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Biochemistry

Function of yeast cytoplasmic C_1-tetrahydrofolate synthase

(*Saccharomyces cerevisiae*/heterologous gene expression/*ADE3*/purine biosynthesis)

JAE MAHN SONG AND JESSE C. RABINOWITZ*

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

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ABSTRACT **The protein product of the *ADE3* gene of the yeast *Saccharomyces cerevisiae* has been identified as the cytoplasmic trifunctional C_1-tetrahydrofolate (THF) synthase, which possesses 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5) activities. However, it has been suggested that the *ADE3*-encoded C_1-THF synthase does not play a role in providing the enzymes involved in the generation of one-carbon intermediates in the biosynthesis of the purine bases but functions in maintaining the structural integrity of the enzyme complex involved in purine biosynthesis [Barlowe, C. K. & Appling, D. A. (1990) *Mol. Cell. Biol.* 10, 5679–5687]. This hypothesis is based on their finding that the presence of the full-length ADE3 C_1-THF synthase, whether catalytically active or not, is correlated with the Ade$^+$ phenotype. In contrast to their results, our deletion analysis of the *ADE3* gene indicates that the presence of either the synthetase or dehydrogenase/cyclohydrolase domains of C_1-THF synthase is enough to complement the adenine requirement in *ade3* strains. These results are also consistent with those obtained in heterologous expression of spinach and *Clostridium acidiurici* monofunctional synthetases in *ade3* strains. Heterologous expression studies show that the high synthetase activity may be correlated with the increased growth in medium lacking adenine. These results suggest that the catalytic activity of the C_1-THF synthase is involved in purine biosynthesis.**

The one-carbon (C_1) units carried by tetrahydrofolate (THF) are enzymatically interconvertible to various states of oxidation from methanol (5-methyl-THF) through formaldehyde (5,10-methylene-THF) to formate (5,10-methenyl-THF, 10-formyl-THF, 5-formyl-THF, and 5-formimino-THF) (1) and finally CO_2 (2, 3). The principal source of the C_1 metabolite is 5,10-methylene-THF, normally derived from glucose via glycine (1), although it is also formed in a nonenzymic reaction by condensation of formaldehyde with THF. The THF derivatives serve as donors of C_1 units in a variety of biosynthetic reactions involving amino acids (methionine, serine, and glycine), pyrimidines (thymine), vitamins (pantothenic acid), purine bases (inosinic acid), and the initiation of protein synthesis in bacteria and organelles.

An enzymatic activity resulting in the formation of "active" formate (10-formyl-THF) in the presence of THF and MgATP was found in almost all cells examined, including those of bacteria, plants, yeasts, and animals (1). The only exception, to date, is the absence of the enzyme from *Escherichia coli* and other enteric bacteria (4, 5). However, the enzymes from yeast (6) and vertebrate species (1), unlike the monofunctional enzyme in prokaryotes, were shown to be trifunctional. They possessed, in addition to the 10-formyl-THF synthetase (SYN) (EC 6.3.4.3) activity, 5,10-methenyl-THF cyclohydrolase (CYC) (EC 3.5.4.9) and 5,10-methylene-THF dehydrogenase (DEH) (EC 1.5.1.5) activities (Fig. 1). The trifunctional enzyme was named C_1-THF synthase. The genes for the monofunctional SYN have been cloned and sequenced from *Clostridium acidiurici* (7), *Clostridium thermoaceticum* (8), and spinach (9). The genes yielding the trifunctional enzymes have been cloned and sequenced from the yeast cytoplasm (*ADE3*) (10) and mitochondria (*MIS1*) (11) and several other eukaryotic sources (12, 13).

It has been assumed that SYN or its corresponding domain on the trifunctional enzyme of eukaryotic origin is responsible for the synthesis of 10-formyl-THF, the essential donor of C-2 and C-8 of purine bases of the nucleic acid of cells (1, 14). It was therefore surprising to obtain evidence for the noncatalytic function of the *ADE3* gene product of the yeast *Saccharomyces cerevisiae* (15). The evidence is based on the observation that whereas deletion of the *ADE3* gene causes adenine auxotropy, presumably as a result of the lack of cytoplasmic 10-formyl-THF, point mutations inactivating any or all three activities of C_1-THF synthase do not result in a requirement for adenine. Heterologous expression of the *C. acidiurici* gene encoding a monofunctional SYN in an *ade3* deletion strain did not restore growth in the absence of adenine, even though the monofunctional SYN was catalytically competent *in vivo* (15). It was therefore proposed that the product of the *ADE3* gene plays a critical role in purine biosynthesis not related to its enzymatic activity. It was suggested that the gene product, C_1-THF synthase, functions in maintaining a structural complex composed of the enzymes involved in purine biosynthesis.

