, and nonparental ditype segregations of *SUP2* and *ade3*. One gets these phenotypic ratios, rather than 2+:2-, 3+:1-, and 4+:0- because of the presence of the homozygous *ade2-1* allele. Of the spores bearing *SUP2*, 50% should bear the nonsuppressible *ade3* allele and should hence require adenine. In the crosses involving the TW strains the diploids were heteroallelic for *trp5*. Both heteroalleles, *trp5-2* and *trp5-48*, are ochre alleles and suppressible by *SUP2*. The presence of *SUP2* can thus be monitored directly by the detection of tryptophan independent clones.

TABLE 2

Analysis for suppressibility by SUP2 of 27 ade3 alleles

Relevant genotype of the diploids is of the form $\frac{ade2-1^*}{ade2-1^*} \frac{+}{ade3-x} \frac{SUP2}{+}$.						
<i>SUP2</i> - bearing parent	<i>ade3</i> - bearing parent	<i>ade3</i> allele number	Number of tetrads (+:- phenotypes) 2:2 1:3 0:4			Phenotypes of <i>SUP2</i> - bearing spores AD+:AD-
C200-10B	BW824	11	5	0	0	14:0
	BW852	13	6	0	0	12:0
	BW886	17	7	0	0	14:0
	BW939	19	.	.	.	3:0
	BW947	20	2	0	0	8:0
	BW1248	31	3	0	0	13:0
	BW1378	36	3	0	0	15:0
	NH31-85	47	.	.	.	15:0
	HAP-6-547	75	8	0	0	24:0
	HAP-6-772	78	7	1	0	29:0
X1687-16C-R1	TW69	87	10	0	0	21:0
	TW120	88	10	0	0	22:0
	TW140	90	4	0	0	17:0
	TW621	94	10	0	0	20:0
	TW913	96	9	0	0	21:0
	TW1001	99	10	0	0	23:0
	TW1009	100	.	.	.	21:0
	TW1039	101	.	.	.	10:0
	56	56	10	0	0	20:0
	NH43-23	54	2	0	0	10:1
	HAP-6-337	73	4	3	1	13:2
	HAP-6-469	74	4	3	1	14:5
	HAP-6-972	81	0	2	0	6:6
	HAP-2-72	84	1	1	0	8:4
	TW130	89	3	7	0	15:8
	TW713	95	2	10	4	14:31
	TW964	97	2	5	2	10:10

* Alleles known to be suppressible by *SUP2*.

In the top portion of Table 2 are data indicating suppressibility by *SUP2* for 19 alleles at the *ade3* locus. Two criteria indicate that these alleles are suppressible. All tetrads segregated 2:2 for AD+:AD-. (Only tetrads in which all four spores germinated are included in these ratios.) In addition all spore clones bearing *SUP2* are AD+. (These data include clones from tetrads in which not all four spores germinated.) In the cross between C200-10B and HAP-6-772 one 1:3 tetrad was observed. As all 29 spores bearing *SUP2* are AD+, the most reasonable explanation of this tetrad is that it is a "false tetrad" (JOHNSTON and MORTIMER 1959) arising from chance association of spores. An alternative explanation is that it arose by gene conversion. The data for alleles 19 and 20 are insufficient to prove that these alleles are suppressible. Further evidence for the classification of these alleles as ochre mutations will be presented in the section on revertibility.

In the lower portion of Table 2 are data indicating nonsuppressibility for 8 *ade3* alleles. These 8 alleles were classified as nonsuppressible according to two criteria. 1) Segregation patterns of 2+:2-, 1+:3- and 0+:4- were obtained. 2) *SUP2* bearing spores which were phenotypically AD- were found. Not all crosses meet both criteria. For the allele for which the data are most seriously open to question, *ade3-54* (NH43-23), supplementary data have been obtained in the next series of crosses and are presented in Table 3.

Prior to mapping, a number of strains of genotype *ade2-1 ade3-x* were crossed to a wild-type strain in order to isolate the *ade3* alleles free of *ade2-1* and in a and α mating types. These strains carrying *ade3-x* and free of *ade2-1* were employed in the crosses to *SUP2* reported in Table 3. The relevant genotype of these diploids is indicated at the top of the table. The ochre allele *ade2-1* is heterozygous in these crosses. For this reason the expected segregation ratios are different from those predicted for diploids in the previous table. If the *ade3* allele is suppressible by *SUP2*, 4:0, 3:1, and 2:2 segregations should be observed in the ratio 1:16:19. All suppressor bearing spores should be phenotypically AD+. Significant observations if the *ade3* allele is suppressible are 1) the absence of 1:3 and 0:4 tetrads, 2) the presence of 4:0 and 3:1 tetrads, and 3) the absence of suppressor bearing spores which are phenotypically AD-.

