Isolation of a *Saccharomyces cerevisiae* Long Chain Fatty Acyl:CoA Synthetase Gene (*FAA1*) and Assessment of Its Role in Protein *N*-Myristoylation

Robert J. Duronio, Laura J. Knoll, and Jeffrey I. Gordon

Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. Regulation of myristoylCoA pools in *Saccharomyces cerevisiae* plays an important role in modulating the activity of myristoylCoA:protein N-myristoyltransferase (NMT), an essential enzyme with an ordered Bi Bi reaction that catalyzes the transfer of myristate from myristoylCoA to ≥12 cellular proteins. At least two pathways are available for generating myristoylCoA: de novo synthesis by the multifunctional, multisubunit fatty acid synthetase complex (FAS) and activation of exogenous myristate by acylCoA synthetase. The *FAA1* (fatty acid activation) gene has been isolated by genetic complementation of a *faa1* mutant. This single copy gene, which maps to the right arm of chromosome XV, specifies a long chain acylCoA synthetase of 700 amino acids. Analyses of strains containing *NMT1* and a *faa1* null mutation indicated that *FAA1* is not essential for vegetative growth when an active de novo pathway for fatty acid synthesis is present. The role of *FAA1* in cellular lipid metabolism and protein N-myristoylation was therefore assessed in strains subjected to biochemical or genetic blockade of FAS. At 36°C, *FAA1* is required for the utilization of exogenous myristate by NMT and for the synthesis of several phospholipid species. This requirement is not apparent at 24 or 30°C, suggesting that *S. cerevisiae* contains another acylCoA synthetase activity whose chain length and/or temperature optima may differ from Faalp.

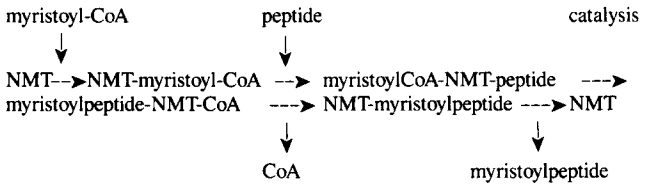
PROTEIN N-myristoylation refers to the co-translational (Wilcox et al., 1987; Deichaite et al., 1988) covalent attachment of myristate (C14:0) to the amino-terminal Gly residue of a variety of eukaryotic and viral proteins (reviewed in Towler et al., 1988*a*; James and Olson, 1990; Gordon et al., 1991). Myristate comprises a small fraction of cellular fatty acids (1–5% fatty acids; Boyle and Ludwig, 1962; Orme et al., 1972; Awaya et al., 1975). Its contribution to the biological function of certain N-myristoylproteins is great. For example, assembly of a number of retroviruses and picornaviruses that infect mammalian cells (including human immunodeficiency virus-1 and polio virus) depends upon addition of myristate to one of their capsid proteins (Gottlinger, 1989; Bryant and Ratner, 1990; Chow et al., 1987; Marc et al., 1989; Moscufo et al., 1991). Proteins involved in regulating cellular growth and signal transduction, such as the *src* family of tyrosine kinases and the GTP-binding α subunits of many heterotrimeric G proteins, require attachment of myristate for full expression of their biological activities (e.g., Cross et al., 1984; Kamps et al., 1985; Jones et al., 1990; Linder et al., 1991). Protein N-myristoylation is catalyzed by myristoyl-CoA protein N-myristoyltransferase (NMT)[1] (EC 2.3.1.97). Insertional mutagenesis or deletion of the gene encoding *Saccharomyces cerevisiae* NMT (*NMT1*) results in recessive lethality, indicating that this enzyme activity is essential for vegetative growth (Duronio et al., 1989). *S. cerevisiae*, *Candida albicans*, and human NMTs have overlapping yet distinct peptide substrate specificities (Towler et al., 1987*a*,*b*; 1988*a*,*b*; Duronio et al., 1991*b*; 1992; Wiegand et al., 1992). Together these observations have focused attention on the possibility that regulation of protein N-myristoylation in vivo may provide a new therapeutic strategy for treatment of viral and fungal infections as well as neoplastic processes.

The genetic manipulability of *S. cerevisiae* makes it an attractive model system for identifying factors that modulate this eukaryotic protein modification. Metabolic-labeling studies have identified ~12 proteins in this yeast that incorporate exogenous myristate (Duronio et al., 1991*a*). Five of the polypeptides are known. The functionally interchangeable ADP-ribosylation factors (Arf1p and Arf2p) have a role in protein secretion. Disruption of both *ARF* genes is lethal (Stearns et al., 1990*a*,*b*). Vacuolar sorting protein 15 (Vps15p) is a 1,455 residue serine/threonine kinase that is essential for growth at 37°C and is critically involved in protein sorting to the yeast vacuole (Herman et al., 1991). The nonessential *CNB1* gene encodes a 16-kD homolog of the mammalian, calcium binding, regulatory subunit of calcineurin, a type 2B phosphoprotein phosphatase (Cyert et al., 1991). *GPA1* is a haploid essential gene that encodes the α subunit of a

1. *Abbreviations used in this paper*: CER, cerulenin; MYR, myristic acid; NMT, myristoylCoA:protein N-myristoyltransferase; ORF, open reading frame; O6, 6-oxatetradecanoic acid; PAL, palmitic acid; YPD, yeast peptone dextrose.

heterotrimeric G protein involved in mating pheromone signal transduction. Genetic studies have shown that Gpalp is responsible for suppression of this signal (reviewed in Blumer and Thorner, 1991). Consequently, deletion of *GPA1* or $Gly^2 \rightarrow Ala^2$ mutagenesis of its protein (which blocks N-myristoylation) activates the mating response (Stone et al., 1991).

Studies of the regulation of protein N-myristoylation in *S. cerevisiae* have focused on characterization of purified NMT and mutations of *NMT1*. NMT is a monomeric 455-residue protein with functionally distinguishable myristoyl-CoA and peptide-binding sites (Towler et al., 1987*a*,*b*; Heuckeroth et al., 1988; Duronio et al., 1989; Rudnick et al., 1990, 1991; Kishore et al., 1991). Kinetic and biophysical studies have demonstrated cooperative interactions between these two sites. The enzyme has an ordered Bi Bi reaction mechanism (Rudnick et al., 1991):



This mechanism predicts that, in vivo, myristoyl-CoA binds to NMT before the enzyme interacts with its nascent polypeptide substrate. Therefore, any perturbation in the levels of intracellular myristoyl-CoA pools, or the ability of NMT to gain access to them, would be expected to have a profound effect on the efficiency of protein N-myristoylation. Recent studies using a *nmt1* mutant allele (*nmt1-181*) support these predictions. Strain LK181 was isolated as a temperature sensitive, myristic acid auxotroph: growth at the nonpermissive temperature is only possible when media are supplemented with $\geq$500 μM myristate (Meyer and Schweizer, 1974). This phenotype is a result of a single missense mutation of *NMT1* that changes Gly^{451} to Asp (Duronio et al., 1991a). In vitro studies of the purified mutant protein revealed a 10-fold increase in its apparent K_m for myristoyl-CoA at 36°C relative to wild type (Duronio et al., 1991*a*). Supplementation of media with myristate masks this defect apparently by increasing intracellular levels of myristoyl-CoA. *nmt1-181* mutant strains can therefore be used as a sensitive indicator of how myristoyl-CoA pools are regulated in vivo.

The de novo pathway for production of long chain acyl-CoAs in *S. cerevisiae* requires acetyl-CoA carboxylase (Acc1p, E.C. 6.4.1.2; Mishina et al., 1980), which produces malonyl-CoA for utilization by the $\alpha_6\beta_6$ fatty acid synthetase complex (FAS). FAS is a multifunctional 2.4 $\times$ 10^6 Da enzyme, composed of the products of the unlinked *FAS1* (β) and *FAS2* (α) loci (Burkl et al., 1972; Kuhn et al., 1972; Schweizer et al., 1986; Chirala et al., 1987; Mohamed et al., 1988; Siebenlist et al., 1990). Eight catalytic activities are associated with the complex (β-ketoacyl synthase, β-ketoacyl reductase, and the 4'-phosphopantetheine prosthetic group with the α subunit; acetyl transacylase, malonyl transacylase, malonyl and palmitoyl transferases, dehydratase, and enoyl reductase with the β subunit; Schweizer et al., 1970, 1978, 1986; Stoops and Wakil, 1978; Lynen, 1980; Singh et al., 1985). FAS produces palmitoyl-CoA and stearoyl-CoA as its principal products (Lynen, 1980). Myristoyl-CoA is a minor product representing 3–5% of the total acyl-CoAs produced by FAS in vitro and in vivo (Singh et al., 1985). The antibiotic cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-*trans,trans*-dodecadienamide, irreversibly inhibits *S. cerevisiae* FAS by covalently modifying the SH group of the cysteine residue of its β-ketoacyl-(acyl-carrier-protein) synthase activity (EC 2.3.1.41; cf, Vance et al., 1972; Kawaguchi et al., 1982; Funabashi et al., 1989). Cerulenin does not inhibit fatty acid elongation or desaturation systems present in *S. cerevisiae* (Awaya et al., 1975). Therefore, wild type strains of *S. cerevisiae* cannot grow in the presence of 25 μM cerulenin at 24, 30, and 36°C without supplementing glucose-rich medium with C12-C18 fatty acids (Awaya et al., 1975; Duronio et al., 1991*a*). Treatment of strains containing *nmt1-181* with cerulenin also causes growth arrest at these temperatures. This arrest can be reversed by adding exogenous myristate but not palmitate to the medium, suggesting that metabolic interconversion of C16:0 to C14:0 is not sufficient to restore myristoyl-CoA pools to a level that permits growth of this mutant (Duronio et al., 1991*a*). These observations suggest that the de novo pathway for fatty acid biosynthesis contributes to myristoyl-CoA pools that are used by NMT.

The ability of *exogenous* myristate to rescue strains containing *nmt1-181* grown at the nonpermissive temperature in the presence or absence of cerulenin suggests that supplementation of intracellular myristoyl-CoA pools by extracellular sources of C14:0 should depend upon activation of the fatty acid. AcylCoA synthetase (EC 6.2.1.3) catalyzes thioester formation between coenzyme A and free long chain fatty acids in a two step reaction:

$$\text{Free fatty acid} + \text{ATP} \xrightarrow{Mg^{2+}} \text{acyl-AMP} + PP_i$$

$$\text{Acyl-AMP} + \text{CoA} \longrightarrow \text{acylCoA} + \text{AMP}$$

Triacsin inhibitors of mammalian acylCoA synthetase have been identified, but their effects on the *S. cerevisiae* enzyme were not examined (Tomoda et al., 1991). However, mutations of a *S. cerevisiae* gene encoding an acyl-CoA synthetase have been identified in connection with studies on the mechanism of repression of acetyl-CoA carboxylase by exogenous long chain fatty acids (Kamiryo et al., 1976, 1977*a*). The decrease in acetyl-CoA carboxylase activity reflects a reduction in the cellular content of the enzyme and requires activation of the long chain fatty acids to their CoA derivatives. Mutants were identified that could not grow at 37°C on medium containing 25 μM cerulenin and palmitate, but that could grow in the absence of cerulenin. The mutations causing this phenotype were mapped to a single locus designated *faa1* (fatty acid activation). These mutants were found to be defective in acyl-CoA synthetase activity, which apparently accounts for their failure to repress acetyl-CoA carboxylase or to use exogenous palmitate as the sole source of fatty acids when the de novo pathway for fatty acid synthesis is blocked. Strains containing *nmt1-181* and *faa1* mutations do not grow at 36°C on glucose rich medium containing 500–1,000 μM myristate, in contrast to strains containing *nmt1-181* and wild type *FAA1* which do (Duronio et al., 1991*a*). These observations support the notion that Faa1p is important for supplementing intracellular myristoyl-CoA pools from exogenous myristate.

To definitively assess the role of Faalp in regulating intracellular pools of myristoyl-CoA, we have isolated the *FAA1* gene by complementation of the *faa1* phenotype, characterized its protein product, and subsequently created strains with *faa1* null mutations. Genetic and biochemical analyses of these strains indicate that the product of the single copy *FAA1* gene affects the utilization of exogenous fatty acids for protein N-myristoylation and phospholipid synthesis in *S. cerevisiae* at some but not all temperatures. Our analyses suggest that this yeast may contain other acylCoA synthetase activities that modulate protein N-myristoylation and supply acylCoAs for phospholipid biosynthetic pathways.