Our results do not support the proposed hypothesis and are not consistent with the proposed noncatalytic function of the *ADE3*-encoded C_1-THF synthase in purine biosynthesis.

MATERIALS AND METHODS

Strains and Media. *S. cerevisiae* strains used were GT48 (*MATa ade3-130 ser1-171 ura3-52*) and GT49 (*MATa ade3-30 ser1-171 ura3-52 trp1 leu2 his3 his4*). SD medium is 0.17% yeast nitrogen base/0.5% ammonium sulfate/2% glucose and was supplemented with the following nutrients where indicated (final concentration in mg/liter): adenine, 20; histidine, 20; serine, 400; uracil, 20; leucine, 30; tryptophan, 20; glycine, 20; and formate, 1000. Yeast transformations were performed by treatment with lithium acetate (16). Yeast DNA was prepared as described (17). *E. coli* DH1 (*recA1 endA1 gyrA96 thi hsrR17 supE44 relA1*) was used to propagate plasmids.

Plasmids. Plasmid pJS8A is a derivative of centromeric plasmid YCp50 and carries the yeast *URA3* and *ADE3* genes (18). Plasmid pBRYE2 is a derivative of pBR322 containing the *ADE3* gene (10) and was used to construct integrating

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Abbreviations: THF, tetrahydrofolate; SYN, 10-formyl-THF synthetase; CYC, 5,10-methenyl-THF cyclohydrolase; DEH, 5,10-methylene-THF dehydrogenase.

*To whom reprint requests should be addressed.

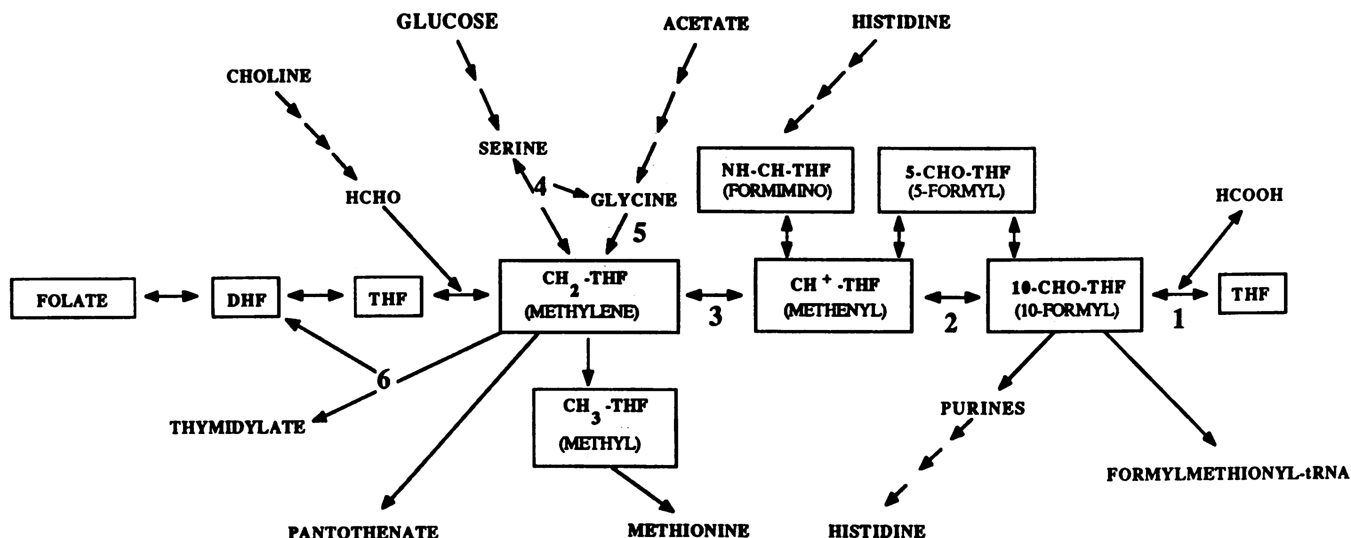

FIG. 1. THF coenzymes in C_1 metabolism. Pathways that utilize or generate folate coenzymes are indicated schematically by arrows. Enzymes shown are as follows: 1, SYN; 2, CYC; 3, DEH; 4, serine hydroxymethyltransferase (EC 2.1.2.1); 5, glycine decarboxylase complex; 6, thymidylate synthase (EC 2.1.1.45). DHF, dihydrofolate.

