Cloning and Characterization of *FAD1*, the Structural Gene for Flavin Adenine Dinucleotide Synthetase of *Saccharomyces cerevisiae*

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Received 21 July 1994/Returned for modification 8 September 1994/Accepted 19 October 1994

The *FAD1* gene of *Saccharomyces cerevisiae* has been selected from a genomic library on the basis of its ability to partially correct the respiratory defect of *pet* mutants previously assigned to complementation group G178. Mutants in this group display a reduced level of flavin adenine dinucleotide (FAD) and an increased level of flavin mononucleotide (FMN) in mitochondria. The restoration of respiratory capability by *FAD1* is shown to be due to extragenic suppression. *FAD1* codes for an essential yeast protein, since disruption of the gene induces a lethal phenotype. The *FAD1* product has been inferred to be yeast FAD synthetase, an enzyme that adenylates FMN to FAD. This conclusion is based on the following evidence. *S. cerevisiae* transformed with *FAD1* on a multicopy plasmid displays an increase in FAD synthetase activity. This is also true when the gene is expressed in *Escherichia coli*. Lastly, the *FAD1* product exhibits low but significant primary sequence similarity to sulfate adenylyltransferase, which catalyzes a transfer reaction analogous to that of FAD synthetase. The lower mitochondrial concentration of FAD in G178 mutants is proposed to be caused by an inefficient exchange of external FAD for internal FMN. This is supported by the absence of FAD synthetase activity in yeast mitochondria and the presence of both extramitochondrial and mitochondrial riboflavin kinase, the preceding enzyme in the biosynthetic pathway. A lesion in mitochondrial import of FAD would account for the higher concentration of mitochondrial FMN in the mutant if the transport is catalyzed by an exchange carrier. The ability of *FAD1* to suppress impaired transport of FAD is explained by mislocalization of the synthetase in cells harboring multiple copies of the gene. This mechanism of suppression is supported by the presence of mitochondrial FAD synthetase activity in *S. cerevisiae* transformed with *FAD1* on a high-copy-number plasmid but not in mitochondria of a wild-type strain.

Previous biochemical analyses of respiration-defective *pet* mutants of *Saccharomyces cerevisiae* (30) led to the identification of two complementation groups lacking α -ketoglutarate dehydrogenase (KGDC), as a result of mutations in the *KGD1* and *KGD2* genes for the dehydrogenase (21) and transsuccinylase (22) components of the complex, respectively. The biochemical screens also disclosed that some complementation groups in the mutant collection are composed of strains deficient in both KGDC and pyruvate dehydrogenase (PDC). Most of the mutants displaying a pleiotropic loss of both activities have been ascertained to be blocked in either the biosynthesis or attachment of lipoic acid to the apoproteins. One complementation group (G178), however, had a distinctly different phenotype. The absence of KGDC and PDC activity in G178 mutants could not be explained by the absence of lipoic acid but, rather, was correlated with a defect in lipoamide dehydrogenase, the only enzyme common to both dehydrogenase complexes (20).

The results of complementation tests made it unlikely that mutations in *LPD*, the structural gene for lipoamide dehydrogenase (4, 23), were responsible for the enzyme deficiency in G178 mutants. The presence of the *LPD* transcript and the apoprotein in G178 mutants also excluded the possibility of the mutations affecting the expression of the lipoamide dehydrogenase subunit. These and other data strongly hinted that the loss of lipoamide dehydrogenase activity was related to a deficiency of the flavin adenine dinucleotide (FAD) coenzyme

and that the mutations, therefore, were likely to affect some aspect of flavin nucleotide metabolism.

To learn more about the nature of their biochemical lesion, we have isolated a yeast nuclear gene capable of correcting the respiratory defect of G178 mutants. In this communication we present evidence that the cloned gene, which we have designated *FAD1*, codes for yeast FAD synthetase, the enzyme responsible for converting flavin mononucleotide (FMN) to FAD (19). The partial restoration of mitochondrial respiration in G178 mutants by *FAD1* is shown to be due to extragenic suppression rather than to true complementation, thereby indicating that the mutations in G178 mutants are in some other nuclear gene, whose function we propose to be exchange of flavin nucleotides across the mitochondrial membrane.

MATERIALS AND METHODS

Strains of *S. cerevisiae* and growth media. The genotypes of the mutant and wild-type yeast strains used in this study are listed in Table 1. The following media were used for routine cultivation of yeast: YPD (2% glucose, 2% peptone, 1% yeast extract), YPGal (2% galactose, 2% peptone, 1% yeast extract), YEPG (3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract), and WO (2% glucose, 0.67% yeast nitrogen base without amino acids) (Difco). Solid media had the same compositions but in addition contained 2% agar. Amino acid and other auxotrophic requirements were added as needed to final concentrations of 20 μ g/ml.

Transformation of *S. cerevisiae*. E536/AL1 (*ade2 leu2 fad1*), obtained from a cross of the original isolate aE536 to LL20, was grown in 10 ml of YPGal to an approximate density of 10^7 cells per ml, and the entire culture was transformed by the procedure of Beggs (2), with a yeast genomic library consisting of partial *Sau3A* fragments of *S. cerevisiae* D273-10B nuclear DNA (7 to 10 kb in length) ligated to the *Bam*HI site of the shuttle vector YEpl3 (3). The transformation yielded a single respiration-competent and leucine-independent transformant (E536/AL1/T1), whose plasmid was used to subclone and characterize the *FAD1* gene.

