Myristic Acid Auxotrophy Caused by Mutation of *S. cerevisiae* Myristoyl-CoA:Protein N-Myristoyltransferase

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**Abstract.** The *S. cerevisiae* myristoyl-CoA:protein N-myristoyltransferase gene (*NMT1*) is essential for vegetative growth. *NMT1* was found to be allelic with a previously described, but unmapped and unidentified mutation that causes myristic acid (C14:0) auxotrophy. The mutant (*nmt1-181*) is temperature sensitive, but growth at the restrictive temperature (36°C) is rescued with exogenous C14:0. Several analogues of myristate supplementation of growth at 36°C by exogenous fatty acids is blocked by a mutation affecting the acyl-CoA synthetase gene. The *nmt1-181* allele contains a single missense mutation of the 455 residue acyltransferase that results in a Gly451→Asp substitution. Analyses of several intragenic suppressors suggest that Gly451 is critically involved in NMT catalysis. In vitro kinetic studies with purified mutant enzyme revealed a 10-fold increase in the apparent *Kₐ* for myristoyl-CoA at 36°C, relative to wild-type, that contributes to an observed 200-fold reduction in catalytic efficiency. Together, the data indicate that nmt-181 represents a sensitive reporter of the myristoyl-CoA pools utilized by NMT.

MYRISTOYL-CoA:protein N-myristoyltransferase (*NMT1*; EC 2.3.1.97) catalyzes the cotranslational (Wilcox et al., 1987; Dechaite et al., 1988) covalent linkage of myristate, a rare (Orme et al., 1972; Awaya et al., 1975) 14-carbon saturated fatty acid, to the NH₂-terminal glycine residue of a variety of proteins having diverse functions (reviewed in Towler et al., 1987b; Heuckeroth et al., 1989), indicating that one or more essential cellular proteins function. In vitro kinetic studies have revealed that the MYR, myristic acid; PAL, palmitic acid.

1. Abbreviations used in this paper: CER, cerulenin; EMS, ethylmethane sulfonate; NMT, myristoyl-CoA:protein N-myristoyltransferase; MYR, myristic acid; PAL, palmitic acid.
al., 1988). Cooperative interactions between these sites contribute to the remarkable specificity of the reaction mechanism. Presentation of the wrong chain length acyl-CoA to NMT produces dramatic alterations in the peptide binding site, which reduce peptide affinity and preclude efficient transfer of the acyl chain (Heuckereth et al., 1988). NMT exhibits a more stringent selection in vitro against longer chain length acyl-CoAs than shorter chain length species (Towler et al., 1987a; Rudnick et al., 1990). Biophysical studies have shown that S. cerevisiae NMT can form a high-affinity acyl-CoA-enzyme intermediate in the absence of its peptide substrate. (Rudnick et al., 1990). Subsequent kinetic studies revealed an ordered Bi-Bi reaction mechanism for NMT, with myristoyl-CoA binding prior to peptide, and CoA releasing before myristoyl-peptide (Rudnick et al., 1991).

How S. cerevisiae regulates its intracellular pools of myristoyl-CoA, where these pools reside, and how NMT gains access to them is unclear. NMT must “avoid” inhibition by other acyl-CoAs (e.g., palmitoyl-CoA), and therefore these factors could be important in regulating the efficiency and specificity of protein N-myristoylation in vivo (Rudnick et al., 1990). The principal fatty acid biosynthetic enzymes in S. cerevisiae, acetyl CoA carboxylase, and the acβ, fatty acid synthetase complex, are encoded by ACCL (Mishina et al., 1980) and the FASL (β) and FAS2 (α) loci (Schweitzer et al., 1986, 1987; Chirala et al., 1987; Mohamed et al., 1988), respectively. The major products of these cytoplasmic enzymes are palmitoyl-CoA and stearoyl-CoA (Lynen, 1980), although myristoyl-CoA is also produced (Lynen, 1969).

Mutations of S. cerevisiae that result in auxotrophy for saturated fatty acids were identified 20 yr ago, and mapped to three unlinked loci (Henry and Fogel, 1971; Kuhn et al., 1972). These mutations cause temperature-sensitive growth arrest that can be complemented with exogenous C14:0, myristoyl-CoA, where these pools residue, and how NMT and specificity of protein N-myristoylation in vivo (Rudnick et al., 1980) and the FAS2 (α) and FAS2 (β) loci (Schweitzer et al., 1986, 1987; Chirala et al., 1987; Mohamed et al., 1988), respectively. The major products of these cytoplasmic enzymes are palmitoyl-CoA and stearoyl-CoA (Lynen, 1980), although myristoyl-CoA is also produced (Lynen, 1969).