Materials and Methods

Strains and Media

All yeast strains were constructed using standard methods (Sherman et al., 1986) and are listed in Table I. YPD (1% yeast extract, 2% peptone, 2% dextrose) was supplemented with 500 μM myristate (YPD-MYR), 500 μM palmitate (YPD-PAL) with or without 25 μM cerulenin (Sigma Chemical Co., St. Louis, MO), or combinations of cerulenin and fatty acids at these concentrations (i.e., YPD-CER; YPD-MYR/CER; YPD-PAL/CER). YPD-O6 contains 500 μM 6-oxatetradecanoate (O6; Heuckeroth et al., 1988). YPD-MYR, YPD-PAL, YPD-O6, YPD-MYR/CER, and YPD-PAL/CER also contained 1% (wt/vol) Brij 58 (Sigma Chemical Co.) to help solubilize the fatty acids. Myristate and palmitate were purchased from NuCheck Prep (Elysian, MN). O6 was synthesized as in Kishore et al. (1991).

Cloning of FAA1

The *faa1* strain YB241 (Table I) was grown in YPD at 30°C until an A_{600} of 2.5 was achieved. Spheroplasts were then prepared and transformed (Hinnen et al., 1978) with a library of *S. cerevisiae* genomic DNA (constructed in YEp24; see Carlson and Botstein, 1982). Ura^+ transformants were transferred to YPD-MYR/CER plates and incubated at 36°C for 1–2 d. Plasmid DNA was recovered (Hoffman and Winston, 1987) from transformants that grew under these conditions. *FAA1* was sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977). Nested deletions (pBluescript Exo/Mung Bean DNA sequencing system; Stratagene, La Jolla, CA) of *FAA1* and synthetic oligonucleotide primers were used to define the sequence of both strands of the cloned DNA.

Database Searching

Homology searches were performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al., 1990). The following non-redundant databases were searched: NBRF/PIR (Release 29.0), SWISS-PROT (Release 19.0), GenPept (translated Genbank, Release 64.3), and GPUpdate (GenPept daily update: Sept 23, 1991).

FAA1 Locus Alterations

Alterations of the *FAA1* locus were generated by transforming diploid strain YB100 (Table I) to histidine prototrophy with linear DNA containing the desired mutation (Rothstein, 1983). The *faa1::HIS3* allele was made by replacing a 341-bp fragment of the *FAA1* coding region (beginning at the EcoRI site at nucleotide 1063 as seen in Fig. 1 *B*) with a 1.7-kb EcoRI-BamHI restriction fragment (Fig. 1 *A*) containing *HIS3* (Struhl, 1985). The *faa1Δ1.9::HIS3* allele was made by replacing a 1.9-kb region of *FAA1* bordered by BglII sites with a 1.7-kb BamHI-BamHI fragment containing *HIS3* (Fig. 1 *A*). Each change in the *FAA1* locus was confirmed by Southern blot hybridization analysis.

Blot Hybridization Analyses

Yeast genomic DNA was isolated (Denis and Young, 1983), digested with a series of restriction endonucleases, fractionated by electrophoresis through a 0.8% agarose gel, and transferred to Hybond-N+ membrane (Amersham International, U.K.). Total yeast RNA was isolated (Elder et al., 1983) and fractionated through a 1.5% agarose/formaldehyde gel (Sambrook et al., 1989), and transferred to Hybond-N+. These filters were probed with a [^{32}P]dATP-labeled, 4.2 kb, ScaI-ScaI fragment containing the entire *FAA1* coding region. Blots were hybridized in a solution containing 5× SSPE, 5× Denhardt's (see Sambrook et al., 1989), 0.1% SDS (DNA blot) or 0.5% SDS (RNA blot), and 20–40 μg/ml denatured, sheared salmon sperm DNA at 65°C for 18 h. They were subsequently washed at 65°C with 2× SSC (DNA blot) or 0.1× SSC (RNA blot) and then subjected to autoradiography at −70°C.

Mapping of FAA1

Filters containing electrophoretically separated *S. cerevisiae* chromosomes were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). A set of three nylon filters containing prime lambda clones (see Results) were obtained from Linda Riles and Maynard Olson (Department of Genetics, Washington University). Both sets of filters were probed with the 4.2-kb ScaI-ScaI *FAA1* fragment (see above) in a solution containing 0.5 M NaCl, 0.1 M Na_2HPO_4, 5 mM Na_2EDTA, 1% sarkosyl, 100 μg/ml denatured, sheared salmon sperm DNA. After an overnight incubation at 65°C, the filters were subsequently washed once at 24°C with 1 mM Tris-HCl, pH 8.0, plus 1% sarkosyl followed by 1 mM Tris-HCl, pH 8.0 (also at 24°C).

Metabolic Labeling of Yeast Cellular Proteins and Lipids

Strains YM2061 and YB363 (Table I) were grown in YPD at either 24, 30, or 36°C until they reached an A_{600} of 1.5. They were then incubated for 1 h with either [9,10(n)-^{3}H]myristate (100 μCi/ml of culture, 54Ci/mmol;

Table I. Yeast Strains

Strain	Genotype	Reference
BJ405	*MATa trp1 prb1-1122 prc1-126 pep4-3*	Hemmings et al., 1981
YM2061	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met LEU2::GAL1-lacZ*	Flick and Johnston, 1991
YB100	*MATa/α ura3-52/ura3-52 his3Δ200/his3Δ200 ade2-101/ade2-101 lys2-801/lys2-801 met/+ +/tyr1-501 LEU2::GAL1-lacZ/LEU2::GAL1-lacZ*	Duronio et al., 1989
YB218	*MATa nmt1-181 ura3-52 his3Δ200 ade2-101 lys2-801 met LEU2::GAL1-lacZ*	Duronio et al., 1991a
YB241	*MATa faa1 ura3-52 his3Δ200 ade2-101 lys2-801 met*	Duronio et al., 1991a
YB360	*MATa/α FAA1/faa1Δ1.9:HIS3 ura3-52/ura3-52 his3Δ200/his3Δ200 ade2-101/ade2-101 lys2-801/lys2-801 met/+ +/tyr1-501 LEU2::GAL1-lacZ/LEU2::GAL1-lacZ*	This work
YB363	*MATafaa1Δ1.9::HIS3 ura3-52 his3Δ200 ade2-101 lys2-801 met LEU2::GAL1-lacZ*	
YB364	*MATα faa1Δ1.9::HIS3 ura3.52 his3Δ200 ade2-101 lys2-801 tyr1-501 LEU2::GAL1-lacZ*	This work
fas1	*MATα fas1-70*	Schweizer et al., 1978
fas2	*MATα fas2-38*	Schweizer et al., 1978
YB366	*MATα fas2-38 ura3-52 his3Δ200 ade2-101 met*	This work
YB371	*MATa nmt1-181 faa1Δ1.9::HIS3 ura3-52 his3Δ200 ade2-101 lys2-801 met LEU2::GAL1-lacZ*	This work

Amersham Corp.) or [9,10(n)-^{3}H]palmitate (100 μCi/ml of culture, 35.6 Ci/mmol; NEN Research Products, Dupont, Wilmington, DE). Cellular proteins were prepared for SDS-PAGE (Laemmli, 1970) and fluorography exactly as described (Duronio et al., 1991*a*). Lipids were extracted from yeast spheroplasts (Hinnen et al., 1978) using the method of Bligh and Dyer (1959), dried, and stored at −20°C. Lipids were resuspended in 500 μl chloroform:methanol (1:1). Aliquots representing material prepared from an equal number of cells were spotted onto Silica Gel 60 high performance thin layer chromatography plates (Merck, Rahway, NJ) adjacent to purified lipid standards (Sigma Chemical Co.). Phospholipids were separated in a single dimension using methyl acetate:2-propanol:chloroform:methanol:0.25% aqueous KCl (25:25:28:10:7). Standards were visualized by iodine vapor. Radiolabeled lipids were identified by spraying the plates with En^{3}Hance (New England Nuclear/Dupont) and performing fluorography at −80°C.

Long Chain Acyl:CoA Synthetase Activity Assay

This assay is a modification of an assay described in Kishore et al. (1991). Briefly, reactions (150 μl) containing 10 mM Tris, pH 7.4, 2 mM CoA, 2 mM ATP, 10 mM $MgCl_2$, 3 mM DTT, 0.05% Triton X-100, 100 μM EGTA, 0.5 μM [^{3}H]myristic acid (39.3 Ci/mmol; NEN Research Products, Dupont), and fractions prepared from *S. cerevisiae* (up to 100 μg protein) were incubated for 20 min at 25°C. The reactions were subsequently quenched with an equal volume of 5% TCA/methanol, cooled on ice for 5 min, and centrifuged at 10,000 *g* for 3 min. [^{3}H]Myristoyl-CoA and [^{3}H]-myristic acid were resolved by C4 reverse-phase HPLC (Vydac, 5 μm × 4.6 mm) using an isocratic gradient of 60% 20 mM KPO_4, pH 5.5, and 40% acetonitrile (flow rate = 2 ml/min). Tritiated products were quantitated using an in-line scintillation counter (model CR; Radiomatic Instruments and Chemical Co., Inc.). Under these conditions, [^{3}H]myristoyl-CoA typically eluted at 5 min and [^{3}H]myristic acid at 10 min.

Purification of Faalp

A three step protocol was used for purification of acylCoA synthetase from lysates of *S. cerevisiae*.

Preparation of a 10,000 g Supernatant Fraction

Strain BJ405 (Table I) was grown in 4 liters of YPD at 30°C to an A_{600} of 1, at which time an additional 80 g of glucose was added to maintain log phase growth. Yeast were harvested at an A_{600} ~6 with a yield of 50 g wet cells. All subsequent manipulations were carried out at 4°C. Cells were resuspended in an equal volume of homogenization buffer (0.2 M Tris-HCl, pH 8.1, 6 mM DTT, 4 mM EDTA, 4 mM benzamidine, 1 mM PMSF, 8 μM leupeptin, 4 μM pepstatin A, 10% glycerol, 0.1% Brij 35) and disrupted using 500 μm glass beads and a Biospec Bead Beater (Biospec Products, Bartlesville, OK). Five 1-min homogenization periods were interspersed with 1-min incubations on ice. The homogenate was spun at 10,000 *g* for 30 min. Approximately 96% of the acylCoA synthetase activity was recovered in the 10,000 *g* supernatant.

P11 Cellulose Phosphate Chromatography

The 10,000 *g* supernatant (70 ml) was diluted to 400 ml with 1 mM EDTA, 1 mM PMSF, and then stirred for 2 h with 10 g of P11 cellulose phosphate (P11) resin (Whatman BioSystems Ltd., Kent, England) that had been preswollen and equilibrated in buffer A (20 mM KPO_4, pH 7.4, 1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.01% Triton X-100). The batch-bound P11 resin was poured into a 30 × 1.6 cm column. The 60 ml P11 column was subsequently washed with 2 vol of buffer A followed by 2 vol buffer B (100 mM KPO_4, pH 7.4, 1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.01% Triton X-100). AcylCoA synthetase was eluted with buffer C (250 mM KPO_4, pH 7.4, 1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.01% Triton X-100), and 5-ml fractions collected for assays of enzymatic activity.

CoA-Agarose Affinity Chromatography

P11 fractions containing the highest specific activity of enzyme were pooled (final volume = 30 ml) and diluted to 300 ml with 2 mM ATP (to stabilize enzyme activity), 1 mM EDTA, and 1 mM PMSF. Material was then applied to a 7-ml column (14 × 0.8 cm) of CoA-agarose Type 5 (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with buffer A. The column was washed with 2 vol buffer A supplemented with 2 mM ATP. Activity was eluted using a linear gradient from buffer A + 2 mM ATP to buffer D (400 mM KPO_4, pH 7.4, 2 mM ATP, 1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.01% Triton X-100). 2-ml fractions were collected and assayed for enzyme activity and by SDS-PAGE (Laemmli, 1970).