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TABLE 1. Genotypes and sources of *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
D273-10B/A1	α <i>met6</i>	28
CB11	a <i>ade1</i>	27
LL20	α <i>his3-11,15 leu2-3,112</i>	17
W303	α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	R. Rothstein ^a
E536	α <i>met6 fad1</i>	This study
aE536	a <i>ade1 fad1</i>	E536 $\times$ CB11
E536/AL1	α <i>ade1 leu2-3,112 pet</i>	aE536 $\times$ LL20
W303 Δ FAD1/H	α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 fad1::HIS3</i> a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 FAD1</i>	This study
W303 Δ FAD1/L	α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 fad1::LEU2</i> a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 FAD1</i>	This study
W303 Δ FAD1/T1	α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 fad1::LEU2</i> + pSG178/T1	This study

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Preparation of mitochondria and nucleic acids from *S. cerevisiae*. Mitochondria were isolated from *S. cerevisiae* grown in liquid YPGal by the method Faye et al. (6), except that Zymolyase 20000 (ICN) instead of Glusulase was used to prepare spheroplasts. Total yeast RNA, prepared by the procedure of Laughon and Gesteland (10), was enriched for polyadenylated RNA by chromatography on poly(U) Sepharose 4B (Pharmacia). Total yeast DNA was obtained by lysis of spheroplasts with 2% sodium dodecyl sulfate–0.1 M NaCl–1 mM EDTA and extraction with phenol-chloroform-isoamyl alcohol (1:1:0.2). After precipitation with alcohol, nuclear DNA was separated from other nucleic acids by centrifugation in CsCl.

Disruption of *FAD1*. To construct disrupted alleles of *FAD1*, pSG178/ST10 (see Fig. 2) was digested with *Bcl*I. The linearized plasmid was ligated either to a 1.7-kb *Bam*HI fragment containing *HIS3* or to a 3-kb *Bgl*II fragment containing *LEU2*. The two disrupted alleles were isolated as linear fragments and were used to introduce the genes into the chromosomal DNA of respiration-competent haploid and diploid strains by the one-step gene replacement procedure of Rothstein (24).

Expression of *FAD1* in *E. coli*. To express *FAD1* in *Escherichia coli*, the entire coding sequence was transferred to pATH26, a vector containing the *trpE* promoter, regulatory sequences, and translational initiation codon. To achieve an in-frame fusion of *FAD1* to the translational frame of pATH26, the coding sequence was amplified by PCR with the two primers 5'-gagcacaatctgcagttgag caag and 5'-tgtatgataggtacacggtaac. The first primer is identical to the sequence reported in Fig. 3, from –9 to +15 except for the substitution of a C for the A at nucleotide +1. This single base change destroys the ATG initiation codon of the gene and creates a *Pst*I site which is in the same register as the *Pst*I cloning site in pATH26. The second primer is complementary to nucleotides 1060 to 1083 in Fig. 3 and also has a single change of a C for an A at nucleotide 1069, creating a *Bam*HI site. The PCR product was digested with a combination of *Pst*I and *Bam*HI and ligated to pATH26, linearized with the same two enzymes. This construct, referred to as pSG178/ST16, codes for a protein with a sequence identical to the sequence of the *FAD1* product starting from the second amino acid (glutamine) but has nine extra amino-terminal residues (MQTPK LACL) that are not present in the native protein. Clones expressing the fusion protein were grown as follows. *E. coli* RR1 cells transformed with pATH26 containing the *FAD1* coding sequence were grown in minimal medium (M9) containing 0.5% Casamino Acids, 20 μ g each of thiamine and ampicillin per ml to mid-log phase. The *trpE* promoter was induced by the addition of indoleacrylic acid, and growth continued for an additional 4 h until stationary phase was reached. The cells were harvested, washed in 20 mM Tris-HCl, pH 7.5, and suspended in twice their wet weight of 20 mM Tris-HCl, pH 7.5. Following digestion with 0.5 mg of lysozyme for 30 min, the suspension was sonically irradiated in three bursts of 1 min each with a Branson Ultrasonifier. The suspensions were centrifuged at 15,000 rpm for 15 min in a Sorvall centrifuge, and the clarified supernatants were collected and used to assay FAD synthetase as described in the legend to Fig. 6.

Manipulation of nucleic acids and miscellaneous methods. Standard methods were used for the transformation of *E. coli*, preparation of recombinant plasmids from *E. coli*, restriction enzyme mapping, and isolation and ligation of restriction fragments (13). The sequence of *FAD1* and flanking regions was determined by the method of Maxam and Gilbert (14) using 5'-end-labeled single-stranded restriction fragments. For the Northern (RNA) hybridizations, RNA was separated by electrophoresis in 1% agarose gels under nondenaturing conditions. Following transfer to diazobenzoyloxymethyl-paper, the blots were hybridized to nick-translated DNA probes. Southern blot analysis of yeast genomic DNA was done under previously described conditions (15). Proteins were separated electrophoretically in the buffer system of Laemmli (9).