Metabolic Labeling Studies

Strains YM2061 and YB218 were grown to an A600 of 0.95 in YPD broth at 24°C. A 2-ml aliquot of each culture was metabolically labeled with either [9,10(n)3H]myristic acid (39.3 Ci/mmol; NEN Research Products, Dupont, Boston, MA; 50 µCi/ml of culture) or [15S]methionine (1.149 Ci/mmol, NEN Research Products, Dupont; 1.0 µCi/ml of culture) for 30 min at 24°C and analyzed as below. At the same time that this aliquot was removed, the remaining cells in each culture were shifted to 36°C and 2-ml aliquots of each were removed 60, 120, and 240 min later. These were incubated with the radiolabeled compounds for 30 min at 36°C, chilled on ice for 5 min, collected by centrifugation for 5 min at 4,000 g, and then washed once with 1 ml PBS. The cells were subsequently disrupted by vortexing for 2 min with 500-μm glass beads in a 100-μl solution containing 240 mM Tris-HCl, pH 6.8, 2% SDS, 0.4% β-mercaptoethanol, and 10% glycerol. The mixture was then incubated at 100°C for 5 min. Cellular debris was removed by centrifugation at 12,000 g for 5 min. An equal mass

Materials and Methods

Yeast Strains and Media

The genotypes of all Saccharomyces cerevisiae strains used in this study are listed in Table 1. YPD (% yeast extract, 2% peptone, 2% dextrose) was used as the standard growth medium with 2% galactose replacing dextrose
Accumulation of pH-Fatty Acids in S. cerevisiae

described above for the metabolic labeling experiments. E. coli lysates were prepared in the same way, except that the glass bead treatment was omitted.

Prepared in the same way, except that the glass bead treatment was omitted. Fluorography was performed by treating gels with ENHANCE (NEN Research Products, Dupont).

Western Blotting

Unlabeled whole cell lysates were prepared from S. cerevisiae as described above for the metabolic labeling experiments. E. coli lysates were prepared in the same way, except that the glass bead treatment was omitted. Lysates were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose. NMT was detected in yeast lysates with a rabbit antisemur designated 53N2, and in E. coli lysates with a rabbit anti-S. cerevisiae NMT antisemur that had been affinity purified over a column containing the purified 53-kD enzyme covalently bound to cyanogen bromide-activated Sepharose (Pharmacia-LKB, Piscataway, NJ) (Rudnick et al., 1990). Mouse Co was detected with rabbit antisemur 307-1 (Duronio et al., 1990). Antigen-antibody complexes were visualized with [125I]protein A (Burntette, 1981).

Accumulation of [H]Fatty Acids in S. cerevisiae

Incorporation of radiolabeled fatty acids into whole cells was quantitated using the method of Kamiyo et al. (1976) with minor modifications. Strain YM2061 was grown at 30°C in YPD to an A600 = 1.9. Duplicate 1-ml aliquots were then incubated with 50 µCi of either [9,10(n)3H]myristate, [9,10(n)3H]palmitate, or [9,10(n)3H]tetradecanoate acid or [9,10(n)3H]13-oxatetradecanoate acid (Research Products, Dupont), [9,10(n)3H]6-oxa-tetradecanoate acids, or [10,11(n)3H]13-oxatetradecanoate acid (Johnson et al., 1990) for 30 min at 30°C. The final concentrations and specific activity of each fatty acid are described in the legend to Fig. 5. Labeled cells were collected by centrifugation and washed three times with 1 ml PBS. 10-µl aliquots of each sample were added to 400 µl Protosol (NEN Research Products, Dupont) and the mixture was incubated for 1 h at room temperature to solubilize the cells. The resulting solution was subsequently added to 10 ml of ACS scintillation cocktail (Research Products, Inc., Mount Prospect, IL). Each assay was performed in duplicate.

Cloning of NMT Mutant Alleles and DNA Sequencing

A yeast centromere plasmid (pBB170) was constructed with a URA3 gene for prototrophic selection and a 6.36-kb segment of the NMT1 locus, the latter subeloned into the polylinker region of pRS316 (Sikorski and Hieter, 1988). The 6.36-kb fragment contains 2.5 kb of genomic DNA upstream and 2.5 kb downstream of the NMT1 coding sequences (demarcated by a 5'SpeI site and a 3'PstI site). Cleavage of pBB170 at unique HpaI and HindIII sites removed a 2.22-kb fragment encompassing the entire NMT1 gene. Then 2.3 kb of DNA 5' and 1.8 kb of DNA 3' of the NMT1 open reading frame. This DNA was used to clone genomic NMT1 alleles by transforming yeast strain dwr-1 (Ort-Weaver and Storzak, 1983). Recircularized plasmid DNA was recovered by transformation of E. coli with crude nucleic acid preparations of yeast (Hoffman and Winston, 1987). DNA sequencing (Sanger et al., 1977) of the NMT1 coding region was performed with seven equally spaced oligonucleotide primers representing nucleotides 500-516, 718-734, 913-929, 1077-1116, 1300-1316, 1503-1519, and 1703-1719 of the minus strand (see Duronio et al., 1989). Derivatives of pBB170 with nmtl mutations were designated as follows: pBB171 (nmtl-81, Asp451), pBB176 (nmtl-81, Asp451), pBB181 (nmtl-181,61, Lys453/Asp451), and pBB190 (nmtl-181,61, Lys453/Asp451).

