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 myristoyl-CoA pools in *Saccharomyces cerevisiae* plays an important role in modulating the activity of myristoyl-CoA:protein N-myristoyltransferase (NMT), an essential enzyme with an ordered Bi Bi reaction that catalyzes the transfer of myristate from myristoyl-CoA to ≥12 cellular proteins. At least two pathways are available for generating myristoyl-CoA: de novo synthesis by the multifunctional, multisubunit fatty acid synthetase complex (FAS) and activation of exogenous myristate by acyl-CoA 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 acyl-CoA synthetase of 700 amino acids. Analyses of strains containing NMTI 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 acyl-CoA 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., 1988a; 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) (EC 2.3.1.97). Insertional mutagenesis or deletion of the gene encoding *Saccharomyces cerevisiae* NMT (NMTI) 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., 1987a,b; 1988a,b; Duronio et al., 1991a,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., 1991a). Five of the polypeptides are known. The functionally interchangeable ADP-ribosylation factors (Arflp and Arf2p) have a role in protein secretion. Disruption of both *ARF* genes is lethal (Steams et al., 1990a,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 CNBI 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...
heterotrimeric G protein involved in mating pheromone sig-

tual transduction. Genetic studies have shown that Gpαl

is responsible for suppression of this signal (reviewed in

Blumer and Thorner, 1991). Consequently, deletion of Gpαl

or Gly^{2}→ 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., 1987a,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):

\[
\begin{align*}
\text{myristoyl-CoA} & \quad \text{peptide} \quad \text{catalysis} \\
\downarrow & \quad \downarrow \\
\text{NMT} \quad \rightarrow \quad \text{NMT-myristoyl-CoA} & \quad \rightarrow \quad \text{myristoylCoA-NMT-peptide} & \rightarrow \quad \text{NMT} \\
\downarrow & \quad \downarrow \\
\text{myristoylpeptide-NMT-CoA} & \quad \rightarrow \quad \text{NMT-myristoylpeptide} & \rightarrow \quad \text{NMT} \\
\downarrow & \\
\text{CoA} & \quad \text{myristoylpeptide}
\end{align*}
\]

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 tempera-
ture sensitive, myristic acid auxotroph: grow at the non-

permissive temperature is only possible when media are

supplemented with ≥500 μM myristate (Meyer and Schweizer,

1974). This phenotype is a result of a single missense muta-
tion of NMT1 that changes Gly^{545} to Asp (Duronio et al.,

1991a). In vitro studies of the purified mutant protein reveal

a 10-fold increase in its apparent K_{m} for myristoyl-CoA

at 36°C relative to wild type (Duronio et al., 1991a).

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

(Accl, E.C. 6.4.1.2; Mishina et al., 1980), which produces

malonyl-CoA for utilization by the acyl-CoA synthetase

complex (FAS). FAS is a multifunctional 2.4 x 10^{6} Da

d enzyme, composed of the products of the unlinked FASI (β)

and FAS2 (α) loci (Burki et al., 1972; Kuhn et al., 1972;

Schweizer et al., 1986; Chira et al., 1987; Mohamed et al.,

1988; Siebenlist et al., 1990). Eight catalytic activities are

associated with the complex (β-ketoacyl synthase, β-keto-

dacyl reductase, and the 4'-phosphopantetheine prosthetic

group with the α subunit; acetyl transacylase, malonyl trans-

acylase, malonyl and palmitoyl transferases, dehydrogenase,

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-dodeca-2,4-dienamide, 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 cerule-
nin at 24, 30, and 36°C without supplementing glucose-rich

medium with CI2-C18 fatty acids (Awaya et al., 1975; Du-

ronio et al., 1991a). Treatment of strains containing nmt1-181

with cerulenin also causes growth arrest at these tempera-
tures. This arrest can be reversed by adding exogenous my-