plasmid pJS24 carrying a partial deletion in the DEH/CYC domain of the *ADE3* gene by cleaving with *Xho* I and *Bgl* II restriction endonucleases, converting to blunt ends with the Klenow fragment of DNA polymerase I, and ligating (Fig. 2). The spinach SYN gene was placed under the control of the yeast *ADH1* promoter by cloning a *Bam*HI–*Xho* I fragment from pJMN1 (9) into the expression vector YEp24(*ADH1*) to yield multicopy plasmid pJS45 (Fig. 3). Plasmid pJS45 was then used to construct integrating plasmid pJS46 by removing 2μ DNA. Centromeric fusion plasmid pJS47 was constructed by fusing the *C. acidiurici* SYN gene to the amino-terminal region of the *ADE3* gene: a *Sph* I–*Sst* I fragment from pUC19(SYN3.1) was cloned into centromeric plasmid pJS8A-A-1 (Fig. 3). Multicopy plasmid pU1-CaS, obtained from D. A. Appling, carries the yeast *URA3* and the *C. acidiurici* SYN gene placed under the control of the *ADH1* promoter (15).

Localization of the *ade3-130* Deletion Region. The *ade3-130* mutation was genetically mapped as a deletion (19), but its deletion boundary has not yet been determined. In this study, the physical location of the *ade3-130* deletion region was determined by gap repair (20). The centromeric plasmid pJS8A was gapped by digestion with *Pvu* II. All Ura^+ colonies of *ade3-130* strain GT48 transformed with the gapped plasmid required adenine for growth. The DNA prepared from yeast transformants was transformed into *E. coli* DH1 and the plasmid prepared from DH1 cells was then characterized to determine the location of the *ade3-130* deletion region by restriction mapping (Fig. 2). This showed that the *ade3-130* mutation is a 6000-base-pair (bp) deletion including the *ADE3* coding sequence, consistent with enzymatic studies that the *ade3-130* mutant contains no C_1-THF synthase activity beyond the level of mitochondrial C_1-THF synthase (10, 11). The deletion extends about 200–450 bp from the *Sst* II site on the left and about 250-500 bp from the *Bgl* II site on the right (Fig. 2).

Determination of Growth Rate. Cultures were grown in 125-ml flasks at 30°C in a shaking water bath. Yeast cells carrying the *URA3*-bearing plasmids were grown in medium lacking uracil (SD supplemented with adenine, histidine, and serine). The strain carrying no *URA3*-bearing plasmid (pJS24) was grown in SD supplemented with adenine, histidine, serine, and uracil. Growth rates of all strains were determined in SD medium supplemented with the indicated nutrients. Cells grown to an OD_{600} of 1 were harvested and washed in SD and were then resuspended in SD and used to inoculate SD supplemented with the appropriate nutrients. Growth was monitored by measuring the OD_{600}.