Enzyme assays. Succinate-cytochrome *c* reductase activity was measured at

25°C by monitoring the reduction of cytochrome *c* at 550 nm. The 1-ml reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 40 μ M oxidized cytochrome *c* (horse heart, type III; Sigma), 15 mM sodium succinate, 3×10^{-4} M KCN, and 10 to 100 μ g of mitochondrial protein. Spectra of cytochromes were determined on detergent extracts of mitochondria (29). For the immunological detection of proteins by the Western (immunoblot) method, samples were separated on 12% polyacrylamide gels (9) and were transferred to nitrocellulose. The blot was incubated with diluted antiserum, and antigenically reactive proteins were visualized by a secondary reaction with ¹²⁵I-protein A. Protein concentrations were measured by the method of Lowry et al. (12).

Nucleotide sequence accession number. The sequence in Fig. 3 has been assigned GenBank accession number U12331.

RESULTS

Phenotype of G178 mutants. Complementation group G178 of the *pet* mutant collection used in this study (30) consists of three independent isolates obtained by mutagenesis of *S. cerevisiae* D273-10B/A1 with ethylmethane sulfonate. The mutants exhibit a leaky growth phenotype on rich medium containing glycerol as a carbon source. Growth on this nonfermentable substrate is restored when the mutants are crossed to *rho*⁰ testers, indicating that the respiration-deficient phenotype is due to recessive mutations in a nuclear gene.

The severity of the respiratory deficiency depends to some extent on the nuclear context of the mutations. For example, E536 displays considerably more growth on glycerol than does E536/AL1, which has the same mutant allele but in a different nuclear background. This is also evidenced by the levels of mitochondrial respiratory carriers in the two strains. Spectra of mitochondria of the original isolate E536 indicate a considerably higher content of cytochromes *a*, *b*, and *c* than that of the derivative mutant E536/AL1 (data not shown).

Biochemical screens of tricarboxylic acid cycle enzymes revealed that E536 and E536/AL1 have less than 10% of wild-type KGDC and PDC activities (Table 2). The near-complete absence in the mutants of overall dehydrogenase activities is due to a parallel decrease in lipoamide dehydrogenase (Table 2), the only component common to both complexes. The possibility that the lipoamide dehydrogenase defect might be caused by mutations in *LPD*, the structural gene for this enzyme (4, 23), was excluded on the basis of the ability of a known *lpd* mutant to complement the respiratory defect of G178 mutants. The absence of lipoamide dehydrogenase activity was also unlikely to be due to any obvious effect of the mutation on the expression of *LPD*. Northern blot hybridization of total and poly(A)-enriched RNA from wild-type and mutant cells indicated equivalent steady-state concentrations of *LPD* mRNA. Even though the mitochondrial concentration

TABLE 2. KGDC, PDC, and lipoamide dehydrogenase activities in wild-type and mutant mitochondria

Strain	Sp act ($\mu\text{mol}/\text{min}/\text{mg}$) ^a		
	KGDC	PDC	Lipoamide dehydrogenase
W303-1A	0.14	0.12	0.27
E536/AL1	<0.01	<0.01	0.01
E536/AL1/ST4	0.11	0.11	0.16

^a KGDC and PDC were assayed at 25°C by measuring the reduction of NAD⁺ in the presence of α -ketoglutarate and pyruvate, respectively (25). Lipoamide dehydrogenase was assayed in a buffer containing 20 mM NaPO₄, 0.1 mM KCN, 14 μM NAD⁺, 30 μM NADH, and 2 mM lipoamide. The reaction was started by addition of mitochondria, and oxidation of NADH was measured at 340 nm. The reaction rates were corrected for blank rates obtained in the absence of lipoamide (less than 10%).

of lipoamide dehydrogenase protein estimated from Western blot analyses is some two times lower in the mutant (data not shown), this was not sufficient to account for the virtually complete absence of enzyme activity.

The results of these studies suggested that the biochemical lesion responsible for the mutant phenotype was more likely to be involved in the synthesis, transport, or attachment of the FAD coenzyme to the apoprotein. This conclusion was further strengthened by the finding that G178 mutants lack succinate dehydrogenase, an enzyme known to also have FAD as its cofactor (data not shown). To test this possibility, the concentrations of FAD, FMN, and riboflavin in respiration-competent and mutant yeast strains were compared. Mitochondria

FIG. 1. HPLC analysis of flavin nucleotides in wild-type and mutant mitochondria. Mitochondria were prepared from the respiration-competent haploid strain D273-10B/AL1, from the G178 mutant E536/AL1, and from the same mutant transformed with pSG178/ST7. The mitochondria were suspended in 10 mM potassium phosphate buffer at a protein concentration of 5 to 10 mg/ml and incubated at 80°C for 15 min. The denatured proteins were removed by centrifugation at $105,000 \times g$ for 15 min. Extracts representing 70 μg of D273-10B/AL1 mitochondria, 62 μg of E536/AL1 mitochondria, and 102 μg of E536/AL1/ST7 mitochondria were adjusted to 30% methanol and separated on a $\mu\text{Bondapak C}_{18}$ column (Waters Inc.). The reverse-phase column was developed isocratically with 25% methanol in 10 mM potassium phosphate buffer, pH 6, at a flow rate of 2 ml/min. The elution times of flavin nucleotides were determined by chromatography of known standards. Flavin nucleotides were detected spectrophotometrically at 450 nm, and fluorimetrically with the excitation wavelength set at 450 nm and the emission wavelength at 530 nm. The tracings shown in the figure were recorded with the fluorescence monitor. The values reported for FAD and FMN are expressed in nanomoles per milligram of mitochondrial protein.