Automated Sequential Edman Degradation

Fractions from the CoA-agarose column were reduced, denatured, and separated by SDS-PAGE. After electrophoretic transfer to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Continental Water Systems, Bedford, MA), individual bands were excised with the filter and introduced into gas phase sequencer (model 470A; Applied BioSystems, Foster City, CA). Phenylthiohydantoin-amino acids derived from each cycle of Edman degradation were identified with an in line detector (model 120A; Applied BioSystems).

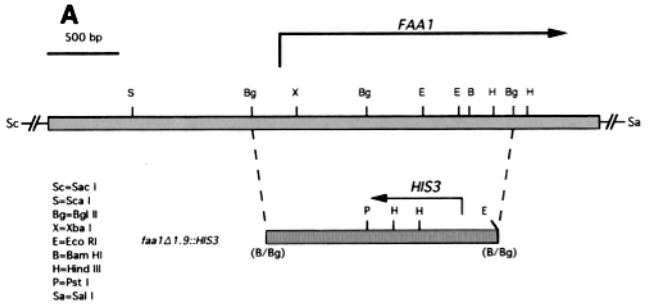
Results

Isolation of FAA1 by Complementation Cloning and Definition of the Primary Structure of Its Protein Product

Since the *faal* mutation identified by Kamiryo et al. (1976) causes an easily scored phenotype, we attempted to clone *FAA1* by genetic complementation. The *faal* strain YB241 (Table I) is unable to grow on YPD containing 500 μM myristate and 25 μM cerulenin at 36°C (Duronio et al., 1991*a*). YB241 spheroplasts were transformed to Ura$^+$ with a yeast genomic library constructed with the high copy 2 μ circle vector YEp24 (Carlson and Botstein, 1982).

Approximately 20,000 of these transformants were transferred to YPD-MYR/CER plates and incubated at 36°C for 1–2 d. Two transformants were isolated that formed colonies under these selective conditions. Ura$^-$ mitotic segregants of these isolates, obtained after nonselective growth in YPD medium, were unable to grow on YPD-MYR/CER medium, suggesting that the two isolates contained a plasmid capable of complementing the *faal* mutation. Restriction endonuclease mapping indicated that each isolate's plasmid contained an identical 5.8-kb insert. Retransformation of strain YB241 with this plasmid DNA permitted growth on YPD-MYR/CER at 36°C, demonstrating that this recombinant plasmid was responsible for complementing the *faal* mutation. To determine whether the 5.8-kb insert mapped to the *faal* locus, *HIS3* was integrated at the genomic position from which this cloned DNA was derived (yielding the *faal::HIS3* allele; see Materials and Methods). Analysis of 25 tetrads from a cross between this *faal::HIS3* strain and YB241 revealed only parental ditypes, indicating that the cloned DNA is tightly linked to the *faal* locus and most likely contains the *FAA1* gene.

We located a region within the cloned 5.8-kb segment of genomic DNA that could still complement the *faal* mutation by placing several restriction fragments into a centromere plasmid and determining whether they allowed growth of YB241 at 36°C on YPD-MYR/CER. Two plasmids containing fragments that divide the 5.8-kb segment at the BamHI site (cf., Fig. 1 *A*) were unable to complement the *faal* mutation. Another plasmid containing all of the sequences 3′ of the XbaI site also failed to complement *faal*. These findings suggested that a functional open reading frame spanned the XbaI and BamHI sites. A 4.8-kb SacI-SalI fragment (indicated in Fig. 1 *A*) did permit growth of YB241 on YPD-MYR/CER at 36°C. Nucleotide sequence analysis of a 4-kb region of this DNA revealed a long open reading frame (ORF) of 2,103 bp that specified a 700 amino acid protein



C

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FAA1   1 ..................MVAQY..TVPVGKAANEHETAPRRNYQCREKPLV  32
RACS   1 MEVHELFRYFRMPELIDIRQYVRTLPTNTLMGFGAFAALTTFWYATRPKA  50

      33 RPPNTKCSTVYEFVLECFQKNKNSNAMG.........WRDVKEIHEESKSV  74
      51 LKPPCDLSMQSVEVTGTTEGVRRSAVLEDDKLLLYYYDDVRTMYDGFQRG 100

      75 MKKVDGKETSVEKKWMYYELSHYHYNSFDQLTDIMHEIGRGLVKIGLKPN 124
     101 IQVSNDGPCLGSRK....PNQPYEWISYKQVAEMAECIGSALIQKGFKPC 146

     125 DDDKLHLYAATSHKWMKMFLGAQSQGIPVVTAYDTLGEKGLIHSLVQTGS 174
     147 SEQFIGIFSQNRPEWVTIEQGCFTYSMVVVPLYDTLGTDAITYIVNKAEL 196

     175 KAIFTDNS.....LLPSLIKPVQAAQDVKYIIHFDSISSEDRRQSGKIYQ 219
     197 SVIFADKPEKAKLLLEGVENKLTPC..LKIIVIMDSYDNDLVERGQKC.. 242

     220 SAHDAINRIKEVRPDIKTFSFDDILKLGKESCNEIDVHPPGKDDLCCIMY 269
     243 ..............GVEIIGLKALEDLGR..VNRTKPKPPEPEDLAIICF 276

     270 TSGSTGEPKGVVLKHSNVVAGVGG...ASLNVLKFVGNTDRVICFLPLAH 316
     277 TSGTTGNPKGAMVTHQNIMNDCSGFIKATESAF..IASPEDVLISFLPLAH 325

     317 IFELVFELLSFYWGACIGYATVKTLTSSSVRNCQGDLQEFKPTIMVGVAA 366
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     416 LIFHKIQSSLGGKVRLMITGAAPVSATVLTFLRAALGCQFYEGYGQTECT 465

     563 ASTTILDPANFELGVAGDLTGCVTVKLVDVEELGYFAKNNQGEVWITGAN 512
     466 AGCCLSLPGDWTAGHVGAPMPCNYIKLVDVEDMNYQAAKGEGEVCVKGAN 515

     513 VTPEYYKNEEETSQALTSDGWFKTGDIGEWEANGHLKIIDRKKNLVKTMN 562
     516 VFKGYLKDPARTAEALDKDGWLHTGDIGKWLPNGTLKIIDRKKHIFKLAQ 565

     563 GEYIALEKLESVYRSNEYVANICVYADQSKTKPVGIIVPNHAPLTKLAKK 612
     566 GEYIAPEKIENIYLRSEAVAQVFVHGESLQAFLIAIVVPDVEILPSWAQK 615

     613 LGIMEQKDSSINIENYLEDAKLIKAVYSDLLKTGKDQGLVGIELLAGIVF 662
     616 RGFQG......SFEELCRNKDINKAILEDMVKLGKNAGLKPFEQVKGIAV 659

     663 FDGEWTPQNGFVTSAQKLKRKDILNAVKDKVDAVYSSS..            700
     660 HPELFSIDNGLLTPTLKAKRPELRNYFRSQIDELYSTIKI           699
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Figure 1. Sequence analysis of *FAA1*. (*A*) Restriction map of the *FAA1* locus. Bent arrows indicate ORFs and the direction of transcription. The stippled bar represents the 4,015 bp of sequence shown in *B*. The dashed lines indicate the portion of the *FAA1* locus replaced in the mutant allele by the restriction fragment carrying the *HIS3* gene (hatched bar). Restriction sites destroyed during subcloning are shown in parentheses. The restriction fragment used in all of the hybridization experiments begins at the indicated ScaI site and extends ≈4.2 kb to the right. (*B*) The nucleotide sequence of *FAA1* and the predicted amino acid sequence of its primary translation product. A putative DAT binding site (−266) and a "TATA" box (−95) are underlined. (*C*) Alignment of *FAA1* (*top*) and rat acyl:CoA synthetase (*RACS*, *bottom*) using the Gap program of the GCG software package (gap weight 3.00, gap length weight 0.10; Devereux et al., 1984).

B

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-1670 CCACGTAGAATATATAAGACCAATAAATTCCTTTATTGTACGGATATTCTGCATTACAAA -1611
-1610 TGAGTCTTCTGCTTTATGCGGATCGTCAGCTTATATTGCCTTCGTACAGAAAATGAAAAT -1551
-1550 TCACTACTGGAAACGGGTGAGCGGATGTATGAGTGTATAGTATACACCGGTTTTTTTTA -1491
-1490 CCCTTTCTAAGACCGGAGCTGGCGACAGTAAGTGATAAGCGGCCGTTGGCGGCCGTGGGT -1431
-1430 GAGTTATGCATGGGAACCAGCATGAGCCAGCGATGTTCACTATAAAGAGATTAAATGTAG -1371
-1370 AAAGTGCTGAGAGACCGATCGATTGGTAAACTTTTCAAAAGGTGGCCTTCTCCACACTTT -1311
-1310 CTTTTTCCCTCGTTCACTCTGCCTTGATCACATGCTTTTTCTATGGTTAACTTTGAAAA -1251
-1250 ATTCGTTCCATATCGCCCGCCACAAGTTAAAACCAGATATGCGTGGGATATAGTAGGGTG -1191
-1190 GACTGTTAAGTTAGCTATAATGGGCTTGTACTCTTTTTGAATACAAAAACCACCTTTCTA -1131
-1130 CACCTTAATACAAGCGGTGGTTGGAAAGCATTCTTGATATATATAATTATATGAGTTTTG -1071
-1070 AAAAAAAGTACTGGCATCATTTAAATAAGCGGGCTAAACTTTGTTTATGTTTAGGTTAAC -1011
-1010 GATAGACATATTTATTTTTTTTTCTAGGAATAAAAATGAAGAACGGGAAGAAAAGGAAAA -951
-950  ATGAAAATGAAGAATATGAAGAGTAAACCTACTTAAAAAAAAAAACCATACTTTAGTATT -891
-890  TAATGTAGCTACCACGGCGTATTCGATACACTATTATTTTTCTTGACTTAGTATGTCAAT -831
-830  TGTAGGTAGCCTCATGATGCCCTCTTGATCTAAGCGCGGTGAAAGTCCGCAGGCAATTGC -771
-770  TATAATACTGACAAAATTTATAAGGGGAGAGGGTCTTTTTTCGGTCCGCACGGAGCGGGC -711
-710  GAAGAAAACGTAGCGGTGAGAAAAGAATACGTCACCAAAGTATCAGAAACGTCACTGCGT -651
-650  TGTAGATGTAAGACCGATCCACTTTGCCAGCTGCTTACGCTGCGGAAAGTAAACAGACCT -591
-590  AGCTAGCGGACCGATAGAGCCGGCTGAACTTTATAACAAATGCGCCTTCTAACAAGCGAT -531
-530  GAAGCCATGCTTAGCGCGGCAAAGAGAATTACTTAGAATATGGATGATGCAGCCCTGTCA -471
-470  TATTCTTCCATAAGGGAATGACAAGGACAATAGTAAAGTCGATCACGCCCTGTCAGGAAC -411
-410  ATGATCTCCGCCATGTGAATATTTATGCATGATACAGGCACGAATACATCATATAATACG -351
-350  AATGACACAGGGGCACCCACCCATCGCATATCAGGAGAACTTCCCTGTGCATACGGGACA -291
-290  GCAGAGATAGGCTGTTCTCGCGCGTTTTTTTTTTTTTTTGCTTTTTTTGGTTCCTTTCG -231
-230  CGGATATCGTATTATCGATATTACAACAAAAATGTTTGGAGATCTCCTTCGCTGTCTTTA -171
-170  AGTAAAAGGCTTTAAACCGCTTATTTTTCTCTCTTTTTTTCCTTTTACCCCTCACTTGTC -111
-110  GTGAGACAATTGTAGTATATTTAGTAAGTATAGAAAGTTCTTGTTGTTAAAAAACTCGTT -51

 -50  AGGATACAATAAAAACTAGAACAAACACAAAAGACAAAAAAAGACAACAATATGGTTGCT  9
                                                          MetValAla  3

  10  CAATATACCGTTCCAGTTGGGAAAGCCGCCAATGAGCATGAAACTGCTCCAAGAAGAAAT  69
   4  GlnTyrThrValProValGlyLysAlaAlaAsnGluHisGluThrAlaProArgArgAsn  23