Whole Cell Mutagenesis

Approximately 106 YB218 and 107 YB219 cells that had been grown in YPD at 24°C were washed with 200 mM sodium phosphate (pH 7.0), and then incubated in 200 mM sodium phosphate (pH 7.0) with or without 3% (vol/vol) ethylmethane sulfonate (EMS) for 1 h at room temperature. Surviving colonies were streaked onto YPD plates at roughly 1 × 107 cells/plate for YB218 and 2 × 106 cells/plate for YB219. The plates were incubated at 36°C for 3 d. The spontaneous reversion rate was <10-8. EMS-treated YB218 cells formed colonies at the restrictive temperature with a frequency of 5 × 10-5, while the frequency for EMS-treated YB219 was ∼5 × 10-4. Since two separate cultures were mutagenized and expanded, identical mutations within cells derived from each culture are presumed to be independent events (see Results).

Site-directed Mutagenesis

Placement of a 2.37-kb Bell-HindIII NMT1 fragment into BamHI-HindIII-digested pK36 (Sikorski and Hieter, 1988) created plasmid pBB163. A lysine codon at position 451 of NMT1 was introduced into this construct by the method of Kunkel (1985) using the mutagenic oligonucleotide 5'-GGGGATCCATAGAATGTCAGAAGAGGATAAAGCGAAA-3' and the desired GGT (Gly451) to AAG (Lys451) change was present. The resulting plasmid was designated pBB182.

A GALI-NMT fusion construct was made by a different method. A 779-bp region (representing nucleotides 214-993) of the 2.1-kb BamHI-HindIII genomic NMT fragment (Duronio et al., 1989) was amplified using the polymerase chain reaction (Saiki et al., 1988) and a mutagenic oligonucleotide (5'-GGGGATCCATAGAATGTCAGAAGAGGATAAAGCGAAA-AAATT-3'). This procedure introduced a BamHI restriction enzyme site just upstream of the initiator AUG codon of NMT1. The BamHI site allowed us to link the NMT gene to the GALI promoter in plasmid pBM272, a derivative of pBM150 (Johnston and Davis, 1984). DNA sequencing of this construct (pBB128) confirmed the desired promoter fusion. A G to A transition in the wobble position of Val26 codon (GTC) was also detected, presumably resulting from misincorporation by the Taq polymerase during amplification. A GALI-nmtl-181 fusion (pBB173) was constructed by subcloning a 730-bp Mul-HindIII fragment containing the Asp451 codon into a derivative of pBB128 lacking the BamHI site (pBB139).

Genomic Library Transformation of nmtl-181

Lithium acetate–competent YB218 was transformed with a Yep24-based genomic library (Carlson and Botstein, 1982) and plated onto synthetic media lacking uracil. As soon as colonies were visible, the plates were shifted to 36°C. Plasmid DNA was isolated and analyzed from colonies that continued to grow at the restrictive temperature.

Coexpression of NMT and Co in E. coli

NMT alleles containing mutations at codon 451 were subcloned into the E. coli NMT-expression vector pBB131 as 370-bp Mul-HindIII restriction fragments. The Asp451, Asp451, and Lys453 derivatives of pBB131 were designated pBB172, pBB185, and pBB186, respectively. These plasmids were each cotransformed into E. coli strain JM101 with pBB132, a mouse Co expression vector (Duronio et al., 1990). Colonies resistant to Luria broth plus 100 µg/ml ampicillin and 100 µg/ml kanamycin were isolated. Restriction analysis of plasmid DNA confirmed the presence of both constructs in each double transformant. NMT species and Co were co-induced and labeled with [9,10(n)3H]-myristic acid (39.3 Ci/mmol, NEN Research Products, Dupont, 50 µCi/ml of culture) at 36°C exactly as described in Duronio et al. (1990). For experiments conducted at 24°C, NMT synthesis was induced for 80 min before induction of Co. This NMT substrate was subsequently labeled for 60 min with C14:0. (Note that these induction periods were twice as long as those used for cultures grown at 36°C).

Purification of nmtl-181 from E. coli

Four one-liter cultures of E. coli strain JM101 containing the nmtl-181 expression vector pBB172 are grown in Luria broth plus 100 µg/ml kanamycin sulfate at 24°C to an A600 of 0.9. Expression of nmtl-181 was induced by the addition of isopropyl-1-1-thiogalactopyranoside (Sigma Chemical Co.) to a final concentration of 1 mM. After a 2-h incubation at 24°C, nmtl-181 was purified to apparent homogeneity from these cells exactly as described in Duronio et al. (1990). For experiments conducted at 24°C, NMT synthesis was induced for 80 min before induction of Co. This NMT substrate was subsequently labeled for 60 min with C14:0. (Note that these induction periods were twice as long as those used for cultures grown at 36°C).