ristate but not palmitate to the medium, suggesting that

metabolic interconversion of CI6:0 to CI4:0 is not sufficient
to restore myristoyl-CoA pools to a level that permits growth

of this mutant (Duronio et al., 1991a). 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 con-
taining nmt1-181 grown at the nonpermissive temperature in

the presence or absence of cerulenin suggests that supple-
mentation of intracellular myristoyl-CoA pools by extracellu-
lar sources of CI4:0 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:

\[
\text{Mg}^{2+} \quad \text{Free fatty acid + ATP} \quad \rightarrow \quad \text{acyl-AMP + PP,}
\]

\[
\text{Acyl-AMP + CoA} \quad \rightarrow \quad \text{acylCoA + AMP}
\]

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 syn-
thetase have been identified in connection with studies on the

mechanism of repression of acetyl-CoA carboxylase by ex-
genous long chain fatty acids (Kamiryo et al., 1976, 1977a).
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 deriva-
tives. 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 design-

ated faa1 (fatty acid activation). These mutants were found
to be defective in acyl-CoA synthetase activity, which appar-

ently accounts for their failure to repress acetyl-CoA carbox-
ylase 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.,

1991a). These observations support the notion that Faalp

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 FAAI gene by complementation of the faal phenotype, characterized its protein product, and subsequently created strains with faal null mutations. Genetic and biochemical analyses of these strains indicate that the product of the single copy FAAI 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-PAL), 500 μM palmitate (YPD-PAL), or without 25 μg/mL cerulinein (Sigma Chemical Co., St. Louis, MO), or combinations of cerulenein and fatty acids at these concentrations (i.e., YPD-CER; YPD-MYR/CER; YPD-PAL/CER). YPD-06 contains 500 μM 6-oxatetradecanoate (O6; Heuckeroth et al., 1988). YPD-MYR, YPD-PAL, YPD-06, 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 FAAI

The faal strain YB241 (Table I) was grown in YPD at 30°C until an OD600 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 that grew under these conditions. FAAI was sequenced using the dye terminator method (Sanger et al., 1977). Nested deletions (pBluescript Exo/Mung Bean DNA sequencing system; Stratagene, La Jolla, CA) of FAAI 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).

Table I. Yeast Strains

| Strain  | Genotype                                      | Reference         |
|---------|-----------------------------------------------|-------------------|
| BJ405   | MATa trp1 prb1-1122 prcl-126 pep4-3           | Hemmings et al., 1981 |
| YM2061  | MATa ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | Flick and Johnston, 1991 |
| YB100   | MATa ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | Duronio et al., 1989 |
| YB218   | MATa nmt1-181 ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | Duronio et al., 1991a |
| YB241   | MATa faa1 ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | Duronio et al., 1991a |
| YB360   | MATa faa1 ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | This work         |
| YB363   | MATa faa1 ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | This work         |
| YB364   | MATa faa1 ura3-52 his3a200 ade2-101 lys2-801 met LEU2::GAL1-lacZ | This work         |
| fas1    | MATa fas1-70                                   | Schweizer et al., 1978 |
| fas2    | MATa fas1-70                                   | Schweizer et al., 1978 |
| YB366   | MATa fas1-70                                   | This work         |

The following non-redundant databases were searched: NBRF/PIR (Release 290), SWISS-PROT (Release 90), GenPept (translated GenBank, Release 64.3), and G/PUpdate (GenPept daily update: Sept 23, 1991).

FAAI Locus Alterations

Alterations of the FAAI locus were generated by transforming diploid strain YBI00 (Table I) to histidine prototrophy with linear DNA containing the desired mutation (Rothenstein, 1983). The faa1::HI53 allele was made by replacing a 341 bp fragment of the FAAI coding region (beginning at the EcoRI site at nucleotide 1063 as seen in Fig. 1B) with a 1.7-kbp EcoRI-BamHI restriction fragment (Fig. 1A) containing HI53 (Sraul, 1985). The faa1::HI53 allele was made by replacing a 1.9-kbp region of FAAI bordered by BglII sites with a 1.7-kbp BamHI-BamHI fragment containing HI53 (Fig. 1A). Each change in the FAAI 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 [32P]dATP-labeled, 4.2 kb, Scal-Scal fragment containing the entire FAAI coding region. Blots were hybridized in a solution containing 5x SSPE, 5x 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 0.1x SSC (DNA blot) or 0.1x SSC (RNA blot) and then subjected to autoradiography at ~70°C.