If the *ade3* allele is not suppressible by *SUP2*, 2:2, 1:3 and 0:4 segregations in the ratio of 19:16:1 should be seen. One half of the *SUP2* bearing spores should be AD-. If the *ade3* allele is not suppressible the pertinent observations are 1) the presence of 1:3 and 0:4 tetrads, 2) the absence of 4:0 and 3:1 tetrads and 3) most importantly, the detection of spores of genotype *SUP2 ade2-1 ade3-x* or *SUP2 ade3-x* which are phenotypically AD-.

In the top portion of Table 3 are data indicating suppressibility for 9 *ade3* alleles. All spores carrying *SUP2* are AD+, no 1:3 or 0:4 tetrads are seen. Data indicating nonsuppressibility by *SUP2* for 13 *ade3* alleles are seen in the lower portion of Table 3. Two criteria indicate nonsuppressibility by *SUP2*. 1) Significant numbers of *SUP2* bearing spores are AD-. 2) Tetrads exhibiting ratios of 1:3 and 0:4 are found.

Three diploids gave unexpected segregation patterns, namely those involving *ade3-58*, *ade3-59*, and *ade3-39*. One 3:1 tetrad was observed in the segregations

TABLE 3

Analysis for suppressibility by SUP2 (ochre) of 22 ade3 alleles

Relevant genotype of the diploids is of the form				<i>ade2-1*</i>	+	<i>SUP2</i>	
				+	<i>ade3-x</i>	+	
<i>SUP2</i> - bearing parent	<i>ade3</i> bearing parent	<i>ade3</i> allele number	4:0	Number of tetrads (+:- phenotypes) 3:1 2:2 1:3			Phenotypes of <i>SUP2</i> - bearing spores Ad+ : Ad-
X1687-16C-R1	α <i>ade3</i> -8	8	0	1	4	0	14:0
	α <i>ade3</i> -33	33	0	3	2	0	13:0
	α <i>ade3</i> -37	37	0	2	4	0	14:0
	α <i>ade3</i> -45	45	0	3	2	0	14:0
	α <i>ade3</i> -47	47	1	3	2	0	20:0
	α <i>ade3</i> -48	48	0	0	2	0	11:0
	α <i>ade3</i> -50	50	0	1	5	0	16:0
	α <i>ade3</i> -51	51	0	2	0	0	4:0
C200-10B	α <i>ade3</i> -22	22	0	6	3	0	18:0
X1687-16C-R1	α <i>ade3</i> -3	3	0	0	1	5	6:7
	α <i>ade3</i> -7	7	0	0	5	2	11:7
	α <i>ade3</i> -10	10	0	0	1	1	6:7
	α <i>ade3</i> -26	26	0	0	2	1	7:4
	α <i>ade3</i> -38	38	0	0	3	3	6:7
	α <i>ade3</i> -39	39	0	2	6	1	13:5
	α <i>ade3</i> -40	40	0	0	3	4	4:8
	α <i>ade3</i> -41	41	0	0	2	1	5:7
	α <i>ade3</i> -54	54	0	0	1	2	4:3
	α <i>ade3</i> -58	58	0	1	3	3	7:10
	α <i>ade3</i> -59	59	0	1	1	0	6:4
	α <i>ade3</i> -63	63	0	0	3	5	5:9
	α <i>ade3</i> -67	67	0	0	3	4	6:11

* Alleles known to be suppressible by *SUP2*.

from diploids in which *ade3*-58 or *ade3*-59 was one parent. Yet spores of genotype *SUP2 ade3*-58 and *SUP2 ade3*-59 are phenotypically AD-. The most likely explanation is that these 3:1 tetrads are "false tetrads." As only two nonsuppressible, unlinked markers were segregating in these crosses the efficiency of detection of "false tetrads" was low.