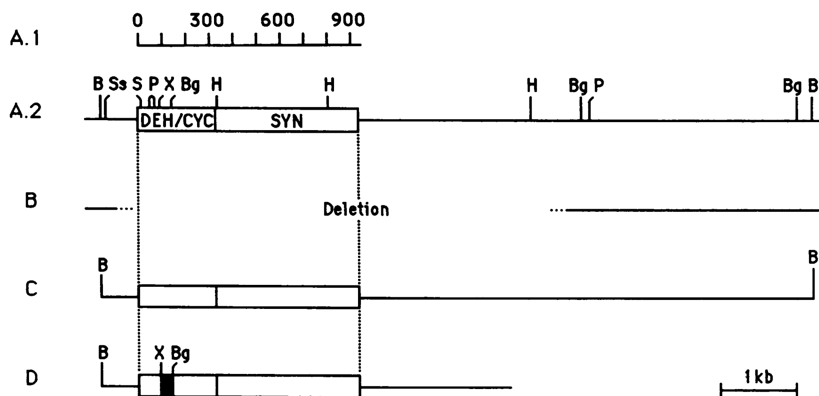

FIG. 2. Positions of deletions in the *ADE3* gene (945 codons) relative to amino acid positions. White boxed regions define the locations of ADE3 domains. Restriction sites: B, *Bam*HI; Bg, *Bgl* II; H, *Hin*dIII; P, *Pvu* II; S, *Sph* I; Ss, *Sst* II; X, *Xho* I. (*A.1*) Amino acid positions in the *ADE3* gene product. (*A.2*) Restriction map of the *ADE3* region. (*B*) Map of the *ade3-130* region. The extent of the deletion in the *ade3-130* allele is analyzed in the text. (*C* and *D*) Maps of pJS8A carrying *ADE3* and pJS24 carrying the deletion allele *ADE3*–ΔDEH/CYC. Black box represents the deletion (gap) generated in the plasmid-borne *ADE3* gene.

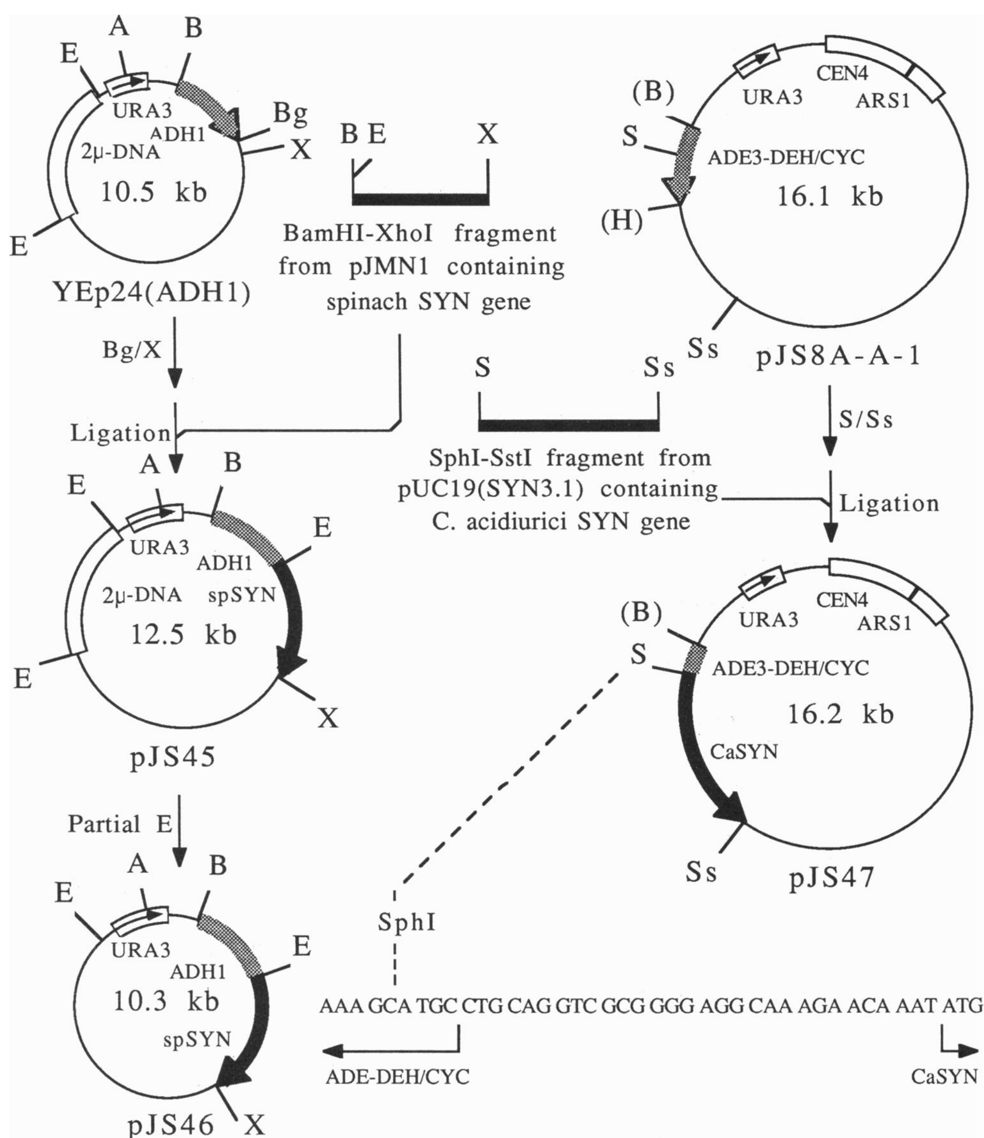

FIG. 3. Plasmids used for heterologous expression in *ade3-130* strains. A fusion of the amino-terminal region of the *ADE3*–DEH/CYC gene with the *C. acidiurici* SYN gene causes the addition of an extra 10 amino acids. Restriction sites: A, *Apa* I; B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; S, *Sph* I; Ss, *Sst* I; X, *Xho* I. The eliminated sites are shown in parentheses.