Mapping of FAAI

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 Scal-Scal FAAI fragment (see above) in a solution containing 0.5 M NaCl, 0.1 M NaHPO4, 5 mM Na2EDTA, 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 OD600 of 1.5. They were then incubated for 1 h with either [9,10(n)3H]myristate (100 μCi/mL of culture, 54 Ci/mmol;
Amersham Corp.) or [9,10(14)C]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., 1991a). 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 En3HANCE (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 MgCl2, 3 mM DTT, 0.05% Triton X-100, 100 μM EDTA, 0.5 μM [H]myristic acid (93.9 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. [H]Mysteryl-CoA and [H]-myristic acid were resolved by C4 reverse-phase HPLC (Vydac, 5 μm × 4.6 mm) using an isocratic gradient of 60% 20 mM KP04, pH 5.5, and 40% acetonitrile (flow rate = 2 ml/min). Titrated products were quantitated using an in-line scintillation counter (model CR; Radiomatic Instruments, 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 faa1 mutation identified by Kamiryo et al. (1976) causes an easily scored phenotype, we attempted to clone FAA1 by genetic complementation. The faa1 strain YB241 (Table I) is unable to grow on YPD containing 500 μM myristate and 25 μM cerulenin at 36°C (Duronio et al., 1991a). 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 faa1 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 faa1 mutation. To determine whether the 5.8-kb insert mapped to the faa1 locus, HIS3 was integrated at the genomic position from which this cloned DNA was derived (yielding the faa1::HIS3 allele; see Materials and Methods). Analysis of 25 tetrads from a cross between this faa1::HIS3 strain and YB241 revealed only parental ditypes, indicating that the cloned DNA is tightly linked to the faa1 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 faa1 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 faa1 mutation. Another plasmid containing all of the sequences 3' of the XbaI site also failed to complement faa1. These findings suggested that a functional open reading frame spanned the XbaI and BamHI sites. A 4.8-kb SacI-Sall 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.

**Purification of Faalp**

A three step protocol was used for purification of acyl-CoA 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 A600 of 1, at which time an additional 80 g of glucose was added to maintain lag phase growth. Yeast were harvested at an A600 of +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 30 s of incubation on ice. The homogenate was spun at 10,000 g for 30 min. Approximately 96% of the acyl-CoA 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 pre-swollen and equilibrated in buffer A (20 mM KPO4, 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 KPO4, pH 7.4, 1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.01% Triton X-100). Acyl-CoA synthetase was eluted with buffer C (250 mM KPO4, 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**

P1 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 KPO4, 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).
**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 by of sequence shown in B. The dashed lines indicate the portion of the FAA1 locus subcloned 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 shown in B. The dashed lines indicate the portion of the FAA1 locus subcloned areshown in parentheses. The restriction fragment used replaced in the mutant allele by the restriction fragment carrying the HIS3 gene (hatched bar). Restriction sites destroyed during subcloning are shown in B. Thedashed lines indicate theportion of the FAA1 locus site and extends =4.2 kb to the right. (C) Alignment of FAA1 (top) and rat.
with a $M_r$ of 77,816 (Fig. 1 B). The location of this ORF agrees 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:

$$\text{luciferin} + \text{ATP} \rightarrow \text{luciferyl-AMP} + \text{PP},$$
$$\text{luciferyl-AMP} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{hv}.$$

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 [3H]myristate to [3H]myristoylCoA. For example, 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- and 53-kD polypeptides shown in fraction 46 established their identities as Faalp and vacuolar H+ -translocating ATPase, respectively.