FIG. 2. Restriction map of pSG178/T1 and derivative plasmids. The partial map of the nuclear DNA insert in pSG178/T1 shows the locations of the restriction sites for *Bam*HI (B), *Hind*III (H), and *Kpn*I (K). The unique *Hind*III and *Sph*I (S) sites in the vector YEp13 are also marked for orientation purposes. The location of *FAD1* is indicated (arrow). The regions subcloned in YE351 are represented by horizontal lines in the upper part of the figure. + and -, complementation of E536/AL1 and lack of complementation, respectively.

were heat denatured under conditions known to release all flavins except those covalently attached to proteins (19), and the extracted flavin nucleotides were separated by reverse-phase chromatography. Elution of FAD, FMN, and riboflavin was monitored both by A_{450} and by fluorescence emission. Representative chromatographic elution patterns, shown in Fig. 1, indicate two opposite effects of the G178 mutation on mitochondrial flavin nucleotides. The first is a three- to four-fold lowering of the mitochondrial concentration of FAD, and the second is a twofold increase in the concentration of FMN. The altered concentrations result in an overall six to seven times lower FAD/FMN ratio in the mutant. Mitochondria of the *rho*⁰ mutant have slightly reduced levels of both nucleotides, but the ratio is not different from that of the wild-type strain (data not shown). These data indicate that the flavin nucleotide imbalance is a specific effect of the G178 mutation and not a general pleiotropic side effect of the respiratory deficiency.

Cloning of *FAD1*, a gene capable of suppressing the respiratory deficiency of G178 mutants. The gene containing the G178 alleles has recently been cloned in our laboratory (unpublished data). As indicated elsewhere in this communication, the encoded product belongs to the family of mitochondrial carrier proteins (18). In prior studies, however, transformation of the G178 mutant E536/AL1 with a yeast genomic library constructed in the shuttle vector YEp13 (3) yielded a respiration-competent clone (E536/AL1/T1) whose respiration-competent phenotype was found to depend on the presence of the episomal plasmid pSG178/T1, reported here.

The nuclear DNA insert of pSG178/T1 was ascertained to be 10 kb long (Fig. 2). To localize the gene responsible for restoring aerobic growth, different regions of the pSG178/T1 insert were transferred to the shuttle vector YEp351 (7) and the new constructs were tested for their ability to transform E536/AL1. The subcloning data indicated that the smallest fragment capable of correcting the respiratory defect of the mutant is defined by 1.7 kb of DNA cloned in the plasmid

-134
5'-GGATCCGTCTATAAGGAAGACCTCGAAAAATCTT

-100 CGAAAGATTTTGTGACTGAGAGTGAAAAAACTGATATTACTTTCTCGG TATAGAGGGCAACATTTGCAAAAAGTAATAAACAAATAGGGAGCACAAAT

+1 Met Gln Leu Ser Lys Ala Ala Glu Met Cys Tyr Glu Ile Thr Asn Ser Tyr Leu His Ile Asp Gln Lys Ser Gln
ATG CAG CTG AOC AAG GCT GCT GAG ATG TGT TAT GAG ATA ACA AAC TCT TAC TTA CAC ATA GAC CAG AAA TCT CAG

+76 Ile Ile Ala Ser Thr Gln Glu Ala Ile Arg Leu Thr Arg Lys Tyr Leu Leu Ser Glu Ile Phe Val Arg Trp Ser
ATA ATA GCA AGT ACA CAA GAA GCG ATA CGG TTG ACA AGA AAA TAC TTA CTA AGT GAA ATT TTT GTA CGT TGG AGT

+151 Pro Leu Asn Gly Glu Ile Ser Phe Ser Tyr Asn Gly Gly Lys Asp Cys Gln Val Leu Leu Leu Leu Tyr Leu Ser
CCA CTA AAT GGG GAA ATA TCA TTC TCG TAC AAC GGA GGA AAA GAT TGC CAG GTA TTA CTA CTG TTA TAT CTG AGT

+226 Cys Leu Trp Glu Tyr Phe Phe Ile Lys Ala Gln Asn Ser Gln Phe Asp Phe Glu Phe Gln Ser Phe Pro Met Gln
TGC TTA TGG GAA TAT TTC TTC ATT AAG GCT CAA AAT TCC CAA TTC GAT TTC GAG TTT CAA AGC TTC CCC ATG CAA
HindIII