Enzyme Assays. Yeast cells carrying plasmids were grown to an OD_{600} of 1 in SD supplemented with appropriate nutrients. Cells were harvested by centrifugation at 4°C. The cell pellets were resuspended in 2 volumes of 50 mM Tris Cl, pH 7.5/10 mM KCl/50 mM 2-mercaptoethanol and were disrupted with glass beads. The lysates were centrifuged at 25,000 × *g* for 30 min. The supernatant fractions were used for enzyme and protein assays. The SYN and DEH activities were assayed as described (11). The activities are expressed in units (μmol of 5,10-methenyl-THF formed or hydrolyzed per min) based on an extinction coefficient of 24,900 $M^{-1} \cdot cm^{-1}$ for 5,10-methenyl-THF. Protein concentrations were determined by the dye-binding assay of Bradford (21) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Deletion Analysis of *ADE3* Gene. It was previously shown that the plasmid-borne amino-terminal portion of the *ADE3* gene, coding for a truncated protein retaining the DEH/CYC domain but not the SYN domain, was sufficient for complementation of the adenine requirement associated with an amber mutation *ade3-26* (18). Since this result is not in agreement with the results of Barlowe and Appling (15) that the Ade^+ phenotype correlates with the presence of the full-length ADE3 C_1-THF synthase whether catalytically active or not, we decided to investigate this issue further.

In the present study, we have constructed and examined the effect of a partial deletion in the amino-terminal portion of the *ADE3* gene that eliminates the DEH/CYC activities but retains the SYN activity in the *ade3-130* deletion strain (Fig. 2*D*). The constructed integrating plasmid, pJS24, carries a deletion of 56 amino acids (from the 115th to the 170th amino acid in the DEH/CYC domain) and thus encodes a slightly smaller protein than the ADE3 protein. The integrating plasmid pJS24 was digested with *Sst* II before transformation to promote integration at the *ade3* site. The gapped plasmid was then transformed into *ade3-130* strain GT48. Thirty-three Ade^+ colonies were obtained on −Adenine medium (see legend to Table 1 for composition), whereas no colony appeared in the control experiment with no plasmid. Integration at the *ade3* site was confirmed by Southern blot analysis (data not shown).

While strain GT48 transformed with vector YCp50 requires adenine for growth even when supplemented with formate, one transformed with the integrating plasmid pJS24 grows well on −Adenine medium and even better when supple-

mented with formate. However, it does not grow on +Glycine +formate medium, a medium in which *ser1 ADE3* strains can grow in the absence of serine by utilizing glycine and formate to synthesize serine (Fig. 1 and Table 1). This could be due to the recovery of the SYN activity and the lack of the DEH activity associated with the cytoplasmic C_1-THF synthase in the transformant with integrated plasmid pJS24. Enzyme assays showed that the transformant with the integrated plasmid pJS24 really retained the SYN activity but still lacked the DEH activity (Table 1). In contrast to the results of Barlowe and Appling (15), these results indicate that the presence of the full-length C_1-THF synthase is not necessary to complement the adenine requirement in *ade3* strains.