Discontinuous Assay of NMT Enzymatic Activity

Details of this assay have been described elsewhere (Towler and Glaser, 1986). Briefly, labeled myristoyl-CoA is first generated using [3H]myristic acid, CoA, ATP, and the nonspecific Pseudomonas acyl-CoA synthetase (EC.6.2.1.3, Sigma Chemical Co.). Purified wild type or mutant NMT and the substrate peptide Gly-Asn-Ala-Ala-Ala-Arg-Arg-NH2 are then added. The resulting [3H]acyl-peptide is separated from the reaction mix-
ture by CI8 reverse phase high pressure liquid chromatography and quantified using an inline Radiomatic detector and Flo-Scint II as fluor. To determine the specific activities of wild type and mutant NMT as a function of temperature, reactions were performed with 0.23 \( \mu \)M myristoyl-CoA and 180 \( \mu \)M peptide at 24°C, 30°C, and 36°C. The data represent the average of two experiments. Myristoyl-CoA \( K_m \) and \( V_{max} \) values were determined at 24°C and 36°C as follows: reactions contained 0.23 \( \mu \)M \(^{3}H\)myristoyl-CoA (generated using the Pseudomonas acyl-CoA synthetase), varying amounts of cold myristoyl-CoA (Sigma Chemical Co.), [final myristoyl-CoA] = 0.25, 0.5, 1, or 2 \( \mu \)M, and 180 \( \mu \)M peptide. Peptide \( K_m \) and \( V_{max} \) values were determined at 24°C and 36°C using 0.23 \( \mu \)M \(^{3}H\)myristoyl-CoA, and varying amounts of peptide (from 20 to 200 \( \mu \)M). Kinetic data represent the results of 4-6 experiments. Note that the critical micelle concentration of myristoyl-CoA is 210 \( \mu \)M (Smith and Powell, 1986).

**Results**

**The Lesion of S. cerevisiae Strain LK181 Is Allelic with NMTI**

We previously probed DNA blots of intact S. cerevisiae chromosomes separated by pulse-field gel electrophoresis to determine that NMTI was located on chromosome XII (Duronio et al., 1989). The current meiotic map of chromosome XII (Mortimer et al., 1989 and Fig. 1) contains several loci in the region suspected to contain NMTI (Duronio et al., 1989). We mapped the NMTI locus by standard tetrad analysis (Mortimer and Hawthorne, 1975) to determine if any of these loci are allelic with NMTI. A single copy of the HIS3 gene was integrated at the NMTI locus in a nondisruptive manner (Orr-Weaver et al., 1981). The resulting NMTI::HIS3 allele was reliably scored as histidine prototrophy in a his3 background. A cross of strain YB137 to YB142 (Table II) revealed no linkage of NMTI::HIS3 to either the iv5 or the ural4 loci located on the distal right arm of chromosome XII (data not shown). Significant linkage was detected between NMTI::HIS3 and the cdc42 locus. No non-parental ditype ascus were observed in over 230 tetrads analyzed, indicating a map distance of 22 centimorgans (cM) from cdc42 (Table II, cross II). Two additional crosses were performed to assess the order of NMTI::HIS3 and cdc42 relative to the centromere. The cross of YB198 to YB201 revealed significant linkage between NMTI::HIS3 and the RDN1::LEU2 and CDC25::URA3 markers (Table II, cross III). NMTI::HIS3 also showed significant linkage to cdc3 in the cross of YB195 to YB137 (Table II, cross I). In all crosses, the NMTI::HIS3 allele was scored relative to auxotrophic markers on chromosomes other than XII. As expected, no linkage was detected between NMTI::HIS3 and any of these loci (Table II). NMTI mapped 48 cM from RDN1, 59 cM from cdc25, and 66 cM from cdc3 (Table II). Based on the previously determined arrangement of these loci on chromosome XII (Johnson et al., 1987), these estimated distances are consistent with the map order CEN2-RDN1-NMTI-CDC42-CDC25-CDC3. A cdc25 suppressor, tfs1, is the only other gene currently mapped to the region between RDN1 and CDC42. tfs1 is not allelic with NMTI since it does not display a similar recombination frequency with CDC42 and RDN1 (Mortimer et al., 1989). Together the data demonstrate that NMTI is not allelic with a previously mapped locus.

LK181 cannot grow on standard rich medium (YPD) at the restrictive temperature (36°C) unless 0.03 % (wt/vol) myristate (equal to 1.3 mM) is present. However, LK181 can grow at a rate comparable to wild type strains at 24°C on YPD lacking exogenous CI4-0. Since LK181 is prototrophic, we constructed a strain (YB191) that retained the temperature-sensitive (ts) phenotype in a genetic background similar to a strain containing NMTI::HIS3 (YB140). Analysis of the meiotic products of a diploid conjugate of strains YB191 and YB140 indicated that the genes responsible for the ts and His" phenotypes were tightly linked. Only parental ditype tetrads were detected of the 47 asci dissected (Table II, cross IV). Furthermore, a centromere plasmid containing only the NMTI coding sequences was able to fully complement the ts phenotype (data not shown). A plasmid that lacked NMTI sequences did not complement, indicating that LK181 contains a defective NMTI gene. The mutant allele was therefore designated nmtl-181.

**Metabolic Labeling of a nmtl-181 Strain with [H]myristate Reveals a Temperature-sensitive Defect in Protein N-myristoylation**