The third exceptional cross is that between X1687-16C-R1 and *ade3*-39. Two 3:1 tetrads were observed. Yet the phenotype of spores of genotype *SUP2 ade3*-39 is clearly AD- (5 examples were detected). A second ochre suppressor capable of suppressing *arg4*-17 as well as *ade3*-39 proved to be segregating in this cross. Of four spores carrying this new suppressor and *ade3*-39 all four were AD+ phenotypically. Further evidence supporting the conclusion that *ade3*-39 is an ochre allele will be presented in the section on revertibility.

The cross of X1687-16C-R1 by *ade3*-54 supplements the data observed for NH43-23 reported in the previous table. The data indicate that *ade3*-54 is not suppressible by *SUP2*, for AD- spore clones were obtained which bore *SUP2*.

Additional ochre suppressors were isolated from 6190D and D609-28C. Selec-

TABLE 4

Analysis for suppressibility by several ochre suppressors of selected ade3 alleles

Relevant genotype of the diploids is of the form				+	+	<i>SUP</i>		
				<i>ade2-1</i> *	<i>ade3-x</i>	+		
<i>SUP</i> - bearing parent	<i>ade3</i> - bearing parent	<i>ade3</i> allele number	4:0	Number of tetrads (+:- phenotypes)			Phenotype of <i>SUP</i> - bearing spores Ad+ : Ad-	
				3:1	2:2	1:3	0:4	
D609-28C-R23	BW852	13	0	4	1	0	0	5:0
	TW140	90	3	3	2	0	0	19:0
D609-28C-R23	BW947	20	0	0	3	3	2	0:5
6190D-R10	NH31-81	46	0	0	3	2	0	4:7
	a <i>ade3-34</i>	34	0	0	6	0	0	6:6

* Alleles known to be suppressible by ochre suppressors present in R23 and R10.

tion was made for simultaneous reversion for *lys1-1* and the *his5* ochre allele present in the strain by plating on a medium lacking histidine and lysine. The ochre suppressor in D609-28C-R23 isolated in this fashion also suppresses *arg4-17* and *ade2-1*, that present in 6190D-R10 also suppresses *ade2-1*.

D609-28C-R23 was crossed to three strains of genotype *ade2-1 ade3-x*. The results are presented in Table 4. As D609-28C-R23 does not carry *ade2-1*, the expected segregation patterns are as for crosses in Table 3. If the *ade3* allele is suppressible, 4:0, 3:1 and 2:2 segregations are expected in the ratio 1:16:19. All suppressor bearing spores will be AD+. If the *ade3* allele is not suppressible, 2:2, 1:3 and 0:4 tetrads in a ratio of 19:16:1 are expected. Fifty percent of the spores carrying the suppressor will be AD-. Alleles 13 and 90 were suppressible, a result in accord with the data obtained for crosses of these alleles to *SUP2*. Allele 20 was not suppressed by the R23 suppressor, though it was suppressed by *SUP2*. This result is not unexpected, for the precise amino acid inserted might very well determine whether the protein produced is active.

6190D-R10, the ochre suppressor bearing derivative of 6190D, was crossed to two different *ade3* alleles. In the cross to NH31-81, *ade2-1* was segregating. The observation of 1:3 tetrads, coupled with detection of suppressor bearing spores of AD- phenotype indicate that this allele (*ade3-46*) is not suppressed by this suppressor. In the cross of 6190D-R10 to *ade3-34*, *ade2-1* was not segregating. As all tetrads showed 2:2 segregation for the adenine requirement and as spore clones of AD- phenotype were observed among suppressor bearing spores, the *ade3-34* is classified as nonsuppressible.

Suppression by "amber" suppressors: The alleles *tyr7-1* and *trp1-1* have been classified as amber alleles (HAWTHORNE and MORTIMER 1968; HAWTHORNE 1969). To obtain amber suppressors the strains D609-28C and 6190D were streaked on a medium lacking tryptophan, phenylalanine, and tyrosine. Several independent TRP+ TYR+ revertants were isolated from each strain, were purified by restreaking on the same medium, and were retested to insure that the clones were TRP+ and TYR+. No mutagen was used in the isolation. One revert-

ant of each strain (D609-28C-R13 and 6190D-R7) was used in further crosses to detect amber alleles among the population of *ade3* alleles. Surprisingly, these two revertants each proved to contain two suppressors, one capable of suppressing *tyr7-1*, the other responsible for suppression of *trp1-1*. These suppressors were provisionally classified as "amber" suppressors, although the data themselves suggest that the suppressors in 6190D-R7 may fall into the class of ochre-amber suppressors previously described by HAWTHORNE and MORTIMER (1968). Site specific suppression of several *ade3* alleles by one or more of these four suppressors was observed.