  70  TATCAATGCCGCGAGAAGCCGCTCGTCAGACCGCCTAACACAAAGTGTTCCACTGTTTAT  129
  24  TyrGlnCysArgGluLysProLeuValArgProProAsnThrLysCysSerThrValTyr  43

 130  GAGTTTGTTCTAGAGTGCTTTCAGAAGAACAAAAATTCAAATGCTATGGGTTGGAGGGAT  189
  44  GluPheValLeuGluCysPheGlnLysAsnLysAsnSerAsnAlaMetGlyTrpArgAsp  63

 190  GTTAAGGAAATTCATGAAGAATCCAAATCGGTTATGAAAAAAGTTGATGGCAAGGAGACT  249
  64  ValLysGluIleHisGluGluSerLysSerValMetLysLysValAspGlyLysGluThr  83

 250  TCAGTGGAAAAGAAATGGATGTATTATGAACTATCGCATTATCATTATAATTCATTTGAC  309
  84  SerValGluLysLysTrpMetTyrTyrGluLeuSerHisTyrHisTyrAsnSerPheAsp  103

 310  CAATTGACCGATATCATGCATGAAATTGGTCGTGGGTTGGTGAAAATAGGATTAAAGCCT  369
 104  GlnLeuThrAspIleMetHisGluIleGlyArgGlyLeuValLysIleGlyLeuLysPro  123

 370  AATGATGATGACAAATTACATCTTTACGCAGCCACTTCTCACAAGTGGATGAAGATGTTC  429
 124  AsnAspAspAspLysLeuHisLeuTryAlaAlaThrSerHisLysTrpMetLysMetPhe  143

 430  TTAGGAGCGCAGTCTCAAGGTATTCCTGTCGTCACTGACCTACTTTGGGCGAGAAA  489
 144  LeuGlyAlaGlnSerGlnGlyIleProValValIleThrAlaTyrAspThrLeuGlyGluLys  163

 490  GGGCTAATTCATTCTTTGGTGCAAACGGGGTCTAAGGCCATTTTTACCGATAACTCTTTA  549
 164  GlyLeuIleHisSerLeuValGlnThrGlySerLysAlaIlePheThrAspAsnSerLeu  183

 550  TTACCATCCTTGATCAAACCAGTGCAAGCCGCTCAAGACGTAAAATACATAATTCATTTC  609
 184  LeuProSerLeuIleLysProValGlnAlaAlaGlnAspValLysTyrIleIleHisPhe  203

 610  GATTCCATCAGTTCTGAGGACAGGAGGCAAAGTGGTAAGATCTATCAATCTGCTCATGAT  669
 204  AspSerIleSerSerGluAspArgArgGlnSerGlyLysIleTyrGlnSerAlaHisAsp  223

 670  GCCATCAACAGAATTAAAGAAGTTAGACCTGATATCAAGACCTTTAGCTTTGACGACATC  729
 224  AlaIleAsnArgIleLysGluValArgProAspIleLysThrPheSerPheAspAspIle  243

 730  TTGAAGCTAGGTAAAGAATCCTGTAACGAAATCGATGTTCATCCACCTGGCAAGGATGAT  789
 244  LeuLysLeuGlyLysGluSerCysAsnGluIleAspValHisProProGlyLysAspAsp  263

 790  CTTTGTTGCATCATGTATACGTCTGGTTCTACAGGTGAGCCAAAGGGTGTTGTCTTGAAA  849
 264  LeuCysCysIleMetTyrThrSerGlySerThrGlyGluProLysGlyValValLeuLys  283

 850  CATTCAAATGTTGTCGCAGGTGTTGGTGGTGCAAGTTTGAATGTTTTGAAGTTTGTGGGC  909
 284  HisSerAsnValValAlaGlyValGlyValGlyAlaSerLeuAsnValLeuLysPheValGly  303

 910  AATACCGACCGTGTTATCTGTTTTTTGCCACTAGCTCATATTTTTGAATTGGTTTTCGAA  969
 304  AsnThrAspArgValIleCysPheLeuProLeuAlaHisIlePheGluLeuValPheGlu  323

 970  CTATTGTCCTTTTATTGGGGGGCCTGCATTGGTTATGCCACCGTAAAAACTTTAACTAGC  1029
 324  LeuLeuSerPheTyrTrpGlyAlaCysIleGlyTyrAlaThrValLysThrLeuThrSer  343

1030  AGCTCTGTGAGAAATTGTCAAGGTGATTTGCAAGAATTCAAGCCCACAATCATGGTTGGT  1089
 344  SerSerValArgAsnCysGlnGlyAspLeuGlnGluPheLysProThrIleMetValGly  363

1090  GTCGCCGCTGTTTGGGAAACAGTGAGAAAAGGGATCTTAAACCAAATTGATAATTTGCCC  1149
 364  ValAlaAlaValTrpGluThrValArgLysGlyIleLeuAsnGlnIleAspAsnLeuPro  383

1150  TTCCTCACCAAGAAAATCTTCTGGACCGCGTATAATACCAAGTTGAACATGCAACGTCTC  1209
 384  PheLeuThrLysLysIlePheTrpThrAlaTyrAsnThrLysLeuAsnMetGlnArgLeu  403

1210  CACATCCCTGGTGGCGGCGCCTTAGGAAACTTGGTTTTCAAAAAAATCAGAACTGCCACA  1269
 404  HisIleProGlyGlyGlyAlaLeuGlyAsnLeuValPheLysLysIleArgThrAlaThr  423

1270  GGTGGCCAATTAAGATATTTGTTAAACGGTGGTTCTCCAATCAGTCGGGATGCTCAGGAA  1329
 424  GlyGlyGlnLeuArgTyrLeuLeuAsnGlyGlySerProIleSerArgAspAlaGlnGlu  443

1330  TTCATCACAAATTTAATCTGCCCTATGCTTATTGGTTACGGTTTAACCGAGACATGCGCT  1389
 444  PheIleThrAsnLeuIleCysProMetLeuIleGlyTyrGlyLeuThrGluThrCysAla  463

1390  AGTACCACCATCTTGGATCCTGCTAATTTTGAACTCGGCGTCGCTGGTGACCTAACAGGT  1449
 464  SerThrThrIleLeuAspProAlaAsnPheGluLeuGlyValAlaGlyAspLeuThrGly  483

1450  TGTGTTACCGTCAAACTAGTTGATGTTGAAGAATTAGGTTATTTTGCTAAAAACAACCAA  1509
 484  CysValThrValLysLeuValAspValGluLeuGluGlyTyrPheAlaLysAsnAsnGln  503

1510  GGTGAAGTTTGGATCACAGGTGCCAATGTCACGCCTGAATATTATAAGAATGAGGAAGAA  1569
 504  GlyGluValTrpIleThrGlyAlaAsnValThrProGluTyrTyrLysAsnGluGluGlu  523

1570  ACTTCTCAAGCTTTAACAAGCGATGGTTGGTTCAAGACCGGTGACATCGGTGAATGGGAA  1629
 524  ThrSerGlnAlaLeuThrSerAspGlyTrpPheLysThrGlyAspIleGlyGluTrpGlu  543

1630  GCAAATGGCCATTTGAAAATAATTGACAGGAAGAAAAACTTGGTCAAAACAATGAACGGT  1689
 544  AlaAsnGlyHisLeuLysIleIleAspArgLysLysAsnLeuValLysThrMetAsnGly  563

1690  GAATATATCGCACTCGAGAAATTAGAGTCCGTTTACAGATCTAACGAATATGTTGCTAAC  1749
 564  GluTyrIleAlaLeuGluLysLeuGluSerValTyrArgSerAsnGluTyrValAlaAsn  583

1750  ATTTGTGTTTATGCCGACCAATCTAAGACTAAGCCAGTTGGTATTATTGTACCAAATCAT  1809
 584  IleCysValTyrAlaAspGlnSerLysThrLysProValGlyIleIleValProAsnHis  603

1810  GCTCCATTAACGAAGCTTGCTAAAAAGTTGGGAATTATGGAACAAAAAGACAGTTCAATT  1869
 604  AlaProLeuThrLysLeuAlaLysLysLeuGlyIleMetGluGlnLysAspSerSerIle  623

1870  AATATCGAAAATTATTTGGAGGATGCAAAATTGATTAAAGCTGTTTATTCTGATCTTTTG  1929
 624  AsnIleGluAsnTyrLeuGluAspAlaLysLeuIleLysAlaValTyrSerAspLeuLeu  643

1930  AAGACAGGTAAAGACCAAGGTTTGGTTGGCATTGAATTACTAGCAGGCATAGTGTTCTTT  1989
 644  LysThrGlyLysAspGlnGlyLeuValGlyIleGluLeuLeuAlaGlyIleValPhePhe  663

1990  GACGGCGAATGGACTCCACAAAACGGTTTTGTTACGTCCGCTCAGAAATTGAAAAGAAAA  2049
 664  AspGlyGluTrpThrProGlnAsnGlyPheValThrSerAlaGlnLysLeuLysArgLys  683

2050  GACATTTTGAATGCTGTCAAAGATAAAGTTGACGCCGTTTATAGTTCGTCTTAATGGATC  2109
 684  AspIleLeuAsnAlaValLysAspLysValAspAlaValTyrSerSerSerLeuEnd  700

2110  AACATTTCCATGATAGGAAAGCCTCATCATACTAAAGCACTTTTCAGTTTTTTGCTTTA  2169
2170  GAACTGCTACCAATTATAAAATAGAATGCACAGGTTATCTTGTTTGGTTTGTTATTATCC  2229
2230  TTTTTTCTTCTTTACCAGTCATATCACCTCATTCACGTATGTTCGTAATTTATAATGTTA  2289
2290  AAAATGGCATATATAGATATATTTATGAACAAACTAAAAAATAAACTAAATTTTT       2344
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with a M_r of 77,816 (Fig. 1 *B*). The location of this ORF agreed with the complementation data: the XbaI site is located 139-bp downstream of the putative initiator methionine codon; the BamHI site is located near the center of the ORF at amino acid 469. A canonical "TATA" box important for transcription initiation (Struhl, 1989) is present 95 nucleotides 5′ to the start of the ORF (underlined in Fig. 1 *B*). A 16-nucleotide-long tract of deoxythymidine residues is also noted. This sequence element is frequently found in the promoter region of *S. cerevisiae* genes and has been implicated in the regulation of transcription (Struhl, 1989). It is also a predicted binding site for the 248 amino acid product of the nonessential *DAT* gene, whose role in the regulation of gene expression is unclear (Winter and Varshavsky, 1989).

The polypeptide encoded by the ORF was used to search a nonredundant protein sequence data base (see Materials and Methods). It had significant sequence similarities to two entries, rat long chain acylCoA synthetase (RACS) and firefly (*Photinus pyralis*) luciferase (EC 1.13.12.7). An alignment of the 700-amino acid *S. cerevisiae* protein and the 699 amino acid RACS is shown in Fig. 1 *C*. The two proteins are 30% identical. When conservative amino acid substitutions are considered the overall similarity increases to 53%. The sequence similarities between this *S. cerevisiae* protein and RACS extend throughout their lengths. Alignments of RACS and firefly luciferase indicate that they have 36% identity and an overall similarity of 55% (Suzuki et al., 1990). Like acyl-CoA synthetase, firefly luciferase catalyzes a two-step reaction mechanism that involves reaction of the carboxyl group of its luciferin substrate with ATP to generate an adenylated intermediate with subsequent release of AMP in the second reaction:

luciferin + ATP ---➤ luciferyl-AMP + PP_i
luciferyl-AMP + O_2 ---➤ oxyluciferin + AMP + CO_2 + *hv*

Suzuki et al. proposed (1990) that the region of greatest similarity between the two enzymes, which spans amino acids 458 and 591 of RACS (Fig. 1 *C*), may represent the ATP binding site of each protein. The corresponding region of the yeast ORF (residues 455 to 588 in Fig. 1 *C*) has 47% identity with rat acylCoA synthetase and 25% identity with firefly luciferase (residues 339-471).