Metabolic labeling studies using \(^{14}H\)myristate have allowed us to identify 10-12 proteins after fractionation of total cell lysates through single dimension reducing and denaturing SDS-polyacrylamide gels (Heuckeroth and Gordon, 1989; Fig. 2 A, lane 3). We assessed whether incorporation of \(^{14}H\)myristate into proteins was affected at the restrictive temperature in cells containing the nmtl-181 allele. Strains YM2061 (NMTI) and YB218 (nmtl-181) were grown at 24°C in YPD until mid-log phase. Aliquots were then withdrawn and incubated for 30 min (at 24°C) with either \(^{35}S\)methionine or \(^{14}H\)myristic acid. Also at this time, the remaining cells were shifted to 36°C. One, two, and four hours after increasing the temperature, aliquots were removed and incubated at 36°C for 30 min with the radiolabeled fatty acid or amino acid. At the end of each labeling period, cellular lysates were prepared and equivalent masses of total cellular protein subjected to SDS-PAGE and fluorography (Fig. 2 A). Incorporation of \(^{14}H\)myristate into several proteins was reduced in strain YB218 compared to YM2061 after a 1-4-h incubation at the nonpermissive temperature. For example, three proteins of 21 kD known to contain myristoyl-glycine (Towler and Glaser, 1986) were labeled much less extensively with exogenous \(^{14}H\)myristate in YB218 (compare lanes 7, 11, and 15 with lanes 8, 12, and 16). By contrast, incorporation of \(^{35}S\)methionine into most cellular proteins increases in the mutant strain during the first hour after temperature elevation (compare lanes 2 and 6). One possible explanation of these results is that N-myristoylation of the three ~21-kD proteins was affected rather than their synthesis. This apparent change in N-myristoylation was not restricted to the group of ~21-kD polyopeptides. Several proteins...
Table I. Yeast Strains

| Strain       | Genotype                                                                 | Source                                      |
|--------------|---------------------------------------------------------------------------|---------------------------------------------|
| C82-1857     | MATα ilv5-1 ura4 asp5 met1 arg1 gal2                                      | Yeast Genetic Stock Center*                 |
| I04BD4-3D    | MATα cdc3-1                                                              | Johnson et al. (1987)                       |
| DJMD2-7C     | MATαcdc42-1 ura3 his4 leu2 gal2 RDN1::LEU2                               | Johnson et al. (1987)                       |
| DJMD12-41A   | MATα cdc3-1 ura3 leu2 CDC25::URA3 RDN1::LEU2                             | J. Pringle                                  |
| YB137        | MATα his3Δ200 lys2-801 ade2-101 NMT1::HIS3 LEU2::pRY181 (GAL1-lacZ)       | This work                                  |
| YB140        | MATα ura3-52 his3Δ200 lys2-801 NMT1::HIS3 LEU2::pRY181                   | This work                                  |
| YB142        | MATα his3Δ200 ilv5-1 ura4 asp5 met1                                      | This work                                  |
| YB195        | MATα cdc3-1 his3Δ200                                                     | This work                                  |
| YB197        | MATα cdc42-1 his3Δ200 lys2-801 ura3 leu2 RDN1::LEU2                        | This work                                  |
| YB198        | MATα cdc42-1 his3Δ200 ade2-101 ura3 leu2 RDN1::LEU2                        | This work                                  |
| YB201        | MATα cdc3-1 his3Δ200 ade2-101 lys2-801 ura3 leu2 RDN1::LEU2               | This work                                  |
| YM2061       | MATα ura3-52 his3Δ200 ade2-101 lys2-801 met· LEU2::pRY181 can1            | This work                                  |
| YB100        | MATα/MA Tα  ura3-52 his3Δ200 ade2-101/ade2-101 lys2-801 met·/+             | Flick and Johnston (1990)                   |
| YB102        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ2.5::HIS3/+             | Duronio et al. (1989)                       |
| YB152        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8::HIS3/+             | Duronio et al. (1989)                       |
| YB143        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8::HIS3/+             | This work                                  |
| LK181        | MATα  nmt1·181                                                           | Meyer and Schweizer (1974)                  |
| YB191        | MATα  nmt1·181 his3Δ200 ade2-101 lys2-801                                 | This work                                  |
| YB206        | MATα  nmt1·181 his3Δ200 ade2-101 lys2-801                                 | This work                                  |
| YB216        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ2.5::HIS3/+-             | This work                                  |
| YB218        | MATα  nmt1·181 ura3-52 his3Δ200 ade2-101 lys2-801 met· LEU2::pRY181         | This work                                  |
| YB219        | MATα  nmt1·181 ura3-52 his3Δ200 ade2-101 lys2-801 met· LEU2::pRY181         | This work                                  |
| YB220        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ2.5::HIS3/+-             | This work                                  |
| YB224        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ2.5::HIS3/+-             | This work                                  |
| A2017        | MATα faa1·1                                                             | T. Kamiryo†                                 |
| YB241        | MATα faa1  ura3-52 his3Δ200 ade2-101 lys2-801 met·                         | This work                                  |
| YB253        | MATα faa1 nmt1·181 ura3-52 his3Δ200 ade2-101 lys2-801 met·                 | This work                                  |
| YB256        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8·81 LEU2::pRY181     | This work                                  |
| YB258        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8·81 LEU2::pRY181     | This work                                  |
| YB268        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8·81 LEU2::pRY181     | This work                                  |
| YB260        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8·81 LEU2::pRY181     | This work                                  |
| YB261        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8·81 LEU2::pRY181     | This work                                  |
| YB276        | MATα  ura3-52 his3Δ200 ade2-101 lys2-801 met· nmt1Δ1.8·81 LEU2::pRY181     | This work                                  |