In Table 5 are reported data for crosses between 6190D-R7 and 8 independently isolated *ade3* alleles. All eight alleles are in genetic backgrounds free of *ade2-1*. If the *ade3* allele is suppressible by only one of the two suppressors segregating in the cross, 4:0, 3:1, and 2:2 segregations should be seen in the ratio 1:4:1. If the suppressor is effective, spores of genotype *SUP ade3-x* will be AD+; if ineffective spores of genotype *SUP ade3-x* will be AD-. If neither suppressor effects suppression of the *ade3* allele all tetrads should segregate 2:2 for AD+:AD-. Phenotypic ratios for spores of genotype *SUP ade3* have been entered in separate columns for the two suppressors segregating in the cross.

One allele, *ade3-26*, was suppressed by the suppressor of *tyr7-1* present in 6190D-R7. It was not suppressed by the suppressor of *trp1-1*. This allele, *ade3-26*, was not suppressed by the ochre suppressor *SUP2*. The remaining seven alleles, for which the data are given in Table 5, were not suppressed by either suppressor present in 6190D-R7. The efficiency of detection of "false tetrads" in these crosses was low as only two nonsuppressible unlinked markers were segregating. This may account for the deviations from expectation in tetrad ratios for crosses involving *ade3-38*, *ade3-41*, and *ade3-58*. In all three crosses it is quite clear that the genotypes *SUP-tyr7-1 ade3* and *SUP-trp1-1 ade3* are phenotypically AD-.

Results of crosses involving 18 independent *ade3* alleles and 6190D-R7 or

TABLE 5

Analysis for suppressibility by ochre-amber suppressors of 8 ade3 alleles

Relevant genotype of the diploids is of the form			$\frac{+ \quad SUP-trp1-1 \quad SUP-tyr7-1}{ade3-x \quad + \quad +}$						
<i>SUP</i> -bearing parent	<i>ade3</i> -bearing parent	<i>ade3</i> -allele number	Number of tetrads (+ : — phenotypes)					Phenotype of spores of genotype	
			4:0	3:1	2:2	1:3	0:4	<i>SUP-trp1-1</i> <i>ade3-x</i> Ad+ : Ad—	<i>SUP-tyr7-1</i> <i>ade3-x</i> Ad+ : Ad—
6190D-R7	a <i>ade3-26</i>	26	1	7	1	0	0	0:4	6:0
6190D-R7	a <i>ade3-34</i>	34	0	0	12	0	0	0:14	0:7
	a <i>ade3-38</i>	38	0	2	12	0	0	0:10	0:8
	a <i>ade3-40</i>	40	0	0	14	0	0	0:11	0:6
	a <i>ade3-41</i>	41	0	1	7	1	0	0:8	0:4
	a <i>ade3-58</i>	58	0	1	11	1	0	0:8	0:5
	a <i>ade3-59</i>	59	0	0	12	0	0	0:7	0:7
	a <i>ade3-67</i>	67	0	0	6	0	0	0:2	0:4

TABLE 6

Analysis for suppressibility by amber or ochre-amber suppressors of 18 ade3 alleles

Relevant genotype of the diploids is of the form $\frac{+}{ade2} \frac{+}{ade3-x} \frac{SUP-trp1-1}{+} \frac{SUP-tyr7-1}{+}$.

<i>SUP</i> - bearing parent	<i>ade3</i> - bearing parent	<i>ade3</i> - allele number	Phenotype of spores of genotype		
			<i>SUP-trp1-1</i> <i>ade3-x</i> Ad+ : Ad—	<i>SUP-tyr7-1</i> <i>ade3-x</i> Ad+ : Ad—	<i>SUP-trp1-1 ade3-x</i> or <i>SUP-tyr7-1 ade3-x</i> Ad+ : Ad—
D609-28C-R13	BW626	7			15:0
6190D-R7	HP73	43	0:3	8:0	
	NH31-85	47	1:0	1:0	6:0
	HAP-6-772	78	3:0	4:0	
	HAP-6-851	79	0:2	6:0	
	TW130	89		5:0	
D609-28C-R13	TW964	97	0:2	6:0	
	BW562	4	0:4	0:2	
D609-28C-R13	BW886	17	0:7	0:5	
	NH31-81	46	0:6	0:3	
6190D-R7	NH43-23	54	0:5	0:6	
	HAP-6-337	73	0:4	0:6	
	HAP-6-469	74	0:4	0:5	
	HAP-6-547	75			0:2
	HAP-6-972	81	0:11	0:8	
	HAP-6-883	83	0:3	0:4	
	HAP-2-72	84	0:5	0:3	
	TW713	95	0:6	0:1	