+301 Arg Leu Pro Thr Val Phe Ile Asp Gln Glu Glu Thr Phe Pro Thr Leu Glu Asn Phe Val Leu Glu Thr Ser Glu
AGA CTT CCA ACT GTT TTC ATT GAT CAA GAA GAA ACT TTC CCT ACA TTA GAG AAT TTT GTA CTG GAA ACC TCA GAG
BclI

+376 Arg Tyr Cys Leu Ser Leu Tyr Glu Ser Gln Arg Gln Ser Gly Ala Ser Val Asn Met Ala Asp Ala Phe Arg Asp
CGA TAT TGC CTT TCC TTA TAC GAA TCA CAA AGG CAA TCT GGT GCA TCG GTC AAT ATG GCA GAC GCA TTT AGA GAT

+451 Phe Ile Lys Ile Tyr Pro Glu Thr Glu Ala Ile Val Ile Gly Ile Arg His Thr Asp Pro Phe Gly Glu Ala Leu
TTT ATA AAG ATA TAC CCT GAG ACC GAA GCT ATA GTG ATA GGT ATT AGA CAC ACG GAC CCA TTT GGT GAA GCA TTA

+526 Lys Pro Ile Gln Arg Thr Asp Ser Asn Trp Pro Asp Phe Met Arg Leu Gln Pro Leu Leu His Trp Asp Leu Thr
AAG CCT ATT CAA AGA ACA GAT TCT AAC TGG CCT GAT TTT ATG AGG TTG CAA CCT CTC TTA CAC TGG GAC TTA ACC

+601 Asn Ile Trp Ser Phe Leu Leu Tyr Ser Asn Glu Pro Ile Cys Gly Leu Tyr Gly Lys Gly Phe Thr Ser Ile Gly
AAT ATA TGG AGT TTC TTA CTG TAT TCT AAT GAG CCA ATT TGT GGA CTA TAT GGT AAA GGT TTC ACA TCA ATC GGC

+676 Gly Ile Asn Asn Ser Leu Pro Asn Pro His Leu Arg Lys Asp Ser Asn Asn Pro Ala Leu His Phe Glu Trp Glu
GGA ATT AAC AAC TCA TTG CCT AAC CCA CAC TTG AGA AAG GAC TCC AAT AAT CCA GCC TTG CAT TTT GAA TGG GAA

+751 Ile Ile His Ala Phe Gly Lys Asp Ala Glu Gly Glu Arg Ser Ser Ala Ile Asn Thr Ser Pro Ile Ser Val Val
ATC ATT CAT GCA TTT GGC AAG GAC GCA GAA GGC GAA CGT AGT TCC GCT ATA AAC ACG TCA CCT ATT TCC GTG GTG

+826 Asp Lys Glu Arg Phe Ser Lys Tyr His Asp Asn Tyr Tyr Pro Gly Trp Tyr Leu Val Asp Asp Thr Leu Glu Arg
GAT AAA GAA AGA TTC AOC AAA TAC CAT GAC AAT TAC TAT CCT GGC TGG TAT TTG GTT GAT GAC ACT TTA GAG AGA

+901 Ala Gly Arg Ile Lys Asn Och
GCA GGC AGG ATC AAG AAT TAA GTACGGTTTAAACGGAGGAAAG AAAATAAAATGCTGATGTTACTATTAGATTAATAAGTTTATATTTTCCG

+993 TTGAGACTATTTAATTTGTATATACAAGCCATGACTTTAATATTATCAA GTTTTCATTCTTGCTGCTTTTGGATATAAAATTAATCATGGGGTTTACCG

+1093 TTGATCCTATCATACAGCCTAGCGCGTGATAGTTAATATTGCTCTTTTG AACGTGTTTCTTTTGCCGAGGGCACTTTCAGTGAACCTTTTCAATT

FIG. 3. Nucleotide sequence of *FAD1*. The sequence reported is that of the sense strand starting from the *Bam*HI site in pSG178/ST7. The open reading frame identified as the *FAD1* gene has been translated. The *Hind*III site inferred to be internal to the gene from the subcloning data and the *Bcl*I site used for the disruption are indicated. The regions corresponding to the primers used for PCR amplification of the gene are underlined.

pSG178/ST7. This region is located between a *Bam*HI site and a *Hind*III site at the extreme right-hand edge of the insert in pSG178/T1, as depicted in Fig. 2. Further dissection of this region by subcloning of the 0.4-kb *Bam*HI-*Hind*III fragment (pSG178/ST8), or of the 1.2-kb *Hind*III fragment (pSG178/ST9), resulted in the loss of complementation, indicating that the gene probably spans the internal *Hind*III site of pSG178/ST7 (Fig. 2).

Approximately 1.2 kb of DNA, starting from the *Bam*HI site up to an *Ssp*I site, including the *Hind*III site determined from the subcloning tests to be essential for restoration of respiration, was sequenced. All the restriction sites used for 5'-end labeling were crossed from neighboring sites, and the sequence was confirmed from the complementary strands. Analysis of the nucleotide sequence revealed the presence of a single reading frame of sufficient length to qualify as a gene. This reading frame is initiated by an ATG codon 135 nucleotides away from the *Bam*HI site (nucleotide +1 in Fig. 3) and ends with an

ochre termination signal 919 nucleotides downstream of the initiation codon. This gene has been named *FAD1* since results presented below indicate that it codes for yeast FAD synthetase.