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‡ See Yocum et al. (1984) for pRY181 (GAL1-lacZ), an integrated plasmid used to measure the activity of the GAL1 promoter.
§ A segregant of strain A-207/B-201 containing either the faa1-201 or faa1-207 allele (Kamiryo et al., 1977). The faa1 allele of A2017 was used in all strains of this study.
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Table II. Meiotic Linkage Analysis of NMT1

| Cross          | Gene pair               | Tetrad type | PD  | NPD | TT  | Map Distance |
|---------------|-------------------------|-------------|-----|-----|-----|--------------|
|               |                         |             |     |     |     |              |
| I. YB195 × YB137 | cdc3-1 vs. NMT1::HIS3  | PD          | 50  | 14  | 118 | 66           |
|               | lys2-801 vs. NMT1::HIS3 | NPD         | 31  | 30  | 124 | unlinked*    |
|               | ade2-101 vs. NMT1::HIS3 | TT          | 40  | 27  | 114 | unlinked     |
| II. YB197 × YB137 | cdc42-1 vs. NMT1::HIS3 | PD          | 132 | 0   | 102 | 22           |
|               | ura3 vs. NMT1::HIS3     | NPD         | 29  | 38  | 173 | unlinked*    |
|               | ade2-101 vs. NMT1::HIS3 | TT          | 44  | 39  | 154 | unlinked     |
| III. YB198 × YB201 | RDN1::LEU2 vs. NMT1::HIS3 | PD          | 77  | 11  | 150 | 48           |
|               | CDC25::URA3 vs. NMT1::HIS3 | NPD       | 65  | 15  | 172 | 59           |
|               | RDN1::LEU2 vs. CDC25::URA3 | TT          | 44  | 29  | 133 | unlinked     |
|               | lys2-801 vs. NMT1::HIS3 | PD          | 44  | 51  | 154 | unlinked     |
|               | ade2-101 vs. NMT1::HIS3 | NPD         | 47  | 0   | 0   | 0            |
| IV. YB191 × YB140 | nmt1-181 vs. NMT1::HIS3 | TT          | 47  | 0   | 0   | 0            |

* PD, parental ditype; NPD, nonparental ditype; TT, tetratype.

† Chi-squared analysis indicated that the deviation of the PD/NPD ratio from 1:1 is not statistically significant for all gene pairs designated "unlinked."

whose masses range from 50–60 kD (see the asterisk in lane 3) also exhibit reduced labeling with [3H]myristate after a 1–2-h incubation of YB218 at 36°C. However, not all proteins exhibited reduced labeling; e.g., a ~116-kD protein was equally labeled in wild type and mutant cells even after a 4-h incubation at 36°C (lanes 15 and 16).

Western blot analysis of unlabeled cellular lysates indicated that the steady-state level of nmt-181 was similar to wild type NMT at all time points (Fig. 2 B). Therefore, the decrease in [3H]myristate labeling observed at 36°C cannot be simply ascribed to degradation of a thermolabile enzyme at the restrictive temperature. The decrease could, however, reflect a reduction in the specific activity of nmt-181 at the restrictive temperature. Since NMT is essential for cell growth, such a reduction in enzyme activity could also account for subsequent growth arrest and the decrease in

Figure 2. Metabolic labeling of nmt1-181 mutant cells. (A) Strains YM2061 (NMT1, odd numbered lanes) and YB218 (nmt1-181, even numbered lanes) were grown to an A_600 = 0.95 in YPD at 24°C. Aliquots were removed at the indicated times after shifting the cultures to 36°C and incubated for an additional 30 min with either 1.0 μCi/ml [35S]methionine (lanes 1, 2; 5, 6; 9, 10; and 13, 14) or 50 μCi/ml [3H]myristic acid (lanes 3, 4; 7, 8; 11, 12; and 15, 16). Cells at t = 0 were labeled at 24°C. Radiolabeled cellular proteins were analyzed by SDS-PAGE and fluorography. Gels were exposed for 7 wk. (B) Unlabeled lysates prepared as in A were transferred to nitrocellulose and probed with a polyclonal rabbit antiserum raised against purified S. cerevisiae NMT (53N2). (odd numbered lanes) YM2061; (even numbered lanes) YB218; (lanes 1 and 2) t = 0; (lanes 3 and 4) t = 60 min after shifting to 36°C; (lanes 5 and 6) t = 120 min; (lanes 7 and 8) t = 240 min.
Figure 3. Phenotype caused by \textit{nmt1-181}. Strains YB218 (\textit{FAA1 nmt1-181, Asp}^{451}), YM2061 (\textit{FAA1 NMT1, Gly}^{451}), YB241 (\textit{faa1 NMT1}), YB253 (\textit{faa1 nmt1-181}), YB258 (\textit{FAA1 nmt1-181,61, Lys293/Asp451}), and YB256 (\textit{FAA1 nmt1-81, Asn}^{451}) were incubated for 2 d at the indicated temperatures after replica plating onto YPD, YPD plus 0.03\% (wt/vol) myristic acid (YPD-MYR), YPD plus 0.03\% (wt/vol) 6-oxatetradecanoic acid (YPD-O6), YPD plus 25 \textmu{}M cerulenin (YPD-CERULENIN), YPD plus 25 \textmu{}M cerulenin and 0.03\% (wt/vol) myristic acid (YPD-MYR/CER), and YPD plus 25 \textmu{}M cerulenin and 0.03\% (wt/vol) palmitic acid (YPD-PAL/CER). All media (except YPD) also contained 1\% (wt/vol) Brij 58. The array of genotypes at the top of the figure corresponds to the patches on each plate.
[3S]methionine incorporation in strain YB218 compared to YM2061 4 h after raising the temperature from 24°C to 36°C (lanes 13 and 14).