To further establish whether the ORF encoded *S. cerevisiae* acyl:CoA synthetase, the enzyme was purified over 300-fold from the protease deficient strain BJ405 (Table I) by subcellular fractionation, P11 cellulose phosphate cation exchange chromatography, and CoA agarose affinity chromatography (Table II). Enzyme activity was monitored by measuring the conversion of [^{3}H]myristate to [^{3}H]myristoylCoA. The fraction from the CoA agarose column that had the highest specific activity contained two polypeptides of ~53 and ~80 kD as revealed by SDS-PAGE and silver staining (Fig. 2 *A*). Edman degradation of the 80-kD polypeptide yielded the sequence XXXQYTVPVGKAAN. This corresponds to residues 1–14 of the primary translation product of the ORF (Fig. 1 *B*). Furthermore, the mass of this

Table II. Purification of S. cerevisiae AcylCoA Synthetase

Fraction	Protein	Activity[a]	Sp. Act.	Purification	Yield
	(mg)	*(U)*	*(U/mg)*	*(fold)*	*(%)*
Homogenate	2,000	100	0.05	1	100
10,000 *g* sup	800	96	0.12	2.4	96
P11 column	5	5.5	1.1	22	5.5
CoA-Agarose	0.02	0.3	16	320	0.32

[a]1 U activity = 1 pmol product formed/min

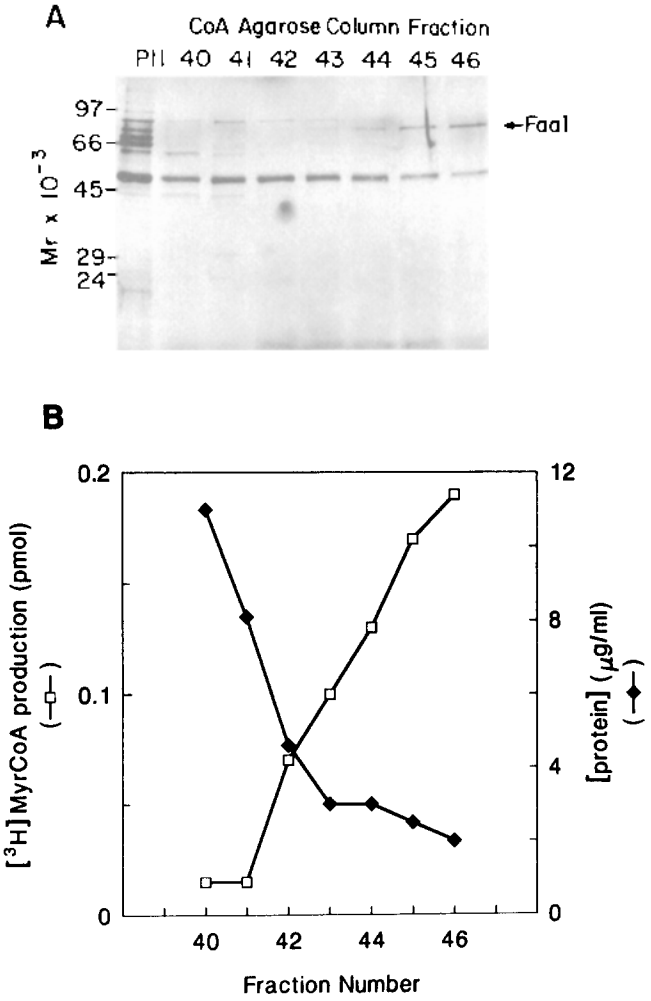

Figure 2. Partial purification of Faalp. (*A*) SDS-PAGE of fractions enriched for acylCoA synthetase activity obtained by CoA agarose chromatography. Chromatography was performed on material pooled from the preceding P11 phosphocellulose step (*P11*).[2] Proteins eluted from the CoA-agarose column in fractions 40-46 were reduced, denatured, and fractionated by electrophoresis through 10% polyacrylamide gels containing SDS (0.1%). The gels were stained with silver. (*B*) Distribution of protein and acylCoA synthetase activity among agarose-CoA column fractions. Note that the increase in myristoylCoA formation follows the increase in abundance of the 80-kD polypeptide. Edman degradation of the 80- and 53-kD polypeptides shown in fraction 46 established their identities as Faalp and vacuolar H^+-translocating ATPase, respectively.

2. The 53-kD *S. cerevisiae* NMT polypeptide has also been purified by P11 chromatography (Rudnick et al., 1990). However, none of the fractions shown in Fig. 2 *A* contained any detectable NMT as judged by (*a*) a sensitive coupled in vitro enzyme assay (Towler and Glaser, 1986; Duronio et al., 1990) and (*b*) Western blot analysis with a monospecific, rabbit anti-NMT sera (Duronio et al., 1991*a*; Knoll et al., 1992).

purified protein (80 kD) is in good agreement with that predicted from the ORF (77,816 Da). Sequence data obtained from the 53-kDa band indicated that this polypeptide was a proteolytic fragment derived from the catalytic subunit of a vacuolar H^+-translocating ATPase encoded by the *VMA1* gene (Hirata et al., 1990). This sequence had no discernible similarities to the predicted yeast protein, to rat acylCoA synthetase or to firefly luciferase. We conclude from these results that the ORF encodes a *S. cerevisiae* acylCoA synthetase that is capable of catalyzing the conversion of myristate to myristoylCoA in vitro and that we had successfully cloned the *FAA1* gene by complementation of the *faa1* mutant allele.

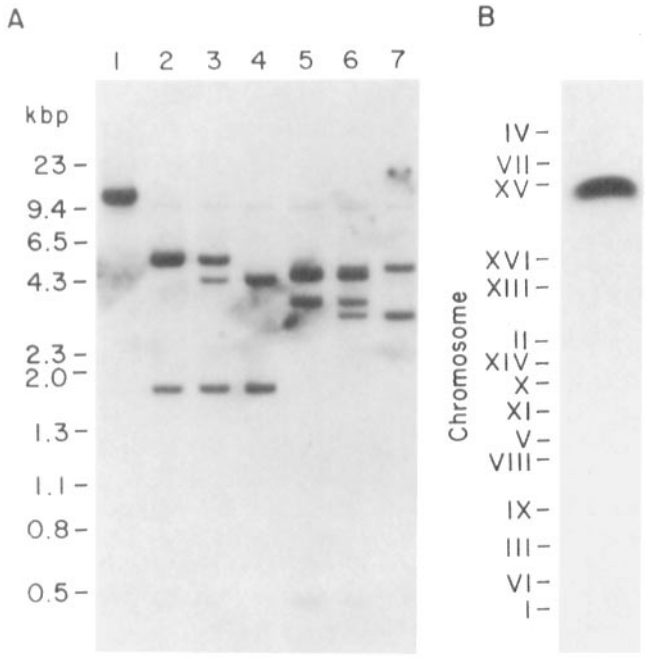

Figure 3. Southern blot analysis of genomic DNA prepared from *S. cerevisiae* strains containing *FAA1* or *faa1Δ1.9:HIS3* mutant alleles. (*A*) DNA was prepared from strains YB100 (*FAA1/FAA1*, lanes *1*, *2*, and *5*), YB360 (*FAA1/faa1Δ1.9::HIS3*, lanes *3* and *6*), and YB363 (*faa1Δ1.9::HIS3*, lanes *4* and *7*), and digested with either SacI (lane *1*), HindIII (lanes *2–4*), or EcoRI (lanes *5–7*). A Southern blot containing the digested DNAs was subsequently probed with a ^{32}P-labeled restriction fragment containing the entire *FAA1* gene. An autoradiograph of the blot is shown. (*B*) Blot of *S. cerevisiae* chromosomes probed with *FAA1* DNA. The position of migration of each chromosome is indicated. (*C*) Schematic diagram showing the genetic map of the distal right arm of chromosome XV in the region containing the *FAA1* locus. Note that the placement of *FAA1* on the genetic map is based on physical mapping data (see text for further details).

FAA1 mRNA Is Derived from a Single Locus Located on Chromosome XV

A variety of blot hybridization studies were performed to determine the location of *FAA1* in the *S. cerevisiae* genome and whether this genome contains any structurally related sequences. Biochemical and genetic studies of another yeast, *Candida lipolytica*, indicate that it contains two functionally distinct long-chain acylCoA synthetases: a phosphatidylcholine-independent acylCoA synthetase I that appears to be used in the synthesis of cellular lipids and a phosphatidylcholine-requiring acylCoA synthetase II that generates acylCoAs that are destined to be degraded by β-oxidation (Kamiryo et al., 1977*b*, 1979; Mishina et al., 1978*a*,*b*). The structures of these proteins and their gene(s) have not been reported to date.

Southern blot studies of DNA isolated from the diploid strain YB100 suggested that there are not multiple copies of *FAA1* dispersed in the *S. cerevisiae* genome (Fig. 3 *A*, lanes *1*, *2*, and *5*). No homologs of *FAA1* were apparent under the hybridization conditions used (see Materials and Methods). This notion was supported by two other observations. First, when blots of total cellular RNA prepared from the *FAA1* haploid strain YM2061 were probed with a restriction fragment containing the entire *FAA1* ORF, a single mRNA species of ~4 kb was detected (Fig. 4). Second, when a blot containing yeast chromosomes that had been separated by pulsed-field gel electrophoresis was incubated with this labeled fragment, only chromosome XV reacted with the probe (Fig. 3 *B*). A set of filters containing 880 recombinant lambda phage clones whose inserts represent 82% of currently mapped DNA covering ~80% of the *S. cerevisiae* genome (Linda Riles and Maynard Olson, personal commu-

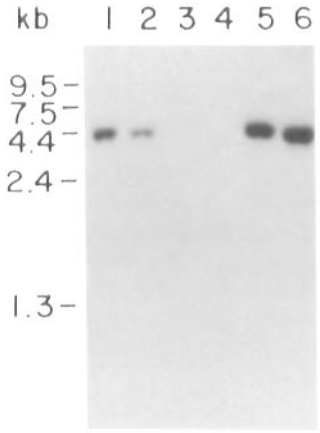

Figure 4. Blot hybridization analysis of RNA prepared from strains of *S. cerevisiae* with *FAA1* or *faa1Δ1.9::HIS3*. RNA was prepared from strains YM2061 (*FAA1*; lanes *1*, *2*, *5*, and *6*) and YB363 (*faa1Δ1.9::HIS3*, lanes *3* and *4*), separated by electrophoresis through a formaldehyde/agarose gel, transferred to a nylon filter, and probed with a ^{32}P-labeled restriction fragment containing the entire *FAA1* gene. Yeast were grown in YPD (lanes *1* and *3*), YPD + 1 mM myristate and 1% (wt/vol) Brij 58 (lanes *2* and *4*), synthetic complete medium (lane *5*), or synthetic complete medium + 1 mM myristate and 1% (wt/vol) Brij 58 (lane *6*). Note that the steady state level of the 4-kb FAA1 mRNA is the same in YM2061 cells grown on YPD alone and on YPD supplemented with 1 mM myristate (compare lanes *1* and *2*) while the steady state level of Faa1 mRNA in cells grown in synthetic medium that completely lacks exogenous fatty acids, or in synthetic medium containing 1 mM myristate, reproducibly differs by less than twofold from that observed in cells grown on rich medium (compare lanes *5* and *6*). These data indicate that accumulation of Faa1p mRNA is not greatly affected by exogenous sources of myristic acid.

nication) was then probed with ^{32}P-labeled *FAA1*. Two overlapping clones were identified, each of which had been previously mapped to the distal right arm of chromosome XV between *CPA1* (a locus encoding the small subunit of carbamoyl-phosphate synthetase A; LaCronte et al., 1965) and *PHR1* (a locus encoding a DNA photolyase involved in pyrimidine dimer repair; Sebastian et al., 1990) (Linda Riles and Maynard Olson, personal communication). The data indicated that *FAA1* is located 10–20-kb centromere distal to *CPA1*, and 100–120-kb centromere proximal to *PHR1* (Fig. 3 *C*). Since *S. cerevisiae* has a total genome average of 0.34 cM/kb (Mortimer et al., 1989), our physical data best support the map order *CEN12-CPA1-(suf13-faa1)-(cdc66-pro2)-PHR1* (Fig. 3 *C*). We have not obtained data on the relative order of *suf13* and *faa1*, but the genes are probably not allelic since the *suf13* mutation functions to suppress translational frameshift mutations (Culbertson et al., 1982).