The amino acid sequence of the product encoded by *FAD1* is not homologous to that of any protein in the most recent data banks. It does, however, have some weak sequence similarity to sulfate adenylyltransferase (5, 11) suggestive of a distant evolutionary kinship (Fig. 4).

Disruption of *FAD1*. Even though G178 mutants harboring *FAD1* exhibit enhanced growth on glycerol, the gene does not fully restore respiratory function to wild-type levels. This is evident from the cytochrome spectrum (data not shown), and the FAD/FMN ratio (Fig. 1), of mitochondria isolated from the transformant. The inability of *FAD1* to completely restore a normal phenotype suggested that *FAD1* acts as a suppressor. This is supported by the recent isolation of a distinct gene that fully restores respiratory capability to G178 mutants by

FIG. 4. Homology of FAD synthetase and sulfate adenylyltransferase. The protein sequence of the yeast FAD synthetase (Sc FAD) is aligned with the sequences of the sulfate adenylyltransferases of *E. coli* (Ec SAT) (11) and *Rhizobium meliloti* (Rm SAT) (5). The alignments are based on the algorithm of Vingron and Argos (31). Only those residues of FAD synthetase identical to, or representing conserved substitutions with, those in at least one of the sulfate adenylyltransferases, have been boxed. Conserved substitutions are defined as R = K, D = E, M = I = L = V, N = Q, F = Y = W, A = G, and T = S. Positions of identity in all three proteins are indicated (asterisks).

complementation (unpublished results). Additional evidence that *FAD1* is a suppressor was obtained by genetic analysis of a diploid strain with one disrupted copy of the gene.

Alleles of *FAD1* disrupted at the *BclI* site with either *HIS3* or *LEU2* were substituted for the normal chromosomal copy of the gene in several different respiration competent yeast strains. Transformation of the haploid strains W303-1A and W303-1B with linear fragments of DNA containing the disrupted *fad1* alleles yielded a few transformant clones, all of which were respiration competent. Since Southern analyses of their genomic DNAs indicated the presence of the wild-type *FAD1* gene, the selectable markers (*HIS3* or *LEU2*) in these strains were concluded to have integrated in some other region of nuclear DNA. These results suggested that inactivation of *FAD1* is probably lethal to *S. cerevisiae*.

The essentialness of *FAD1* for cell viability was confirmed by replacement of only one copy of the gene in the diploid strain

W303. To assess the effect of mutations in *FAD1* on cell viability, randomly picked transformants selected for acquisition of the histidine or leucine independence were forced to sporulate and subjected to tetrad analysis. The results of the tetrad dissections indicated that the heterozygotes produced only two viable spores (Table 3). Furthermore, none of the viable spores recovered from the tetrads carried the marker (*LEU2* or *HIS3*) used for the disruption. Dissections were also done on the heterozygous diploid cells (containing one copy of *fad1::HIS3*) transformed with pSG178/T1. In this case all four meiotic spore progeny harboring the complementing plasmid were viable and respiration competent. Furthermore, segregation tests on the His⁺ Leu⁺ haploid cells confirmed that loss of the plasmid also led to loss of viability.

Diploid transformants, determined from the tetrad dissections to produce only two viable meiotic progeny, were also analyzed by Southern blot hybridization of genomic DNA to

TABLE 3. Tetrad analysis of diploid transformants

Allele and no. of tetrads ^a	No. of tetrads with the following no. of viable spores ^b :		
	2	1	0
<i>fad1::LEU2</i>			
11	11	0	0
10	3	3	4
10	10	0	0
9	8	1	0
8	6	2	0
<i>fad1::HIS3</i>			
10	9	1	0
9	9	0	0
8	7	0	1

^a Five Leu⁺ and three His⁺ clones obtained by transformation of the diploid strain W303 with the *fad1::LEU2* and *fad1::HIS3* alleles, respectively, were sporulated, and the indicated number of tetrads were dissected.

^b No tetrad had more than two viable spores. All the viable spores recovered from the tetrad dissections were Leu⁻ or His⁻.

confirm the presence of the *fad1::HIS3* or *fad1::LEU2* allele. Nuclear DNA, digested with *HindIII*, was hybridized with the nick-translated 1.2-kb *HindIII* fragment containing all but the first 95 codons of the gene. This probe detected only the homologous 1.2-kb *HindIII* fragment in the wild-type parent. The 1.2-kb *HindIII* fragment was also present in the transformants. Each of the transformants, however, had an additional band whose size was consistent with that expected from the maps of the disrupted alleles (data not shown).

To test whether the mutation in E536/AL1 is an allele of the *FAD1* gene, the mutant E536/AL1 was crossed to aW303Δ *FAD1*/T1, a haploid strain containing a disrupted chromosomal copy of *FAD1* and wild-type *FAD1* in the multicopy plasmid pSG178/T1. Following prototrophic selection, diploid cells were purified and grown under nonselective conditions. Diploid cells that had lost the plasmid (Leu⁻) were viable and fully respiration competent. The ability of the G178 mutants to complement a strain carrying a disrupted copy of *FAD1* provides strong evidence that the two mutations are in different genes.