D609-28C-R13 are presented in Table 6. I have not presented segregation ratios for these diploids, for presence of suppression merely results in a shift in the relative frequencies of 2:2, 1:3 and 0:4 tetrads. As the ochre allele *ade2-1* is not suppressible by the "amber" suppressors, at least two spores in each ascus will be AD— whether or not the *ade3* allele is suppressed. As large numbers of tetrads were not dissected, sufficient data for classification of the allele on the basis of segregation patterns were not obtained.

In columns 4 and 5 of Table 6 are presented phenotypic ratios for spore clones of genotype *SUP-trp1-1 ade3-x* (column 4) or genotype *SUP-tyr7-1 ade3-x* (column 5). When the *ade3* allele was suppressed by both suppressors and the precise identity of the suppressor responsible for the suppressed phenotype could not be ascertained, the phenotypic ratio is presented in column 6.

The allele *ade3-7* was suppressed by both of the suppressors present in D609-28C-R13. This allele (*ade3-7*) was not suppressed by the ochre suppressor *SUP2* (Table 3). Alleles *ade3-89* and *ade3-97* are suppressed by the suppressor of *try7-1* present in D609-28C-R13. Neither of these alleles is suppressible by *SUP2*. There is no reason to think that the two suppressors present in D609-28C-R13 are other than amber suppressors. Hence *ade3-7*, *ade3-89* and *ade3-97* have been classified as amber mutations. The alleles *ade3-47* and *ade3-78* responded to the suppressors of *tyr7-1* and *trp1-1* present in 6190D-R7. These two alleles were suppressed by *SUP2* (see Table 2 for data) and are thus ochre alleles.

Additional support for the classification of these two *ade3* alleles as ochre alleles will be presented in the section on revertibility. The two suppressors present in 6190D-R7 apparently have the capacity to suppress both ochre (*ade3-47* and *ade3-78*) and amber (*tyr7-1* or *trp1-1*) mutations and hence fall into the category of ochre-amber suppressors (HAWTHORNE and MORTIMER 1968). Two additional *ade3* alleles were suppressed by the suppressor of *tyr7-1* present in 6190D-R7. Allele 79 is probably an ochre mutation suppressible by the ochre amber suppressor (see section on revertibility) whereas allele 43 may be an amber (see revertibility).

In the bottom portion of Table 6 are data indicating nonsuppressibility for eleven alleles by suppressors present in either 6190D-R7 or D609-28C-R13. Of these, three (*ade3-4*, -17, and -75) have been classified as ochre alleles on other grounds. The other eight have so far eluded classification.

Revertibility: The *ade3* alleles were originally isolated as white *ade2-1 ade3-x* derivatives of a red *ade2-1* strain according to the procedure described by ROMAN (1956). If the *ade3* allele is an ochre allele, mutational events giving rise to ochre suppressors should result in an AD+ phenotype in these *ade2-1 ade3-x* strains, for the *ade2-1* allele is known to be an ochre mutation (HAWTHORNE and MORTIMER 1968; HAWTHORNE 1969). Reversion to the AD+ phenotype in these *ade2-1 ade3* strains should be diagnostic of *ade3* ochre mutations. If the *ade3* allele is not an ochre allele, reversion to the AD+ phenotype in the *ade2-1 ade3-x* strains should require two mutational events and should be a relatively infrequent event.

Fourteen *ade2-1 ade3-x* strains were examined for reversion to the AD+ phenotype. The results are presented in Table 7. Ten strains gave AD+ revertants either spontaneously or upon irradiation with ultraviolet light. Four strains failed to revert to the AD+ phenotype. That ochre suppressors are involved in

TABLE 7

Reversion to AD+ phenotype of ade2-1 ade3-x strains as a test for ade3-x ochre alleles