FAA1 Is a Nonessential Gene of S. cerevisiae

We performed a gene disruption experiment to characterize the role of Faa1p in cell growth, fatty acid metabolism, and protein N-myristoylation. A *faa1* deletion mutation was produced in vitro by replacing a 1.9-kb segment of *FAA1*, which included the initiator Met codon and almost all of the coding region (residues 1–576), with *HIS3* sequences, generating the *faa1Δ1.9::HIS3* allele (Fig. 1 *A*). A *FAA1/faa1Δ1.9::HIS3* heterozygous diploid was constructed by transforming strain YB100 to histidine prototrophy with *faa1Δ1.9::HIS3* DNA. Sporulation and tetrad analysis of this strain (YB360) yielded four viable spores from >90% of dissected asci when grown on YPD medium at 24, 30, and 36°C. Each tetrad segregated in the predicted 2 His$^+$:2 His$^-$ manner. All His$^+$ meiotic segregants were unable to grow at 36°C on YPD containing 25 μM cerulenin and either 500 μM myristate or palmitate, indicating that the *faa1Δ1.9::HIS3* allele blocked the utilization of exogenous long chain fatty acids at this temperature. At 24 and 30°C, however, the *faa1Δ1.9::HIS3* segregants were able to grow on these cerulenin containing media, but at a rate slightly reduced from wild type cells (e.g., see Fig. 5). DNA blot hybridization analysis using two separate restriction enzymes confirmed the presence of the *faa1Δ1.9::HIS3* allele in the *FAA1/faa1Δ1.9::HIS3* heterozygote and one of its His$^+$ meiotic progeny (Fig. 3 *A*). Furthermore, there were no detectable *FAA1* transcripts in RNA prepared from the *faa1Δ1.9::HIS3* strain YB363 (Fig. 4, lanes *3* and *4*). These observations indicate that *FAA1* is not an essential gene when the de novo pathway for fatty acid synthesis is operational at 24, 30, and 36°C.

Further Phenotypic Analyses of Strains Containing a faa1 Null Allele Suggest that S. cerevisiae May Have Other Long Chain acylCoA Synthetase Activities

The absence of any detectable *FAA1* mRNA transcripts in strains containing the *faa1Δ1.9::HIS3* allele suggested that it should have no acylCoA synthetase activity. The specific activity of the enzyme in a 10,000 *g* supernatant fraction prepared from a wild type (strain BJ405) cell lysate was ~0.13 pmol/min/mg protein (using [^{3}H]myristate as the substrate plus the assay conditions described in Materials and Methods). Parallel assays of strains YB241 (*faa1*) and YB363 (*faa1Δ1.9::HIS3*) failed to detect any acylCoA synthetase activity. This negative result was further evaluated in a series of control experiments. Partially purified *S. cerevisiae* acyl-CoA synthetase (the P11 fraction prepared from BJ405; see Fig. 2 *A* and Table II) was added to the 10,000 *g* supernatants prepared from both mutant strains. >95% of the activity was recovered, indicating that these fractions did not contain any inhibitors of the enzyme. Moreover, a series of increasing dilutions of a given quantity of active, partially purified (through the P11 step) wild type enzyme into these supernatant fractions indicated that the limit of detection of acyl-CoA synthetase activity was 1% of the activity in 10,000 *g* supernatants prepared from BJ405 cells. Together, these controls confirm that deletion of the *FAA1* locus reduces cellular long chain acyl-CoA synthetase activity to a level below that which is detectable by the in vitro assay.

To further define the phenotype caused by inactivation of Faa1p, *FAA1* and *faa1Δ1.9::HIS3* containing strains with isogenic backgrounds (YM2061 and YB363, respectively) were replica plated to various types of cerulenin containing media and incubated at 24, 30, and 36°C for 2 d (Fig. 5). The *NMT1 FAA1* strain does not grow on YPD media containing 25 μM cerulenin (YPD-CER) at any of the three temperatures surveyed. Growth of this strain can be rescued at all 3 temperatures by adding myristate or palmitate to a final concentration of 500 μM (YPD-MYR/CER, YPD-PAL/CER in Fig. 5). At 36°C, myristate supplementation appears to be somewhat more advantageous to growth than palmitate. The strain containing the *faa1* null allele was not able to grow at 36°C on YPD containing cerulenin even when the medium is supplemented with myristate or palmitate. These observations indicate that Faa1p is required for utilization of these exogenous fatty acids at 36°C when the de novo pathway for fatty acid synthesis is inhibited by cerulenin. However, at 24 and 30°C the *faa1Δ1.9::HIS3* strain is able to grow in YPD-CER medium supplemented with myristate or palmitate, although the rate is somewhat reduced when compared to the wild type strain. C16:0 appears to allow somewhat better growth of this strain in YPD-CER at 24 and 30°C than C14:0. These results suggest that *S. cerevisiae* contains another metabolic pathway that is used at 24 and 30°C for activation and utilization of exogenous fatty acids that does not require Faa1p. This latter hypothesis was supported by metabolic labeling of exponentially growing strains containing the *FAA1* or *faa1Δ1.9::HIS3* alleles. At 30°C there was no difference between the two strains in the extent of incorporation of label from [^{3}H]myristate into a variety of phospholipid classes including phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) or phosphatidylethanolamine (PE) (Fig. 6). In the absence of Faa1p, there was a two- to fivefold reduction in the incorporation of label into each phospholipid species at 36°C (Fig. 6). These results suggest that Faa1p supplies activated fatty acids for phospholipid synthetic pathways in *S. cerevisiae* (Carman and Henry, 1989) during growth at 36°C.

The Contribution of Faa1p in Regulating Protein N-Myristoylation

The *faa1* null allele allowed us to definitively determine whether Faa1p plays a role in modulating protein N-myristoylation. *nmt1-181 FAA1* or *nmt1-181 faa1Δ1.9::HIS3* strains (YB218, YB371) were grown at 24, 30, or 36°C in various

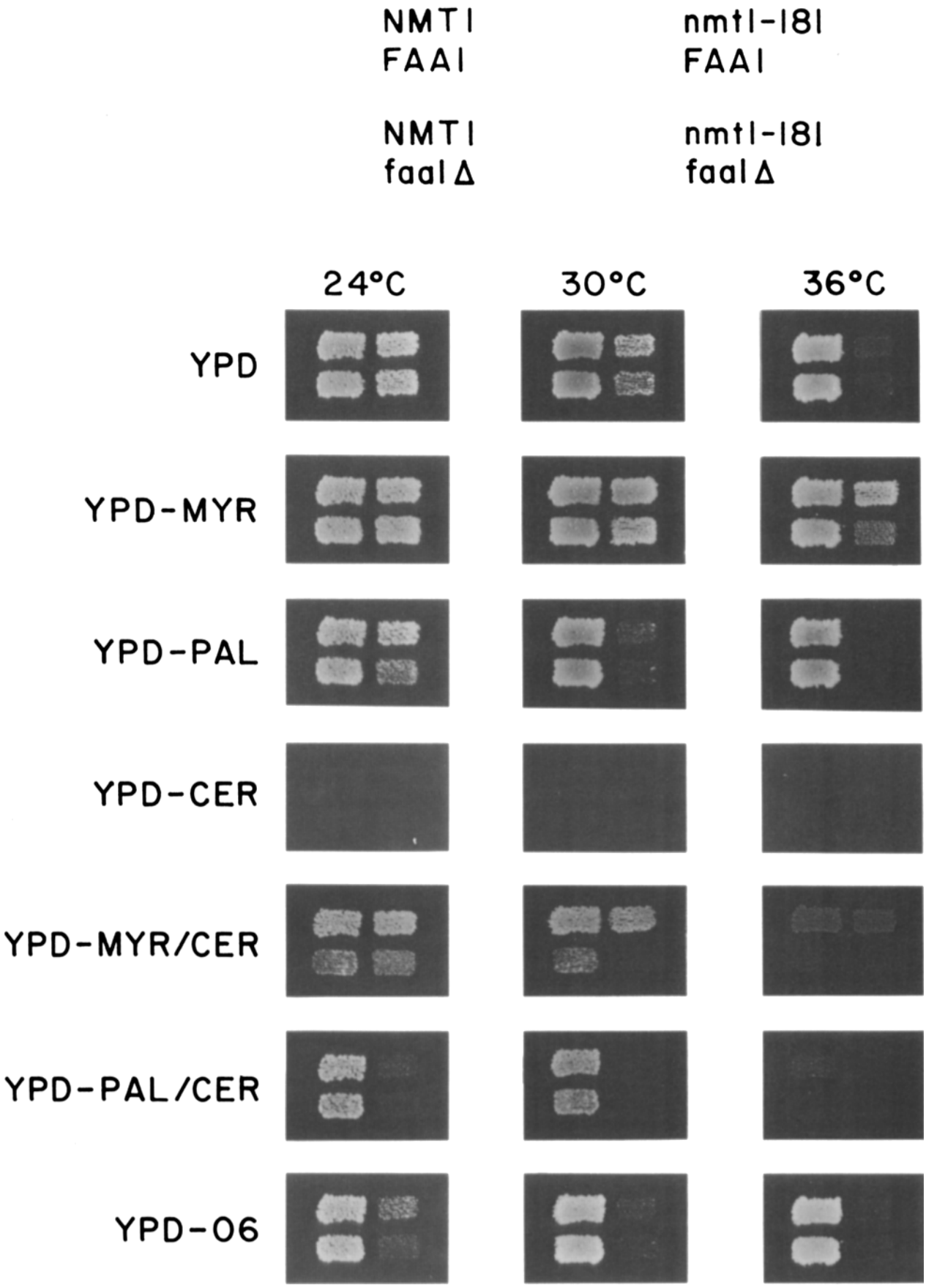

Figure 5. Phenotype of *S. cerevisiae* containing wild type and mutant *NMT1* and *FAA1* alleles. Strains YM2061 (*NMT1 FAA1*), YB218 (*nmt1-181 FAA1*), YB363 (*NMT1 faa1Δ1.9::HIS3*), and YB371 (*nmt1-181 faa1Δ1.9::HIS3*) were replica plated to YPD medium alone or YPD supplemented with 500 μM myristate (MYR), palmitate (PAL), 6-oxatetradecanoic acid (06), and/or 25 μM cerulenin (CER). Plates were incubated at 24, 30, or 36°C for 2 d. The genotypes shown at the top of the figure correspond to the patches on each plate.