Table II. Purification of S. cerevisiae AcylCoA Synthetase

| Fraction     | Protein | Activity* | Sp. Act. | Purification | Yield |
|--------------|---------|-----------|----------|--------------|-------|
| 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  |

*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). 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.
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 (FAAI/FAAI, lanes 1, 2, and 5), YB360 (FAAI/Δ faa1::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 32P-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).

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

A variety of blot hybridization studies were performed to determine the location of FAAI 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 acyl-CoAs that are destined to be degraded by β-oxidation (Kamiryo et al., 1977b, 1979; Mishina et al., 1978a,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-
Fatty acid synthesis is operational at 24, 30, and 36°C. The faa1 null allele is not an essential gene when the de novo pathway is used (YB363; Fig. 4, lanes 3 and 4). These observations indicate that FAA1 is located 10–20-kb centromere distal to CPA1, and 100–120-kb centromere proximal to PHRI (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-(suf3-7;faa1)-(cdc66-pro2)-PHRI (Fig. 3 C). We have not obtained data on the relative order of suf3 and faa1, but the genes are probably not allelic since the suf3 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 Faalp in cell growth, fatty acid metabolism, and protein N-myristoylation. A faal 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/FAA1::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 ascii 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/FAA1::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 [3H]myristate as the substrate plus the assay conditions described in Materials and Methods). Parallel assays of strains YB241 (faal) 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 I) 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 Faalp, FAAI 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 NMIT 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 Faalp 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 Faalp. This latter hypothesis was supported by metabolic labeling of exponentially growing strains containing the FAAI or faa1Δ1.9::HIS3 alleles. At 30°C there was no difference between the two strains in the extent of incorporation of label from [3H]myristate into a variety of phospholipid classes including phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylethanolamine (PE) (Fig. 6). In the absence of Faalp, 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 Faalp supplies activated fatty acids for phospholipid synthetic pathways in S. cerevisiae (Carman and Henry, 1989) during growth at 36°C.

**The Contribution of Faalp in Regulating Protein N-Myristoylation**

The faa1 null allele allowed us to definitively determine whether Faalp plays a role in modulating protein N-myristoylation. nmutl-181 FAAI or nmutl-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 *FAAI* alleles. Strains YM2061 (*NMT1 FAAI*), YB218 (*nmt1-181 FAAI*), YB363 (*NMT1 faa1Δ1.9::HIS3*), and YB371 (*nmtI-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 
[3H]myristate. Strains YM2061 (FAA1) and YB363 (faalΔ1.9::HIS3) were grown at 30 or 36°C in YPD plus [3H]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 chroma-
tography as described in Materials and Methods. An autoradio-
graph of the silica plate is shown. The positions of migration of
lipid standards are indicated. PC, phosphatidylcholine; PE, phos-
phatidylethanolamine; PI, phosphatidylinositol; and PS, phospha-
tidylserine.

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 nmtl-181. Blockade of de novo fatty acid synthesis by 25 μM cerulenin prevents growth of the nmtl-181 FAA1 strain on YPD and YPD containing 500 μM palmitate at all three temperatures (YPD-CER, YPD-PAL/CER; Fig. 5). In

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 [35S]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 nmtl-181 strains. Substitution of oxygen for methylene at C6 (the carboxyl carbon = Cl) 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 [3H]O6 during exponential growth on YPD indicated that the accumulation of analog in the yeast was 1% that of myristate (Duronio et al., 1999a). This could be because of inefficient transport (Duronio et al., 1999a; Bryant et al., 1991) and may explain why [3H]O6 is not incorporated into S. cerevisiae cellular N-myristoylproteins at detectable levels (Duronio et al., 1999a). Despite these findings, supplementing YPD with 500 μM O6 inhibits the growth of nmtl-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., 1999a). 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 nmtl-181/FAA1 strains and indicated that Faalp is not required for O6-induced inhibition of growth of nmtl-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.