Strain	Genotype	Reversion to AD+ in one step	Simultaneous reversion to TRP+, LYS+, AD+	Suppressibility by SUP2
TW49	<i>ade2-1 ade3-85</i>	+	+	UT
TW50	<i>ade2-1 ade3-86</i>	+	+	UT
TW69	<i>ade2-1 ade3-87</i>	+	+	+
TW120	<i>ade2-1 ade3-88</i>	+	+	+
TW130	<i>ade2-1 ade3-89</i>	—	—	—
TW140	<i>ade2-1 ade3-90</i>	+	+	+
TW621	<i>ade2-1 ade3-94</i>	+	+	+
TW713	<i>ade2-1 ade3-95</i>	—	—	—
TW913	<i>ade2-1 ade3-96</i>	+	+	+
TW964	<i>ade2-1 ade3-97</i>	—	—	—
TW1001	<i>ade2-1 ade3-99</i>	+	+	+
TW1009	<i>ade2-1 ade3-100</i>	+	+	+
TW1039	<i>ade2-1 ade3-101</i>	+	+	+
TW986	<i>ade2-1 ade3-98</i>	—	—	UT

the reversions to the AD⁺ phenotype was shown in the following analysis. The parental strain C62-30B carried three ochre alleles, namely *ade2-1*, *trp5-2*, and *lys2*. Selection was made for TRP⁺ LYS⁺ phenotype in the *ade2-1 ade3-x* derivative strains. The TRP⁺ LYS⁺ revertants (presumably suppressed by an ochre suppressor) were scored for the AD⁺ or AD⁻ phenotype. If the TRP⁺ LYS⁺ revertants are AD⁺, an ochre suppressor responsible for suppression of all four alleles (*ade2-1*, *ade3-x*, *trp5-2*, and *lys2*) is indicated. Of fourteen strains tested 10 gave simultaneous reversion for the three requirements and hence for the four alleles. These ten strains were the same strains which reverted to AD⁺ in a single step. Eleven of these fourteen *ade3* alleles have been crossed to the ochre suppressor *SUP2*. The three alleles which were not suppressed by *SUP2*, namely alleles 89, 95, and 97, when present as the *ade2-1 ade3-x* derivative did not give AD⁺ revertants. The eight alleles which were suppressed by *SUP2* were to be found in the class of strains which gave AD⁺ revertants when tested for revertibility in the *ade2-1 ade3-x* state (Table 7). There is thus a perfect correlation in these data between suppressibility by ochre suppressors and the ability to give rise to AD⁺ revertants in *ade2-1 ade3-x* strains.

Reversion studies have been carried out on 14 other strains known to be suppressible by ochre suppressors. Twelve of fourteen gave AD⁺ revertants in the double mutant reversion test described above. Two did not. A similar finding was reported by Esposito (1968). Reversion tests were performed on fourteen additional alleles known not to be suppressed by *SUP2*. In reversion tests of the double mutants of these 14 *ade3-x* alleles no AD⁺ revertants were detected. If an *ade2-1 ade3-x* strain gives AD⁺ revertants, the *ade3-x* allele can be classified as an ochre allele. Failure to detect AD⁺ revertants, however, does not ensure that the *ade3* allele is not an ochre mutation. This reversion test can be used to supplement the previous data for classification of ochre alleles.

On the basis of the criterion of revertibility eight alleles have been classified as ochre alleles, namely *ade3-4*, 19, 20, 46, 70, 79, 85, and 86. The reversion data for alleles 19 and 20 supplement the somewhat sketchy data on segregation patterns reported in Table 2. Allele 39 was classified as an ochre allele on the grounds that it was suppressed by a suppressor of *arg4-17* although not by *SUP2* (Table 3). Support for this assignment of *ade3* as an ochre mutation was obtained from reversion analysis, for *ade2-1 ade3-39* reverts to AD⁺ in one step.

Alleles *ade3-47* and *ade3-78* were classified as ochre alleles, for they were suppressed by *SUP2*. They were also suppressed by both suppressors present in 6190D-R7. Reversion analyses support the classification of these two mutations as ochre alleles. This result lends credence to the classification of the suppressors in 6190D-R7 as ochre-amber suppressors.

Two additional alleles were suppressed by the ochre-amber suppressors of 6190D-R7, namely *ade3-79* and *ade3-43*. Allele 79 was classified tentatively as an ochre allele for it gave AD⁺ revertants in the above test. It is currently being crossed to *SUP2*. The allele 43 has provisionally been classified as an amber allele, for AD⁺ revertants were not isolated in the double mutant reversion test. This assignment awaits confirmation by crosses to known amber suppressors,

FIGURE 2.—Recombinational analyses for crosses between *ade3-98* and *ade3-73* and *ade3* alleles of known map position. The *ade3* alleles indicated in the top line of the figure are arranged in map order (JONES 1972). Alleles 63 and 10 fail to recombine with alleles which lie over them. + indicates presence of AD+ recombinants; 0 indicates absence of recombinants; U T are combinations not tested. All recombinational analyses were meiotic tests.

for two known ochre alleles failed to give AD+ revertants in the double mutant reversion test.