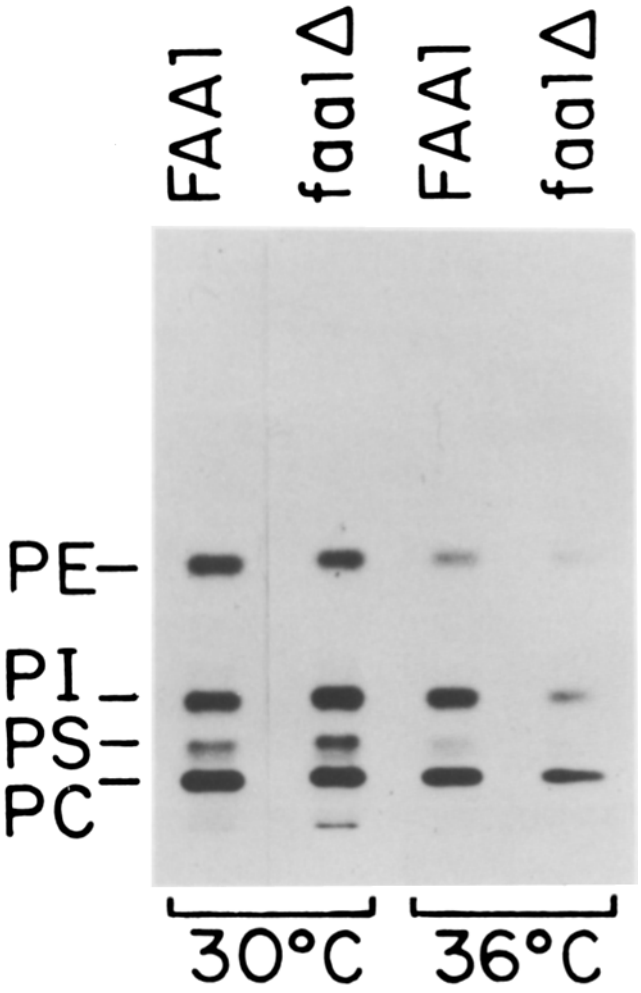

Figure 6. Labeling of cellular lipids by treating exponentially growing strains of *S. cerevisiae* containing a *FAA1* or *faal* null allele with [^{3}H]myristate. Strains YM2061 (*FAA1*) and YB363 (*faalΔ1.9::HIS3*) were grown at 30 or 36°C in YPD plus [^{3}H]myristate. At the conclusion of the 1-h labeling period, cells were harvested, washed, and total cellular lipids extracted. Phospholipids from comparable numbers of cells were separated by thin layer chromatography as described in Materials and Methods. An autoradiograph of the silica plate is shown. The positions of migration of lipid standards are indicated. *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; and *PS*, phosphatidylserine.

fatty acid containing media with or without 25 μM cerulenin (Fig. 5). *nmt1-181* strains cannot grow at 36°C in YPD unless the medium is supplemented with ≥500 μM myristate. Palmitate is unable to support growth under these conditions (see Fig. 5). Removal of Faalp produces an attenuation of growth of the *nmt1-181* strain at 36°C in YPD-MYR, but has little apparent effect on growth at 30°C (Fig. 5). Metabolic labeling studies indicate that exogenous myristate was incorporated into cellular N-myristoylproteins to the same extent when the two strains were grown at 24 and 30°C (Fig. 7, *A* and *B*). However, at 36°C, a pronounced reduction in labeling of all N-myristoylproteins resolved by single dimension SDS-PAGE was noted in the *nmt1-181 faalΔ1.9::HIS3* strain compared to the *nmt1-181 FAA1* strain (compare Fig. 7 *C* with *A* and *B*). The same results were obtained at 24 and 36°C when these strains were grown in YPD containing 25 μM cerulenin (data not shown). The extent of incorporation of [^{35}S]methionine into cellular proteins during the labeling period was equivalent for both strains at each temperature (data not shown). These observations allow us to conclude that Faalp contributes to the myristoylCoA pools used by NMT and that this contribution may be greater at 36°C compared to 30 or 24°C. The Faalp-independent pathway invoked above appears not only to contribute to phospholipid biosynthesis at 24 and 30°C, but to the myristoylCoA pools used by NMT at these two temperatures.

The myristic acid analog 6-oxatetradecanoate (O6) was used to further explore the effect of Faalp on *nmt1-181* strains. Substitution of oxygen for methylene at C6 (the carboxyl carbon = C1) produces an analog whose chain length and bond geometry are similar to those of myristate but whose hydrophobicity is equivalent to dodecanoic acid (C12:0) (Heuckeroth et al., 1988; Kishore et al., 1991). O6 is a substrate for *S. cerevisiae* NMT in vitro (Heuckeroth et al., 1988; Kishore et al., 1991). Metabolic labeling of *S. cerevisiae* with [^{3}H]O6 during exponential growth on YPD indicated that the accumulation of analog in the yeast was 1% that of myristate (Duronio et al., 1991*a*). This could be because of inefficient transport (Duronio et al., 1991*a*; Bryant et al., 1991) and may explain why [^{3}H]O6 is not incorporated into *S. cerevisiae* cellular N-myristoylproteins at detectable levels (Duronio et al., 1991*a*). Despite these findings, supplementing YPD with 500 μM O6 inhibits the growth of *nmt1-181* strains at the permissive temperature (24°C). O6 has no apparent effect on the growth of strains containing *NMT1* (see Fig. 5 and Duronio et al., 1991*a*). The mechanism for this growth inhibition is unknown. The *faal* null mutant now allowed us to assess whether Faalp activity is needed to elicit this phenotype. There were no reproducible differences in the relative growth rates of *nmt1-181 faalΔ1.9::HIS3* and *nmt1-181 FAA1* strains on YPD containing 500 μM O6 at 24°C (Fig. 5), indicating that Faalp is not required for O6-induced inhibition of growth of *nmt1-181* strains. This finding is consistent with our observation that O6 is not a substrate for Faalp and does not inhibit binding of myristate to partially purified enzyme even when present at >10–100 fold molar excess (data not shown). Thus, O6 either does not need to be converted to its CoA derivative to produce growth inhibition, or the other postulated *S. cerevisiae* acylCoA synthetase activity is able to generate O6-CoA at 24°C. If the latter is true, monitoring the conversion of O6 acid to its CoA derivative in vitro may be a useful way of purifying this enzyme.

Biochemical and Genetic Approaches for Examining the Relative Roles of Faalp and FAS in Maintaining MyristoylCoA Pools Used by NMT

Blocking De Novo Fatty Acid Synthesis with Cerulenin. We next determined whether the de novo pathway could contribute sufficient amounts of myristoylCoA, in the absence of Faalp, at 24, 30, and 36°C to satisfy the needs of the mutant enzyme *nmt-181*. Blockade of de novo fatty acid synthesis by 25 μM cerulenin prevents growth of the *nmt1-181 FAA1* strain on YPD and YPD containing 500 μM palmitate at all three temperatures (YPD-CER, YPD-PAL/CER; Fig. 5). In

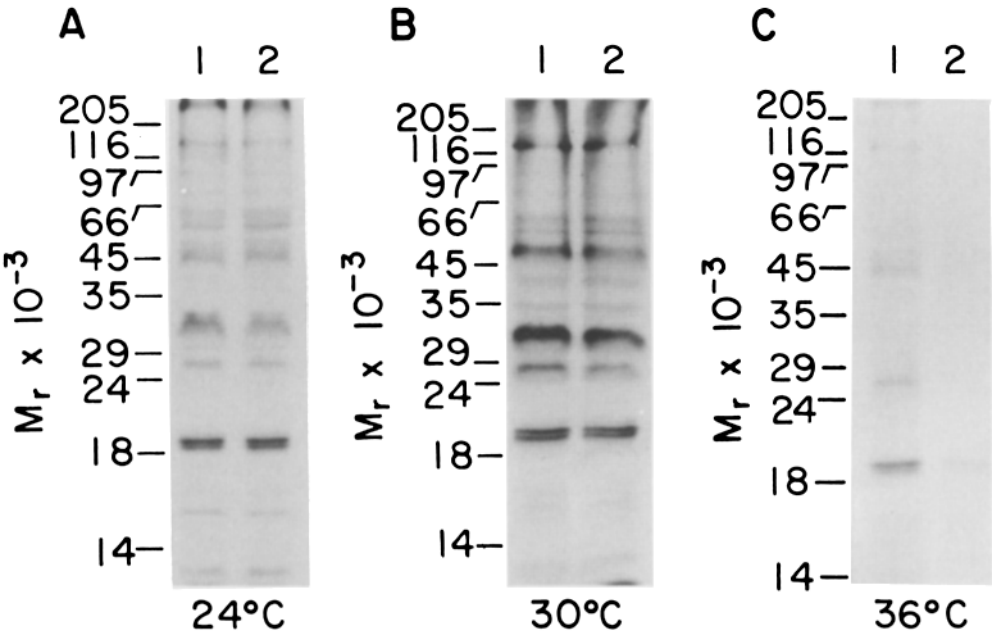

Figure 7. Incorporation of exogenous [^{3}H]myristate into cellular N-myristoylproteins produced in strains of *S. cerevisiae* containing *NMT1* and *FAA1* or *faa1Δ1.9::HIS3*. Cells were labeled for 1 h during exponential growth at 24°C (*A*), 30°C (*B*), or 36°C (*C*) in YPD medium. Cellular lysates were prepared and 100 μg of total cellular protein (Lowry et al., 1951) from each strain was reduced, denatured, and subjected to SDS-PAGE followed by fluorography at −70°C. Lane *1* in all panels contains total cellular proteins from strain YM2061 (*NMT1 FAA1*). Lane *2* contains total cellular proteins from strain YB363 (*NMT1 faa1Δ1.9::HIS3*).

contrast, this strain can grow in YPD-CER when myristate is used as the fatty acid source (Fig. 5). These findings indicate that metabolic interconversion of C16:0 is not sufficient to supplement myristoyl-CoA pools to the extent required to overcome the K_m defect in the mutant *nmt1-181*.

Blockade of de novo fatty acid biosynthesis by cerulenin completely inhibits growth of the *nmt1-181 faa1Δ1.9::HIS3* strain at 36°C on YPD containing 500 μM myristate. However, unlike the *NMT1 faa1Δ1.9::HIS3* strain, the *nmt1-181 faa1Δ1.9::HIS3* strain could not grow at 30°C in YPD-MYR/CER (Fig. 5). Therefore, the *nmt1-181* allele caused an increase in dependence on Faa1p activity at 30°C. At 24°C, the *nmt1-181 faa1Δ1.9::HIS3* strain is viable on YPD-MYR/CER, and grows at a rate that is similar to that of the *NMT1 faa1Δ1.9::HIS3* strain (Fig. 5). Together, these data suggest that de novo fatty acid biosynthesis is required in *nmt1-181* strains at all temperatures in the absence of C14:0 supplementation of the media. At 30 and 36°C there is an additional requirement for Faa1p to activate exogenous myristate.

Blocking De Novo Fatty Acid Synthesis with fas Mutations. A genetic experiment was performed using strains containing the nonconditional *fas1-70* and *fas2-38* alleles, which encode defective β and α subunits, respectively, of fatty acid synthetase (Schweizer et al., 1978). As with cerulenin treated cells, these *NMT1* containing strains cannot grow at any temperature unless YPD medium is supplemented with ≥500 μM myristate or palmitate. (Our results indicate that these *fas* strains grow best when 1 mM myristate is used as the fatty acid source, data not shown.) The *fas1-70* and *fas2-38* strains (Table I) were crossed to a *nmt1-181* strain (YB218) and the resulting diploids sporulated. Tetrads from these diploids were dissected on YPD plates containing 1 mM myristate, and incubated at 30°C for several days. Four viable spores were obtained from ~70% of the tetrads. The segregants from these tetrads were replica plated to YPD and YPD containing 1 mM palmitate or 1 mM myristate and incubated at 24 and 36°C. All haploid progeny could grow on YPD + 1 mM myristate at both temperatures (Fig. 8). For each tetrad, the inability to grow at 24°C in YPD was indicative of the presence of a *fas* allele (Fig. 8) while the inability to grow at 36°C in YPD + 1 mM palmitate was indicative of the presence of the *nmt1-181* allele (e.g., as shown in Fig. 5). These phenotypes independently segregated 2+:2− in both crosses, exactly as expected for unlinked loci (Fig. 8). In this way, the *nmt1-181 fas* segregants could be identified. *nmt1-181 fas1-70* and *nmt1-181 fas2-38* cells were unable to grow at 24°C on YPD-PAL (Fig. 8), a finding that is consistent with results obtained with *nmt1-181 FAS1 FAS2* strains grown on cerulenin. These results confirm the importance of the contribution of the de novo pathway to myristoyl-CoA pools when *nmt1-181* strains are grown at permissive temperatures. The data allow us to conclude that NMT, a cytoplasmic enzyme (Knoll et al., 1992), is able to use the myristoylCoA product of the cytoplasmic FAS complex (Schweizer et al., 1978).

A fas2-38 faa1Δ1.9::HIS3 NMT1 Strain Is Viable

We used the *fas2-38* allele to examine the consequences of eliminating Faa1p activity in a Fas-cell. Tetrads were dissected from a diploid constructed by conjugating *fas2-38 his3Δ200* and *faa1Δ1.9::HIS3 his3Δ200* strains (YB366 and YB363, respectively; Table I). After incubation on YPD plus 1 mM myristate for several days at 30°C, viable meiotic segregants were replicated to YPD and synthetic medium lacking histidine (Sherman et al., 1986) but containing 1 mM myristate. Although the germination rate was somewhat low (i.e., ~50% of the tetrads had less than four spores germinate), His$^+$ segregants were identified that were unable to grow on YPD at 30°C. These viable haploids presumably contain the *fas2-38* and *faa1Δ1.9::HIS3* alleles. This result supports the conclusion that Faa1p activity is not essential at 30°C in the absence of de novo fatty acid synthesis.