Fatty acid containing media with or without 25 μM cerulenin (Fig. 5). nmtl-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 nmtl-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 nmtl-181 faalΔ1.9::HIS3 strain compared to the nmtl-181 FAA1 strain (compare Fig. 7 C with...
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 Km defect in the mutant nmt1-181.

Blockade of de novo fatty acid biosynthesis by cerulenin completely inhibits growth of the nmt1-181 faa1d1.9::HIS3 strain at 36°C on YPD containing 500 μM myristate. However, unlike the NMT1 faa1d1.9::HIS3 strain, the nmt1-181 faa1d1.9::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 Faalp activity at 30°C. At 24°C, the nmt1-181 faa1d1.9::HIS3 strain is viable on YPD-MYR/CER, and grows at a rate that is similar to that of the NMT1 faa1d1.9::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 Faalp to activate exogenous myristate.

**Blocking De Novo Fatty Acid Synthesis with fas Mutations.** A genetic experiment was performed using strains containing the nonconditional fasl-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 fasl-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 fasl-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 FAST 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 myristoyl-CoA product of the cytoplasmic FAS complex (Schweizer et al., 1978).

**A fas2-38 faa1d1.9::HIS3 NMT1 Strain Is Viable**

We used the fas2-38 allele to examine the consequences of eliminating Faalp activity in a Fas-cell. Tetrads were dissected from a diploid constructed by conjugating fas2-38 his3Δ200 and faa1d1.9::HIS3 his3Δ200 strains (YB360 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 faa1d1.9::HIS3 alleles. This result supports the conclusion that Faalp activity is not essential at 30°C in the absence of de novo fatty acid synthesis.
Discussion

The Contribution of Faalp 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 faal were used to examine the effect of eliminating Faalp 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 an (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:m-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 Faalp activity is essential only at 36°C in the presence of cerulenin. At 24 and 30°C the faal null strain was able to grow on YPD-MYR/CER, and at 30°C an faal 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 Faalp. 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 (Duorono et al., 1999) 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 (Acclp) 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 Acclp activity is greater, malonyl CoA concentrations higher, and FAS-mediated production of longer chain fatty acids favored (Hori et al., 1987). The faal-caused phenotypes we observe suggest that Faalp 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, Faalp 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 (Kamiyo 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 unfractonated 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 (Kamiyo et al., 1977b, 1979). AcylCoA synthetase I is associated with microsomes andochondria (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 [1H]myristate was reduced at 36°C but not at 30°C in exponentially growing strains of S. cerevisiae containing NMTI and faalΔ1.9::HIS3 null allele compared to strains containing NMTI 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 NMTI have provided a way to monitor changes in the levels of cellular myristoylCoA. A nmtl-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 NMTI 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 nmtl-181 mutation causes a dependence on exogenously derived C14:0 at 36°C. Although the nmtl-181 faalΔ1.9::HIS3 mutant was viable onYPD-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 nmtl-181 strain to become entirely dependent upon exogenous myristate for growth at 24, 30, and 36°C. Rescue of growth of the nmtl-181 mutant by exogenous myristate in the presence of cerulenin depends on Faalp at both 36 and 30°C, in contrast to NMTI 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 nmtl-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 nmtl-181 faalΔ1.9::HIS3 strain is viable on YPD-MYR/CER at this temperature. In other words, the nmtl-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 nmtl-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., 1986; 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 Kamiyo 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 faa1 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.

We thank Carol Langner for her help with the high performance thin layer chromatographic analysis of labeled cellular lipids. Eckart Schweizer for S. cerevisiae faa1 strains, Linda Riles and Maynard Olson for prime lambda clones used in the physical mapping studies, George Gokel for samples of 6-oxoexetadecanoic acid, and Robin Johnson for excellent technical assistance.