Fine structure mapping: On the basis of data previously presented (JONES 1972), two *ade3* alleles, *ade3-10* and *ade3-63*, were classified as multisite mutants. Mapping data for alleles *ade3-73* and *ade3-98* are presented in Figure 2. The alleles indicated in the top line have been mapped previously and are arranged in their order in the map. Alleles 10 and 63 are indicated as bars. They fail to recombine with all mutations listed above them. Allele *ade3-98* fails to re-

TABLE 8

Assignment of mutational lesions in 56 ade3 mutations

Classification	Suppressible by ochre suppressor(s)	Suppressible by ochre-amber suppressor(s)	Suppressible by amber suppressor(s)	Reversion as <i>ade2 ade3</i>	Alleles showing pattern
Ochre	+	*	*	+	8, 11, 17, 19, 20, 22, 31, 33, 37, 39, 45, 75, 87, 88, 90, 94, 96, 99, 100, 101
	UT†	..	..	+	4, 46, 70, 85, 86
	+	+	..	+	47, 78
	+	..	..	UT	48, 50, 51, 56
	UT	+	..	+	79
	+	..	..	—	13, 36
Amber	—	+	UT	—	7, 26
	UT	+	UT	—	43
	—	UT	+	—	89, 97
Multisite or Complex	based on mapping analysis				10, 63, 98
Unknown	—	—	UT	—	73
	—	—	UT	—	34, 38, 40, 41, 54, 74, 81, 83, 84, 95
	—	—	UT	UT	58, 59, 67

* In order to minimize the number of patterns within the ochre category, crosses which were made but gave no evidence for suppression have not been separated from crosses which were not made. If either result obtained the entry has been left blank.

† UT = Untested.

TABLE 9

Classification of ade3 mutations according to the nature of the mutational lesion

Ochre	Amber	Multisite	Complex	Unknown	Total
34	5	3	1	13	56

combine with all mutations to the right of *ade3-37*, including *ade3-41*, the right-most allele. The bar in Figure 2 marked 98 indicates the extent of the lesion in this mutation. The allele *ade3-73* fails to show recombination with *ade3-63*, *ade3-14* and *ade3-57*. No straightforward explanation for the lesion in this mutant strain can be adduced. The lesion must take in at least two sites, namely *ade3-14* and *ade3-57*. This mutation was obtained from a culture which has been grown in the presence of 2-aminopurine.

Classification: A summary of the assignment of mutational lesions present in *ade3* alleles and the grounds on which the classification was made is given in Table 8. The data in Table 8 have been collected and condensed in Table 9. The allele for which the data are incomplete (*ade3-3*) has been omitted from Table 9.

DISCUSSION

Fifty seven mutations at the *ade3* locus have been crossed to ochre, amber, and ochre-suppressors. 70% (39/56) of the mutations at this locus are nonsense mutations; 61% (34/56) are ochre mutations and 9% (5/56) are amber mutations. Previous estimates of ochre mutations among nonselected alleles in yeast have ranged from 33% (HAWTHORNE and MORTIMER 1963; FINK 1966) to 41% (MANNEY 1964; ESPOSITO 1968), frequencies substantially less than the 61% reported for the *ade3* locus.

If nonsense mutations in eucaryotic systems are polar as they are in procaryotic systems (MARTIN *et al.*, 1966), these data raise the interesting possibility that all mutations at the *ade3* locus which have been recovered to date are polar mutations.

What of the 17 mutations which have not been classified as nonsense mutations? Three are multisite mutations and one has a complex lesion. On a random basis alone one would expect $\frac{2}{3}$ of all deletions to be out of phase deletions and hence to produce frameshifts. If these multisite mutations are out of phase deletions and if frameshifts in eucaryotic systems are polar as they are in procaryotic systems (MARTIN *et al.*, 1966) then the minimum estimate for the frequency of polar mutations at *ade3* is 75%.