**Rescue of nmtl-181 Cells by Exogenous Myristate Requires acyl-CoA Synthetase**

Fig. 3 A further illustrates the temperature sensitivity of nmtl-181 strains in the absence of exogenous fatty acid. Absolutely no growth was detected on YPD medium after a 2-d incubation of strain YB218 (nmtl-181) at 36°C. After a 1-wk incubation at 36°C, most cells could resume growth when shifted to 24°C, indicating that the elevated temperature produces growth arrest rather than absolute lethality. High concentrations of myristate (~1.3 mM) must be added to YPD to achieve a level of growth of the mutant strain at 36°C that is comparable to wild type. At 100 μM, myristate (MYR) only partially rescues growth at the nonpermissive temperature. Neither 10 μM or 1 μM C14:0, nor 0.03% (wt/vol) decanoate (C10:0), dodecanoate (C12:0), or palmitate (C16:0, PAL), support any growth of the mutant at 36°C. C13:0 and C15:0 (0.03 % wt/vol) allow growth at the restrictive temperature but at a level considerably less than that observed with either wild type strains at 36°C or mutant strains placed on YPD-MYR and incubated at 36°C (data not shown; Meyer and Schweizer, 1974). At 30°C, C13:0 supported growth of the mutant nearly as well as myristic acid.

A temperature-sensitive mutation of the *S. cerevisiae* acyl-CoA synthetase gene (faal, Kamiryo et al., 1976, 1977a) was used to demonstrate that rescue of growth at the restrictive temperature requires conversion of myristic acid to a CoA thioester. In vitro studies indicated that the mutant faal enzyme has reduced activity at 24°C compared to wild type, and that this difference becomes more marked at 36°C (Kamiryo et al., 1977a). Yeast strains carrying only the faal or nmtl-181 alleles grew on YPD-MYR at all temperatures surveyed. However, a strain (YB253) with both the faal and nmtl-181 mutations failed to grow on this medium at 36°C (Fig. 3 A).

**nmtl-181 Cells Require De Novo Fatty Acid Synthesis**

These observations suggested that nmt-181 is somehow unable to efficiently gain access to or utilize the pool(s) of myristoyl-CoA available at the nonpermissive temperature, and/or that these pools are not of sufficient size to allow a necessary level of protein N-myristoylation. The data also implied that strains with the nmtl-181 allele could be used to report changes in these pools and define the mechanisms that regulate myristic acid metabolism in this lower eukaryote. Cerculenin (CER) (2,3-epoxy-4-oxo-7,10-dodecadienoylamide) is an antifungal agent that specifically inhibits a component of the *S. cerevisiae* fatty acid synthetase complex (Vance et al., 1972), and consequently blocks de novo long chain fatty acid biosynthesis (Awaya et al., 1975). *S. cerevisiae* is unable to grow in the presence of cerculenin unless an exogenous fatty acid is supplied. Hence, strain YM2061 (NMT1, FAA1) is not able to grow at 36°C on YPD containing 25 μM CER, a concentration that essentially eliminates de novo fatty acid.

*Figure 4.* Dose-response of nmtl-181 growth inhibition by 6-oxatetradecanoate. Strains YM2061 (NMT1) and YB218 (nmtl-181) were streaked onto YPD (0) or YPD supplemented with the indicated amounts of 6-oxatetradecanoic acid and grown for 4 d at 24°C.
synthesis without inhibiting elongation or desaturation systems (Kamiyoi et al., 1976; Awaysa et al., 1975). Growth can be restored when the medium is supplemented with 0.03% (wt/vol) C16:0 (Fig. 3 B). Strain YB241 (nmt1, faa1) fails to grow on YPD-PAL/CER at 36°C, indicating that overcoming the effect of cerulenin requires a functional acyl:CoA synthetase to convert an exogenous fatty acid to its CoA thioester (Kamiyoi et al., 1976, 1977a). Myristate can also satisfy the fatty acid requirements of the wild type strain at 24, 30, and 36°C (Fig. 3 B). However, YB241 (nmt1, faa1) cannot grow at any of the three temperatures on YPD-MYR/CER. YB241 can utilize palmitate at 24°C and 30°C (but not at 36°C) to support growth (Fig. 3 B). This latter finding is consistent with C14:0 being a poorer substrate of the mutant acyl:CoA synthetase than C16:0 (Orme et al., 1972).

YB218 (nmt1-181, faa1) does not grow at 24, 30, or 36°C in the presence of CER with palmitate as the exogenous fatty acid. However, this strain is viable at all temperatures on YPD-MYR/CER. By contrast, strain YB253 (nmt1-181, faa1) fails to grow at any of the three temperatures on YPD-MYR/CER (Fig. 3 B). Together these results allow us to conclude that the nmt1-181 mutation causes a requirement for an active fatty acid synthetase even at the permissive temperature, and that exogenous palmitate (at concentrations up to ~1.3 mM) cannot sufficiently restore endogenous pools of myristoyl-CoA via metabolic interconversion to a level that satisfies the requirements of the mutant acyltransferase.