R. J. Duronio is a recipient of a Spencer T. and Ann W. Olin Predoctoral Fellowship. This work was supported by grants from the National Institutes of Health (AI30188 and AI27179) and from the Monsanto Company.

Received for publication 23 December 1991 and in revised form 6 February 1992.

References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Awaya, J., T. Ohno, H. Ohno, and S. Omura. 1975. Substitution of cellular
fatty acids in yeast cells by the antibiotic cerulenin and exogenous fatty acids. *Biochim. Biophys. Acta*. 409:267-275.

Black, P. N., and J. G. C. Hemmings. 1996. The promoter of the *Escherichia coli* fadA gene encoding an outer membrane protein required for long-chain fatty acid transport. *J. Bacteriol*. 177:435-422.

Black, P. N., B. Said, C. R. Ghosn, J. V. Beach, and W. D. Nunn. 1987. Purification and characterization of an outer membrane-bound protein involved in long-chain fatty acid transport in *Escherichia coli*. *J. Biol. Chem.* 262:1412-1429.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*. 37:911-917.

Blumer, K. J., and J. Thornr. 1991. Receptor-G protein signaling in yeast. *Ann. Rev. Phys*. 53:37-57.

Boyle, J. H., and E. H. Ludwig. 1962. Analysis of fatty acids of continuously cultured mammalian cells by gas-liquid chromatography. *Nature (Lond.*) 196:893-894.

Bryant, M. L., and L. Ratiner. 1980. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA*. 77:523-527.

Bryant, M. L. L. Ratiner, R. J. Duronio, N. S. Kishore, B. Devadas, S. P. Adams, and J. I. Gordon. 1991. Incorporation of 12-methyloctadecanoate into the giant polyprotein precursor of HIV-1 inhibits its proteolytic processing as well as viral production in a chronically infected human lymphoid cell line. *Proc. Natl. Acad. Sci. USA*. 88:2055-2059.

Burki, G., H. J. Castroph, and E. Schweizer. 1972. Mapping of a complex gene locus coding for part of the *Saccharomyces cerevisiae* fatty acid synthetase multienzyme complex. *Mol. Gen. Genet.* 119:315-322.

Burton, D. N., and A. G. Haavik, and J. W. Porter. 1968. Comparative studies of the rat and pigeon fatty acid synthetases. *Arch. Biochem. Biophys*. 126:141-154.

Carlsson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell*. 28:145-154.

Carman, G. M., and S. A. Henry. 1989. Phospholipid biosynthesis in yeast. *Annu. Rev. Biochem*. 58:635-669.

Chirala, S. S., M. A. Kuziora, D. M. Spector, and S. J. Wakil. 1987. Complementation of mutations and nucleotide sequence of FAS1 gene encoding protein N-myristoyltransferase. *J. Cell Biol.* 113:1313-1330.

Chow, M. R., R. F. Gabor, and C. M. Cummins. 1982. Frameshift suppression in *Saccharomyces cerevisiae*. *Science (Wash. D.C.)* 243:796-800.

Clay, J. I., and T. A. H. Fischer. 1982. Sequence analysis programs for the VAX. *Nucleic Acids Res*. 12:387-395.

Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa. 1984. A short sequence in the p60r subunit is required for p60r myristoylation and membrane association and for cell transformation. *Mol. Cell. Biol*. 4:1834-1842.

Culbertson, M. R., R. F. Gabor, and C. M. Cummins. 1982. Frameshift suppression in *Saccharomyces cerevisiae* virus. *Isolation and genetic properties of nongroup-specific suppressors. Genetics*. 102:361-378.

Cyert, M. S., R. Kuniawa, D. Kaim, and J. Thornr. 1991. Modulation of phosphorylation by calcium: biochemical and genetic characterization of yeast calcineurin. In Yeast Cell Biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 224.