Expression of the *FAD1* product in *E. coli* and correlation with FAD synthetase activity. Assays of subcellular fractions from a wild-type yeast strain indicated FAD synthetase to be present in the postmitochondrial supernatant fraction but not in mitochondria, suggesting that FAD synthetase is a cytoplasmic enzyme (Fig. 5). The specific activity of FAD synthetase was approximately four times higher in the postmitochondrial supernatant fraction of a G178 mutant transformed with *FAD1* on a multicopy plasmid. Of additional significance was the finding of low but measurable FAD synthetase in the mitochondrial fraction of the transformant. The latter activity was recovered quantitatively in the soluble matrix fraction following sonic disruption of mitochondria (Fig. 5).

The higher FAD synthetase in the transformant suggested that this enzyme might be encoded by *FAD1*. This possibility was tested by assays of FAD synthetase activity in crude homogenates of *E. coli* transformed with *FAD1* in a high-expression vector. This plasmid construct (pSG178/ST16) contains the entire coding sequence of *FAD1* fused to a short segment of DNA coding for eight amino acids not present in the original protein (see Materials and Methods). Polyacrylamide gel electrophoresis of total soluble proteins of several independent clones of *E. coli* harboring the plasmid with

FIG. 5. FAD synthetase activities of mitochondrial (MITO) and postmitochondrial (PMS) supernatant fractions. The parental respiration-competent yeast strain W303-1A (wild type [WT]) and the transformant aW303Δ*FAD1*/T1 (T1) were grown in YPGal to early stationary phase, and mitochondria were prepared. The mitochondria were layered over 25 ml of a discontinuous sucrose gradient (20, 30, 50, 60, and 80% sucrose) containing 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. The gradient was centrifuged at 24,000 rpm in a Beckman SW24 rotor for 60 min, and the mitochondrial fraction banding at the 30 and 50% sucrose interface was collected. The material was diluted with 10 volumes of 0.4 M sorbitol–20 mM Tris-HCl (pH 7.5) (ST), centrifuged at 79,000 × *g* for 15 min, and resuspended in a small volume of ST. Mitochondrial suspensions at 20 mg of protein per ml in ST were sonically irradiated for 5 s and centrifuged at 105,000 × *g* for 20 min. The supernatant consisting mostly of matrix proteins was decanted, and the pellet consisting of submitochondrial membrane vesicles was washed with an excess of ST and resuspended in the same volume of ST as the starting mitochondria. The postmitochondrial supernatant was centrifuged at 105,000 × *g* for 20 min to remove remaining membrane fragments and was dialyzed for 5 h against 20 mM Tris-HCl, pH 7.5. The postmitochondrial supernatant fraction was assayed for FAD synthetase in 20 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM ATP, 10 μM FMN, and 50 μg of supernatant protein. Mitochondria were assayed under the same conditions except that the amount of protein added was 200 μg. The volumes of submitochondrial membranes and the matrix fractions added to the assay were identical to that of mitochondria. After incubation at 37°C, the reactions were quenched by addition of 180 μl of 10 mM potassium phosphate, pH 6.5, preheated to 80°C. After further incubation at 80°C for 5 min, 65 μl of methanol was added and denatured proteins were removed by centrifugation in a Microfuge for 5 min. The supernatant was collected, and 100 μl was used for high-pressure liquid chromatography (HPLC) analysis. The amount of FAD synthesized was calculated as the difference between the amount of FAD measured at zero time and that after incubation for the indicated times. The values are expressed in nanomoles per milligram of mitochondrial or postmitochondrial supernatant protein. The activities in the mitochondrial membrane and matrix fraction are normalized for the volume of the mitochondria. ○, mitochondria; □, submitochondrial membranes; ●, soluble matrix fraction.

the fusion gene indicated the presence of a protein of the expected size (36 kDa). This protein is absent in control cells transformed with the empty vector (data not shown). The concentration of the protein identified as the product of the fusion gene, however, is not as high as might be expected on the basis of past experience with this expression system (unpublished data). The reason for the suboptimal level of the protein has not been studied but could be due to proteolytic degradation, a common occurrence with yeast proteins produced in *E. coli*.

In *S. cerevisiae*, the conversion of riboflavin to FAD involves two chemical events, each catalyzed by a separate enzyme. In the first step, riboflavin is phosphorylated to FMN by riboflavin kinase (8). The second enzyme, FAD synthetase, converts

FIG. 6. FAD synthetase activity in *E. coli* transformed with *FAD1*. *E. coli* RR1 cells transformed either with the control plasmid pATH26 or with the same plasmid containing the *FAD1* coding sequence were grown as described in Materials and Methods. (A) Extract (40 μg of protein) from control cells transformed with pATH26 was incubated at 37°C in 20 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 5 mM ATP, and 4.5 μM FMN. At the indicated times the reaction was stopped by addition of 180 μl of 10 mM potassium phosphate, pH 6, preheated to 80°C. After 2 min at 80°C, the mixture was adjusted to 25% methanol and denatured proteins were removed by centrifugation. A sample of the supernatant was separated on a reverse-phase C_{18} column as described in the legend to Fig. 1. The differences (Δ) in the amounts of FMN and FAD at 0 and 10 min are expressed in picomoles. (B) Same as panel A, except that the extract (57 μg of protein) was obtained from a clone transformed with pSG178/ST16 containing the *FAD* fusion gene.