Effect of Heterologous Expressions of Monofunctional Synthetases in an *ade3-130* Strain. We have examined the effect of heterologous expression of spinach monofunctional SYN in *ade3-130* strain GT48. Multicopy plasmid pJS45 and integrating plasmid pJS46 carrying the spinach SYN gene placed under the control of the yeast *ADH1* promoter were transformed into strain GT48. The integrating plasmid pJS46 was digested with *Apa* I before transformation, to promote integration at the *ura3* site. Transformants with the integrated plasmid pJS46 require adenine for growth even when supplemented with formate. Surprisingly, the transformant carrying plasmid pJS45 grows well on −Adenine medium and even better when supplemented with formate (Table 1). In contrast, Barlowe and Appling (15) reported that overexpression of the *C. acidiurici* SYN gene in the *ade3-130* strain does not complement the adenine requirement even in medium supplemented with formate. We have constructed a centromeric fusion plasmid, pJS47, carrying the *C. acidiurici* SYN-coding sequence placed after the 11 codons of the *ADE3*−DEH/CYC gene, hoping that it could increase the efficiency of translation initiation. But strain GT48 transformed with plasmid pJS47 still requires adenine for growth even when supplemented with formate. To clarify the difference between the expressions of spinach and *C. acidiurici* SYN genes on multicopy plasmid in *ade3* strains, we have obtained multicopy plasmid pU1-CaS, which carries the *C. acidiurici* SYN gene placed under the control of the *ADH1* promoter (15), from Appling. As reported, strain GT48 carrying this pU1-CaS does not grow on −Adenine medium. However, we found that it grows slowly on −Adenine medium supplemented with formate (Table 1). This is in direct contrast to the results reported by Barlowe and Appling (15).

An alternative phenotypic assay for catalytic function of the ADE3 C_1-THF synthase not related to the adenine requirement is based on the inability of *ade3 ser1* mutants to satisfy their serine requirement by utilizing glycine and formate to synthesize serine (22). To test this catalytic ability of monofunctional synthetases *in vivo*, the integrating plasmid (pJS46), the single-copy plasmid (pJS47), and the multicopy plasmids (pJS45 and pU1-CaS) were transformed into *ade3-30 ser1* strain GT49. The integrating plasmid pJS46 was digested with *Apa* I before transformation, to promote integration at the *ura3* site. The *ade3-30* mutant exhibits wild-type levels of DEH and CYC activities but little SYN activity above that derived from the mitochondrial C_1-THF synthase, and it shows no growth on +Glycine +formate medium (15). All the transformants—including ones carrying the plasmids pJS46 and pJS47, which are unable to complement the adenine requirement in *ade3-130* strains—grow well on +Glycine +formate medium, like the one carrying the *ADE3* plasmid pJS8A (data not shown). This indicates that the monofunctional synthetases, even on an integrating or single-copy plasmid, are catalytically active *in vivo*.

Our most provocative result is the finding that the *ade3-130* strain GT48 transformed with multicopy plasmid pJS45 carrying the spinach SYN gene does not require adenine for growth. Table 1 shows that the expression of SYN genes in *ade3-130* strain GT48 complements the adenine requirement more efficiently in medium supplemented with formate, possibly due to the increase of substrate for SYN enzymes. These results confirm that any domain, SYN or DEH/CYC, of cytoplasmic C_1-THF synthase is enough to complement the adenine requirement in *ade3* strains, and suggest that the catalytic activity of C_1-THF synthase is involved in purine biosynthesis.

To determine whether the growth rate is related to the SYN activity in these transformants, we analyzed crude extracts of the transformants for their enzyme activities (Table 1). The results show that the transformants with multicopy plasmids had higher SYN activity than those with an integrated or single-copy plasmid. Furthermore, the transformants with multicopy plasmid pJS45 carrying the spinach SYN gene exhibited higher SYN activity than those with multicopy plasmid pU1-CaS, carrying the *C. acidiurici* SYN gene. These results suggest that the high SYN activity is correlated

Table 1. Phenotypes of *ade3* deletion strain carrying *ADE3* and heterologous genes

Plasmid	Plasmid copy type	Enzyme activity, units/mg		Growth rate (doubling time, hr)				Relative growth rate on −Adenine +formate*
		SYN	DEH	+Adenine	−Adenine	−Adenine +formate	+Glycine +formate	
Vector								
YCp50	Single	0.008	0.005	2.0	–	–	>24	0
ADE3 gene								
pJS8A	Single	0.184	0.026	2.0	2.0	2.0	5.4	1
pJS24	Integrated	0.269	0.004	2.4	4.1	2.4	>24	1
Spinach gene								
pJS45	Multiple	1.013	0.004	2.5	4.7	2.9	>24	0.9
pJS46	Integrated	0.031	0.006	2.0	–	–	>24	0
C. acidiurici gene								
pU1-CaS	Multiple	0.403	0.005	2.0	–	23	>24	0.1
pJS47	Single	0.051	0.005	2.0	–	–	>24	0

Yeast *ade3-130* strain GT48 was transformed with single-copy plasmids YCp50, pJS8A, and pJS47, integrating plasmids pJS24 and pJS46, and multicopy plasmids pJS45 and pU1-CaS. Enzyme activity was determined on cells grown in +Adenine for strains carrying YCp50, pJS46, and pJS47; −Adenine +formate for strains carrying pJS8A, pJS24, pJS45, and pU1-CaS. Growth rate was determined in SD supplemented with the following nutrients: adenine, histidine, and serine for +Adenine; histidine and serine for −Adenine; histidine, serine, and formate for −Adenine +formate; and adenine, histidine, glycine, and formate for +Glycine +formate. For the growth of the strain carrying no *URA3*-bearing plasmid (pJS24), uracil was added to each medium. –, No sign of growth by 3 days.