Deichateau, L., P. L. Casson, H.-P. Ling, and M. D. Resh. 1988. In vitro synthesis of p60src- myristoylation in a cell-free system. *Mol. Cell. Biol*. 8:4295-4301.

Denis, C. L., and E. T. Young. 1983. Isolation and characterization of the positive regulatory gene ADR1 from *Saccharomyces cerevisiae*. *Mol. Cell. Biol*. 3:360-370.

Devereux, J., P. Haebeler, and O. Smithies. 1982. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.

Duronio, R. J., D. A. Towler, R. O. Heckerthor, and J. I. Gordon. 1989. Disruption of the yeast N-myristoyl transferase gene causes recessive lethality. *Science (Wash. DC).* 243:796-800.

Duronio, R. J., D. A. Rudnick, R. L. Johnson, M. E. Linder, and J. I. Gordon. 1990. Reconstitution of protein N-myristoylation in *E. coli*. Methods: A Companion to Methods Enzymol. 1:253-268.

Durojio, R. J., D. A. Rudnick, J. D. Rosson, and J. I. Gordon. 1991a. Myristic acid auxotrophy caused by mutation of *S. cerevisiae* myristoyl-CoA-protein N-myristoyltransferase. *J. Biol. Chem*. 113:1313-1330.

Durojio, R. J., D. A. Rudnick, S. P. Adams, D. A. Towler, and J. I. Gordon. 1991b. Analyzing the substrate specificity of *S. cerevisiae* myristoyl-CoA: Protein N-myristoyltransferase by Co-expressing it with mammalian G protein α subunits in *E. coli*. *J. Biol. Chem.* 266:10496-10504.

Durojio, R. J., S. O, and J. I. Gordon. 1992. Mutations of human myristoyl-CoA protein N-myristoyltransferase cause temperature sensitive myristic acid auxotrophy in *S. cerevisiae*. *Proc. Natl. Acad. Sci. USA*. In press.

Elder, R. I., E. Y. Loh, and R. W. Davis. 1983. RNA from the yeast transposable element Ty1 has both ends in the direct repeats, a structure similar to retrovirus RNA. *Proc. Natl. Acad. Sci. USA*. 80:2432-2436.

Flick, J. S., and M. Johnson. 1990. Two systems of repression of the GAL promoter in *Saccharomyces cerevisiae*. *J. Biol. Chem*. 261:1497-1499.

Funabashi, H., A. Kawaguchi, H. Tomoda, S. Omura, S. Okuda, and S. Iwasaki. 1989. Binding site of cerulenin in fatty acid synthetase. *J. Biochem*. 105:751-755.
Duronio et al. The Role of FAAI in Protein N-Myristoylation

Schweizer, E., K. Werkmeister, and J. Jain. 1978. Fatty acid biosynthesis in yeast. *Mol. Cell. Biochem.* 21:95-107.

Schweizer, M., L. M. Roberts, H.-J. Holtek, K. Takabayashi, E. Hollerer, B. Hoffmann, G. Muller, H. Kottig, and E. Schweizer. 1986. The pentafucional FAS1 gene of yeast: its nucleotide sequence and order of the catalytic domains. *Mol. Gen. Genet.* 203:479-486.

Sebastian, J., B. Kraus, and G. B. Sancar. 1990. Expression of the yeast PHR1 gene is induced by DNA-damaging agents. *Mol. Cell. Biol.* 10:4630-4637.

Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Siebenlist, U., J. Nix, M. Schweizer, D. Jager, and E. Schweizer. 1990. Mapping of the functional fatty acid synthetase gene FAS2 on chromosome XVI of *Saccharomyces cerevisiae*. *Yeast* 6:411-415.

Singh, N., S. J. Walik, and J. K. Stoops. 1985. Yeast fatty acid synthase: structure to function relationship. *Biochemistry.* 24:6598-6602.