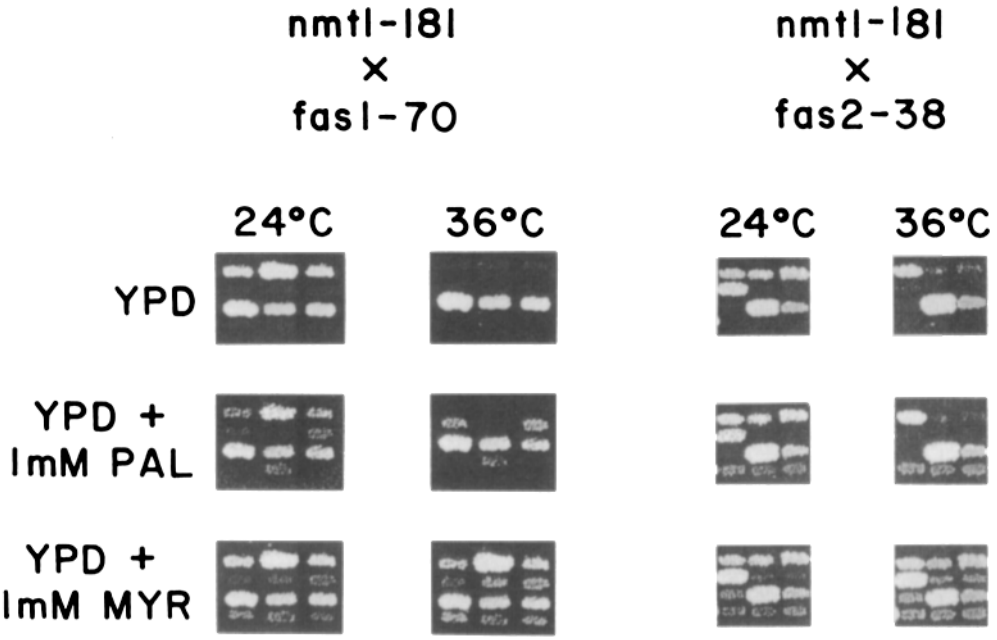

Figure 8. Tetrad analysis of *nmt1-181/NMT1 fas1-70/FAS1* and *nmt1-181/NMT1 fas2-38/FAS2* diploids. Three representative tetratype tetrads derived from each diploid are shown. Each cross is listed at the top of the figure. The genotypes of the strains used to generate the two diploids are listed in Table I as YB218, *fas1*, and *fas2*.

Discussion

The Contribution of Faa1p to Cellular Acyl-CoA Pools

We have cloned the *FAA1* gene of *S. cerevisiae* and characterized its protein product. *FAA1* encodes a long chain acyl-CoA synthetase. Deletion alleles of *faa1* were used to examine the effect of eliminating Faa1p activity on cell viability, lipid metabolism, and protein N-myristoylation. Our results indicate that *FAA1* is a nonessential gene of *S. cerevisiae*. This result is not surprising if one considers that the *S. cerevisiae* FAS complex contains an activity that catalyzes the transfer of the acyl residue from acyl carrier protein to CoA, thereby releasing acylCoA as its end product (Lynen, 1980). In contrast, animal cell fatty acid synthetases contain a specific thioesterase (Burton et al., 1968; Kumar, 1975) that hydrolyzes the growing acyl chain from acyl carrier protein to release free fatty acids as the final product. Consequently, inhibitors of mammalian acyl:CoA synthetase can reduce incorporation of a (exogenous) long chain fatty acid into phospholipids and block cell proliferation (Tomoda et al., 1991).

Several essential *S. cerevisiae* enzymes use acylCoAs as substrates. These include NMT and enzymes that are involved in glycerolipid synthesis, the first step in de novo phospholipid biosynthesis (e.g., acylCoA:dihydroxyacetone-phosphate-*O*-acyltransferase [DHAP acyltransferase, EC 2.3.1.42] and acylCoA:*sn*-glycerol-3-phosphate-*O*-acyltransferase [glycerol-P acyltransferase, EC 2.3.1.15]; [Schlossman and Bell, 1978]). Therefore, in the absence of FAS, acylCoA synthetase should become necessary for activating exogenous fatty acids and be essential for vegetative growth. However, our results clearly show that Faa1p activity is essential only at 36°C in the presence of cerulenin. At 24 and 30°C the *faa1* null strain was able to grow on YPD-MYR/CER, and at 30°C an *faa1* null *fas2* double mutant was viable. This raises the possibility that *S. cerevisiae* contains another acylCoA synthetase activity. This putative enzyme (Faa2p) may have temperature and/or chain length optima that are distinct from those of Faa1p. We have observed strain-dependent growth differences in YPD-MYR/CER at 24 and 30°C: i.e., YB241 fails to grow under these conditions (Duronio et al., 1991*a*) but YB363 does. Preliminary genetic data suggests that allelic variations of loci other than *FAA1* contribute to these phenotypes. It is interesting to speculate that these variations arise from loci encoding other acylCoA synthetases.

AcylCoA synthetases with differing chain length and temperature optima may be advantageous to *S. cerevisiae*. The membrane composition of this yeast changes with different growth temperatures: membrane lipids contain relatively shorter and less fully saturated acyl chains when cells are grown at lower (5–10°C) compared to higher (30–35°C) temperatures (Okuyama et al., 1979; Hori et al., 1987). Regulation of these temperature-dependent changes in acyl chain length is due, in part, to the fatty acid synthetase complex (Okuyama et al., 1979; Hori et al., 1987). In vitro studies using purified *S. cerevisiae* FAS demonstrated that the ratio of C16:C18 fatty acids is higher at 5°C compared to 35°C. However, the level of C14-CoA production did not change with temperature (Singh et al., 1985; Hori et al., 1987). In addition, acetylCoA carboxylase (Acc1p) activity is higher in yeast cells grown at 35°C than in cells grown at 10°C, and fatty acids with longer chain lengths are synthesized by FAS in vitro at higher malonylCoA concentrations. Thus, the FAS effect may reflect the acetylCoA to malonyl-CoA ratio at a given temperature: i.e., at relatively high temperatures Acc1p activity is greater, malonyl CoA concentrations higher, and FAS-mediated production of longer chain fatty acids favored (Hori et al., 1987). The *faa1*-caused phenotypes we observe suggest that Faa1p is the dominant acylCoA synthetase activity at 36°C and may recognize relatively longer chain length fatty acids. Therefore, under conditions where exogenous fatty acids are abundant, Faa1p sup-

plies the cell with the longer chain acylCoAs that are used at high growth temperatures. It does so in lieu of de novo fatty acid synthesis since its acylCoA product represses acetylCoA carboxylase (Kamiryo et al., 1976). Faa2p would have an activity optimum at 24 or 30°C and recognize relatively shorter chain length fatty acids. These hypotheses can be tested by examining the substrate specificities of Faalp at several different temperatures and by surveying unfractionated or fractionated lysates of *faalΔ1.9::HIS3* strains for acylCoA synthetase activities at a variety of temperatures and with a variety of substrates of different chain lengths.

Another possible role for multiple acylCoA synthetases in *S. cerevisiae* would be to direct acylCoAs to cellular pools that have different metabolic fates. As noted above, the yeast *Candida lipolytica* contains two genetically distinct acyl-CoA synthetase activities (Kamiryo et al., 1977*b*, 1979). AcylCoA synthetase I is associated with microsomes and mitochondria (where *sn*-glycerol-3-phosphate *O*-acyltransferase is located) while acylCoA synthetase II is located in microbodies where acylCoAs undergo oxidation (Mishina et al., 1978). *C. lipolytica* strains containing mutations in acyl-CoA synthetase I are unable to incorporate exogenous fatty acids into cellular lipids but can use these fatty acids for β-oxidation. In contrast, acylCoA synthetase II mutants cannot grow on fatty acids as a sole carbon source, but can incorporate exogenous fatty acids into cellular lipids. *S. cerevisiae* may also contain distinct pools of acyl-CoAs. Labeling of phospholipids and N-myristoylproteins with exogenous [^{3}H]myristate was reduced at 36°C but not at 30°C in exponentially growing strains of *S. cerevisiae* containing *NMT1* and *faalΔ1.9::HIS3* null allele compared to strains containing *NMT1* and *FAA1*. These observations raise the possibility that the acyl-CoA pools in *S. cerevisiae* are functionally compartmentalized based on growth temperature or chain length, rather than metabolic fate.

The Role of Faalp in Protein N-Myristoylation

Mutant alleles of *NMT1* have provided a way to monitor changes in the levels of cellular myristoylCoA. A *nmt1-181 faalΔ1.9::HIS3* strain allowed us to examine the contribution of Faalp to the pools of myristoylCoA used by NMT at several different temperatures. In *NMT1* strains, deletion of *faal* had no effect on the extent of incorporation of exogenous myristate into cellular N-myristoylproteins at 24 and 30°C, implying that the putative Faa2p can activate exogenous myristate at these temperatures to a degree sufficient to support N-myristoylation. However, the extent of incorporation was dramatically reduced at 36°C, suggesting that at this temperature Faalp is primarily responsible for supplying myristoylCoA to NMT. The *nmt1-181* mutation causes a dependence on exogenously derived C14:0 at 36°C. Although the *nmt1-181 faalΔ1.9::HIS3* mutant was viable on YPD-MYR at 24 and 30°C, it was unable to grow on this medium at 36°C. This finding is consistent with the metabolic labeling results. We conclude that in the presence of an active FAS complex the relative contribution of Faalp to the myristoyl-CoA pools used by NMT is greater at 36°C than at 24 or 30°C.

By exploiting two means of eliminating FAS activity, we were able to further define the cellular pathways that yield myristoylCoA used by NMT. Eliminating de novo fatty acid synthesis with either cerulenin or *fas1* and *fas2* mutations causes a *nmt1-181* strain to become entirely dependent upon exogenous myristate for growth at 24, 30, and 36°C. Rescue of growth of the *nmt1-181* mutant by exogenous myristate in the presence of cerulenin depends on Faalp at both 36 and 30°C, in contrast to *NMT1* strains, which absolutely depend on Faalp activity only at 36°C when grown in YPD-MYR/CER. Therefore in the *absence* of FAS activity, there is an increase in a *nmt1-181* cell's need for myristoylCoA at 30°C which cannot be solely supplied by the putative Faa2p activity. This is not true at 24°C since the *nmt1-181 faalΔ-1.9::HIS3* strain is viable on YPD-MYR/CER at this temperature. In other words, the *nmt1-181* cell requires both FAS and Faalp activity at 30°C, but does not require Faalp at 24°C (in the absence of FAS) presumably because the postulated Faa2p can provide a necessary amount of myristoyl-CoA from exogenous C14:0.

Finally, the inhibition of *nmt1-181* strains by O6 raises the issue of whether transport of long chain fatty acids into yeast is coupled to acylCoA formation. In *E. coli*, transport of long chain (≥C10) fatty acids into the cell requires a functional acylCoA synthetase (encoded by the *fadD*$^+$ gene) that is loosely affiliated with the inner membrane (Klein et al., 1971; Kameda and Nunn, 1981; Maloy et al., 1981) as well as a 421 residue, multifunctional outer membrane receptor/transporter encoded by the *fadL*$^+$ gene (Nunn et al., 1978, 1979, 1986; Black et al., 1987; Black, 1991; Kumar and Black, 1991). Our data indicate that the O6 inhibitory effect does not require the product of *FAA1*. If this result is interpreted to mean that O6 produces its effect as a free fatty acid, then it follows that conversion of this and perhaps other long chain fatty acids to their CoA derivatives may not be a prerequisite for their transport into *S. cerevisiae*. The results of Kamiryo et al. (1976) support this contention. They observed a ~5-fold increase in the proportion of free fatty acids in preparations of total cellular lipids isolated from *faal* compared to *FAA1* strains of *S. cerevisiae*. If the O6 effect does require conversion of the myristic acid analog to its CoA derivative, then another acylCoA synthetase activity must be operating at 24°C (as suggested by the results of our other studies of *faal* null mutants). The *faal* deletion mutants described in this report should now allow a search to be conducted for additional acylCoA synthetase activities in *S. cerevisiae* and if identified permit an exploration of their role in regulating cellular lipid metabolism and protein N-myristoylation.

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