*Ratio of the doubling time in −Adenine +formate to that in +Adenine.

with the increased growth in medium lacking adenine. This is in direct contrast to the results of Barlowe and Appling (15) that the Ade$^+$ phenotype does not correlate with the catalytic activity of the ADE3 C_1-THF synthase.

Thus, two results shown in this paper are different from those of Barlowe and Appling (15). First, the full-length ADE3 C_1-THF synthase is not required to complement the adenine requirement in *ade3* strains. This was demonstrated by the deletion analysis of the *ADE3* gene, rather than the analysis of the *ade3* point mutants. Second, the expression of monofunctional synthetases in *ade3* deletion strains complements the adenine requirement for growth. The expression of the spinach SYN gene was found to complement the adenine requirement of *ade3* deletion strains even more effectively than the *C. acidiurici* SYN gene. These results are not consistent with the proposed noncatalytic function of the ADE3 C_1-THF synthase involved in purine biosynthesis (15).

Although our results are consistent with the conclusion that the product of the *ADE3* gene, C_1-THF synthase, is involved in the synthesis of purine bases through its catalytic function, some of our experimental observations are puzzling and require additional clarification. Thus, the SYN activity of the transformant with plasmid pU1-CaS containing the *C. acidiurici* SYN gene was lower by a factor of $\approx$2.5 than that observed in the transformant with plasmid pJS45, containing the spinach SYN gene, while the relative growth rate of the former was lower by a factor of 9. In addition, the enzyme level was $\approx$2-fold higher than that observed in the transformant with plasmid pJS8A, containing the *ADE3* gene, or plasmid pJS24, containing the *ADE3*$-\Delta$DEH/CYC gene, while its relative growth rate with each of these transformants was lower by a factor of 10.

These observations may result from the differences in the structures of the subunits of the yeast, spinach, and *C. acidiurici* enzymes and the resulting differences in the interactions and activities of the heterologous subunits in forming active monofunctional and trifunctional enzymes. Indeed, in addition to the differences in the characteristics of the peptides involved in formation of the active enzymes, it should be noted that the *C. acidiurici* SYN is a tetrameric enzyme (23), whereas yeast C_1-THF synthase (6) and spinach SYN (24) are dimeric enzymes, and that the spinach SYN subunit shows structural properties characteristic of enzymes of both eukaryotic and prokaryotic sources (9).

The inconsistencies in the activities of the various strains constructed in this study may also reflect the differences in the particular C_1 metabolites utilized in purine-base biosynthesis; that is, whether formate is available for use in satisfying the C_1-THF requirement via reaction 1 in Fig. 1, or whether the requirement for the C_1-THF metabolite is met entirely through activity of reactions 3 and 2 in Fig. 1. This question may be dependent on the degree of polyglutamylation of the C_1-THF coenzyme present and the affinity of the particular structural forms of the enzymes available for utilization of the particular polyglutamylation forms of the C_1-THF metabolite present. Previous results have shown that the polyglutamates predominate in the cell rather than the monoglutamate (25) and that particular polyglutamates present in cells are characteristic of different species or culture conditions [hexaglutamates predominate in yeast (25), tetra- and pentaglutamates in peas (26), and triglutamates in *Clostridium* (27)]. The polyglutamates have significantly greater affinities for the enzymes than the monoglutamic acid derivatives so commonly used in enzymatic studies (25, 28), and available evidence suggests that the polyglutamyl forms of the various C_1-THF coenzymes present in the cell may function in regulating the activity of particular enzymes with which they interact based on their differential kinetic activity (29, 30), in addition to their role in restricting diffusion of the monoglutamyl-THF coenzymes from cells (31). Unfortunately, the details on the structural forms of the enzymes and coenzymes present in the particular strains under investigation here or by Barlowe and Appling (15) and their affinities for the particular enzyme structures present are not known.

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