Stearns, T. C., M. Williamson, D. Botstein, and R. A. Kahn. 1990a. ADP-ribosylation factor is functionally and physically associated with the Golgi complex. *Proc. Natl. Acad. Sci. USA.* 87:1238-1242.

Stearns, T., R. A. Kahn, D. Botstein, and M. A. Hoyt. 1990b. ADP ribosylation factor is an essential protein in *Saccharomyces cerevisiae* and is encoded by two genes. *Mol. Cell. Biol.* 10:6690-6699.

Stone, D. E., G. M. Cole, M. B. Gropel, and S. I. Reed. 1991. N-myristoylation is required for function of the pheromone-responsive Gap protein of yeast: conditional activation of the pheromone response by a temperature-sensitive N-myristoyl transferase. *Genes & Development.* 5:1969-1981.

Stoops, J. K., and S. J. Walik. 1978. The isolation of the two subunits of yeast fatty acid synthetase. *Biochem. Biophys. Res. Commun.* 84:225-231.

Sturhol, K. 1985. Nucleotide sequence and transcriptional mapping of the yeast pse56-his3-dei gene region. *Nucleic Acids Res.* 13:8587-8601.

Sturhol, K. 1989. Molecular mechanisms of transcriptional regulation in yeast. *Ann. Rev. Biochem.* 58:1051-1077.

Suzuki, H., Y. Kawarabayasi, J. Kondo, T. Ahe, K. Nishikawa, S. Kimura, T. Hashimoto, and T. Yamamoto. 1990. Structure and regulation of rat long-chain acyl-CoA synthetase. *J. Biol. Chem.* 265:8681-8685.

Tomoda, H., K. Igarashi, J.-C. Cyong, and S. Omura. 1991. Evidence for an essential role of long chain acyl-CoA synthetase in animal cell proliferation: inhibition of long chain acyl-CoA synthetase by triacycl caused inhibition of Raji cell proliferation. *J. Biol. Chem.* 266:4214-4219.

Towler, D., and L. Glaser. 1986. Protein fatty acid acylation: Enzymatic synthesis of an N-myristoylglycpeptide. *Proc. Natl. Acad. Sci. USA.* 83:2812-2816.

Towler, D. A., S. E. Eubanks, D. S. Towery, S. P. Adams, and L. Glaser. 1987a. Amino-terminal processing of proteins by N-myristoylation. *J. Biol. Chem.* 262:1030-1036.

Towler, D. A., S. P. Adams, S. E. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. 1987b. Purification and characterization of myristoyl-CoA protein N-myristoyltransferase. *Proc. Natl. Acad. Sci. USA.* 84:2708-2712.

Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser. 1988a. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* 57:69-99.

Towler, D. A., S. P. Adams, S. E. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. 1988b. Myristoyl CoA: Protein N-myristoyltransferase activities from rat liver and yeast posses overlapping yet distinct peptide substrate specificities. *J. Biol. Chem.* 263:1784-1790.

Tance, D., I. Goldberg, O. Mitsuhashi, and K. Bloch. 1972. Inhibition of fatty acid synthetases by the antibiotic cerulen. *Biochem. Biophys. Res. Commun.* 48:649-656.

Weigand, R. C., J. C. Minnery, A. M. Pauley, C. P. Carron, C. Z. Carr, C. Langner, R. J. Duronio, and J. I. Gordon. 1987. The Candida albicans myristoyl CoA-protein N-myristoyltransferase gene: isolation and expression in *S. cerevisiae* and *e. coli*. *J. Biol. Chem.* 262:8591-8598.

Wilcox, C., J.-S. Hu, and E. N. Olson. 1987. Acylation of proteins with myristic acid occurs cotranslationally. *Science (Wash. DC).* 238:1275-1278.

Winter, E., and A. Varshavsky. 1989. A DNA binding protein that recognizes oligo(A)- oligo(T) tracts. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1867-1877.