Fourteen of the 26 previously mapped nonleaky alleles with low reversion rates for which there was no evidence for a multisite or complex lesion (JONES 1972) were crossed to amber or ochre-amber suppressors and to ochre suppressors. Seven of the 14 were not suppressed by any suppressor. These seven alleles comprise more than half (7/13) of the mutations for which the nature of the mutational lesion could not be assigned. It seems clear that the selection for nonleaky alleles with low reversion rates (a choice made to facilitate mapping)

results in a nonrandom sample of alleles for this locus. It is interesting to note that frameshift mutations, with few exceptions, have very low reversion frequencies in bacterial systems, much lower than the frequencies for nonsense mutations (WHITFIELD, MARTIN and AMES 1966). The choice of mutations with low reversion frequencies for mapping purposes may have militated against selection of nonsense mutations. Studies are currently underway to determine whether these 13 alleles of unknown lesion are frameshift mutations to see whether all mutations at the *ade3* locus which have been recovered to date are indeed polar mutations.

If only polar mutations are recovered at the *ade3* locus one would expect no bradytrophs, no temperature sensitive mutations and no osmotic remedial mutations to be recovered among *ade3* alleles. Findings previously reported (JONES 1972) are clearly not in accord with these expectations. For alleles at this locus, however, these characteristics seem not to be reliable indicators of missense mutations and hence cannot be used to argue for or against the hypothesis presented below.

Mutations in the *ade3* locus result in simultaneous loss of activity for three enzymes of tetrahydrofolate metabolism (JONES and MAGASANIK 1967a; LAZOWSKA and LUZZATTI 1970a, 1970b). Two of the enzymes, formyltetrahydrofolate synthetase and methenyltetrahydrofolate cyclohydrolase, are present at reduced levels whereas one of two isoenzymes for methylenetetrahydrofolate dehydrogenase is absent altogether (LAZOWSKA and LUZZATTI 1970a, 1970b).

An hypothesis to explain the genetical and biochemical findings is as follows. 1) That the *ade3* locus is the structural gene for the missing methylenetetrahydrofolate dehydrogenase, 2) that this gene is the proximal cistron of three contiguous cistrons, 3) that these three cistrons are transcribed as a unit, 4) that in order to recover an observable phenotype, namely a requirement for adenine, the cells must be deficient in two or more of the three interconversion enzymes for tetrahydrofolate which are missing in the *ade3* mutant strains, 5) that polar mutations in the gene for the dehydrogenase will result in loss of the dehydrogenase activity and coordinate reduction in the levels of cyclohydrolase and synthetase and will hence lead to an observable phenotype, namely the adenine requirement, 6) that nonpolar mutations will result in loss only of the dehydrogenase and will hence remain undetected.

This hypothesis will explain the very high frequency of nonsense mutations and by extrapolation, polar mutations found among *ade3* alleles. Total loss of activity for the one dehydrogenase in point mutations coupled with a reduction in level of the two other enzymes is explained as a consequence of the polarity (ZIPSER 1970).

One of the peculiar attributes of this hypothesis is that the cells must be deficient in at least two of the interconversion enzymes in order to be detectable as adenine requiring strains. A consideration of the pathway in Figure 1 discloses that the hypothesis described above is not unreasonable on physiological grounds. So long as the cell has ample endogenous supplies of formate and serine, loss of

the dehydrogenase or the cyclohydrolase or the synthetase should not lead to a deficit of 10-formyltetrahydrofolate or 5,10-methenyltetrahydrofolate, for there are two routes of synthesis for each of these derivatives.

The only evidence against the hypothesis comes from LOMAX, GROSS and WOODS (1971) who have isolated mutants deficient in cyclohydrolase and the dehydrogenase and partially deficient in synthetase. These mutants have an adenine requirement. Their strains and assay procedures were different and, consequently, their findings cannot be evaluated until such mutations are derived in our strains.

There are numerous predictions which arise from the hypothesis which I have presented. Mutations which result in deficiency for the synthetase or the cyclohydrolase should map adjacent to *ade3*. There might well be a polarity gradient. If there is a gradient of polarity the synthetase or cyclohydrolase deficient mutants should map at the distal end of the gradient. Enzymatic activities for the synthetase, cyclohydrolase and dehydrogenase should be regulated in a coordinated fashion. If multisite mutations are out of phase deletions, incorporation of one or two frameshift mutations of the same sign (the number needed will depend on the deletion) should revert these deletions. These and other questions are the subject of current investigation.

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