FMN to FAD (26). To determine if *E. coli* cells transformed with *FAD1* in the high-expression vector have increased FAD synthetase activity, crude homogenates cleared of cell debris and particulate material were assayed for their ability to convert FMN to FAD. Following incubation of FMN and ATP in the presence of the soluble protein extracts from cells transformed with the control vector and with plasmid containing the fusion gene, the products were analyzed by HPLC on a reverse-phase C_{18} column under conditions that separate FAD from FMN. The results of these analyses show that extracts obtained from two independent clones transformed with *FAD1* are capable of efficiently converting FMN to FAD (Fig. 6). A similar extract, obtained from control cells transformed with the empty pATH26 vector, shows no FAD synthetase activity.

DISCUSSION

The respiratory deficiency of mutants previously assigned to complementation group G178 has been correlated with an imbalance of mitochondrial flavin nucleotides. Mitochondria of wild-type *S. cerevisiae* have a sixfold higher concentration of FAD relative to FMN. In the mutants, this ratio is reduced to less than one as a consequence of a decrease in FAD and a parallel increase in FMN. The altered stoichiometry of flavin nucleotides results in loss of activities catalyzed by FAD-dependent enzymes such as lipoamide dehydrogenase and succinate dehydrogenase. This effect was first noticed as a pleiotropic absence of KGDC and PDC activity in G178 mutants.

In the course of our attempts to clone the wild-type gene representative of G178 mutants, a plasmid capable of conferring growth of the mutant E536/AL1 on nonfermentable substrates was isolated from a yeast genomic library. Several lines of evidence indicate that the restoration of respiratory function by *FAD1* is due to suppression rather than true complementation of the mutation in E536/AL1. The growth phenotype of the transformant on the respiratory substrate glycerol is intermediate between that of the wild type and that of the mutant. This is also evidenced by a partial restoration of the FAD/FMN ratio in mitochondria to that seen with normal cells. More tellingly, an in situ disruption of *FAD1* is lethal and is complemented by G178 mutants.

The synthesis of FAD from riboflavin involves an initial phosphorylation of riboflavin to FMN, which then reacts with ATP to form FAD (1, 19). In *S. cerevisiae* and most other organisms studied, the two reactions are catalyzed, respectively, by riboflavin kinase (8) and FAD synthetase (26). One means to compensate for the abnormally low concentration of FAD in G178 mutants would be to increase the rate of FAD synthesis. Thus, the suppressor could act by affecting the efficiency of the first or second reaction. The higher concentration of mitochondrial FMN in the mutant argued against an effect of the suppressor on FMN production. Assays of FAD synthetase in subcellular fractions of respiration-competent cells, and of an *fad1* mutant harboring the wild-type *FAD1* gene on a multicopy plasmid, indicated higher FAD synthetase activity in the soluble protein fraction of the transformant. More significantly, while FAD synthetase activity was not detected in mitochondria of the wild-type parental strain, the mitochondrial fraction of the transformant had measurable activity. This presence of FAD synthetase in mitochondria of the transformant is probably due to mislocalization when the enzyme is expressed from a multicopy plasmid. Although uncommon, some cytoplasmic proteins can be inefficiently transported to mitochondria when overproduced (16; unpublished data).

The increased FAD synthetase activity of cells transformed with *FAD1* on a high-copy-number plasmid tended to support the notion that this gene codes either for a protein involved in regulation of the FAD biosynthetic pathway or for the FAD synthetase itself. The latter possibility was more likely in view of the weak but significant primary sequence similarity of the protein encoded by *FAD1* to sulfate adenylyltransferase. The conversion of FMN to FAD occurs via a transfer of the adenylyl group of ATP, a mechanism formally analogous to that catalyzed by sulfate adenylyltransferase. More definitive evidence supporting *FAD1* as the yeast FAD synthetase gene has been obtained by assays of the enzyme in *E. coli* cells transformed with an *FAD1*-bearing plasmid.

The precise nature of the biochemical lesion in G178 mutants is still not completely clear. As indicated above, FAD synthetase is not detected in mitochondria of wild-type *S. cerevisiae*. This would suggest that FMN is normally converted to FAD in the cytoplasmic compartment, from where it is transported to mitochondria. The FAD deficiency of G178 mutants could be a consequence of a flawed flavin nucleotide transport machinery. We have recently succeeded in cloning the gene defective in G178 mutants. The deduced sequence of the protein encoded by this gene indicates it to be a member of a large family of mitochondrial transport proteins that includes the adenine nucleotide, phosphate, and different carboxylate exchange carriers (18). While this evidence makes it fairly certain that the phenotype of G178 stems from a transport defect, it remains to be determined experimentally whether the ligand being affected is FAD or some other metabolite.

ACKNOWLEDGMENTS

This research was supported by research grant HL22174 from the National Institutes of Health. D.M.G. is the recipient of an MRC (Canada) postdoctoral fellowship.

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