**Heteroaom-substituted Analogues of Myristate Elicit Different Phenotypes in S. cerevisiae Containing nmt1-181**

In vitro studies of myristic acid analogues with single sulfur or oxygen for methylene substitutions at positions 3 to 13 (the carbonyl carbon is designated Cl) have shown that such substitutions along the length of the hydrocarbon chain are well accommodated by purified S. cerevisiae NMT (Kishore et al., 1991). These compounds are selectively incorporated into subsets of cellular N-myristoylatedproteins, and once incorporated affect the biologic function of some, but not all, analogue-substituted polypeptides (Heuckeroth and Gordon, 1989; Mumby et al., 1990; Johnson et al., 1990). These changes in function (e.g., membrane association) appear to be specific for both the analogue and the protein. For example, a given N-myristoylatedprotein will undergo changes in subcellular distribution with one, but not another, oxatetradecanoic acid, depending upon the site of oxygen substitution (Johnson et al., 1990).

We evaluated the effects of such analogues on growth of the nmt1-181 mutant by replica platting strains YB218 (nmt1-181, faa1) and YM2061 (nmt1, faa1) onto YPD medium supplemented with 500 μM analogue. Tetradecanoic acids containing a single oxygen or sulfur for methylene substitu-

2. Substitution of an oxygen or sulfur atom for methylene in Cl4:0 yields molecules with modest differences in bond angles and bond distances compared to myristate but with significant alterations in polarity (Heuckeroth et al., 1988, 1990). Oxygen will be more strongly solvated in aqueous solution than sulfur (i.e. greater electronegativity leads to extensive hydrogen bonding). The reduction in hydrophobicity seen with oxatetradecanoic acids relative to Cl4:0 is therefore greater than that obtained with thia-tetradecanoic acids.

O6 but not S9 was available as a radiolabeled compound (Johnson et al., 1990). Therefore, we examined its effect on the mutant in greater detail. The ability of O6 to inhibit growth of YB218 (nmt1-181, faa1) at the permissive temperature was dose dependent (Fig. 4): 500 μM completely prevented growth, and growth rate increased with a progressive reduction in the concentration of analogue in YPD. Growth of strains containing nmt1-181 was only slightly inhibited relative to wild type at 36°C on YPD medium containing 0.03% (wt/vol) C14:0 and 0.03% (wt/vol) O6 (data not shown). Metabolic labeling studies using equal amounts of [3H]myristate or [3H]oxatetradecanoate at equal specific activities indicated that the analogue was not incorporated into any proteins produced by the mutant or wild type strains at 24°C (as judged by prolonged exposure of fluorographs, data not shown). This suggested that O6 was not exerting its effect by incorporation into N-myristoylatedproteins. We subsequently studied the accumulation of O6 in whole yeast cells relative to myristate. Strain YM2061 (nmt1, faa1) was grown to late log phase at 30°C and equal numbers of cells were labeled for 30 min with identical quantities of [3H]myristate, [3H]palmitate, [3H]O6, or [3H]O13, an analogue that partially myristoylates and FAA1, as an analogue that partially rescues growth of strain YB218 at 30°C in an FAA1-dependent fashion. The specific activities of all fatty acids were identical. The amount of tritium present within cells at the conclusion of labeling with C14:0 and C16:0 was similar (6% of the total label added to the medium), and 100-fold higher than O6 (Fig. 5 A). [3H]O13 produced a 10-fold higher level of incorporation of radioactivity than [3H]O6. Addition of 100 or 500 μM [3H]myristate to YM2061 resulted in a large increase in cellular radioactivity compared to levels achieved with 1.6 μM [3H]myristate alone (Fig. 5 B). 100 and 500 μM [3H]O6 produced a level of accumulation of label that was 26- and 28-fold less than the amount observed with 100 and 500 μM [3H]myristate. The respective values obtained with [3H]O13 were 8- and 13-fold less than [3H]-myristate. Control experiments performed under identical conditions revealed that the level of accumulation of [3H]O6 in strain YB218 (nmt1-181, faa1) was very similar to that in YM2061 (nmt1, faa1) (data not shown). Together, these findings indicate that O6 does not efficiently accumulate in
plasmid containing the genomic copy of the mutant allele can be recovered in *E. coli*. nmt1-181 was isolated in plasmid pBB171 from strain YB206 using this technique (see Materials and Methods). DNA sequence analysis of the entire coding region revealed a single G to A transition affecting codon 451 of the 455 residue protein. This changed a glycine codon (GGT) to one encoding aspartic acid (GAT).

The Gly<sup>451</sup>→Asp mutation was shown to be responsible for the nmt1-181 phenotype by engineering a haploid yeast strain (YB216) that contains a deletion of the NMTI genomic locus complemented with pBB171. YB216 exhibited temperature-sensitive myristic acid auxotrophy, although its phenotype was much leakier than strains with a single haploid copy of *nmt1-181*. This suggested that an increase in gene dosage supplied by the centromere plasmid could partially rescue the mutant phenotype. Therefore, we determined if overexpression of nmt1-181 could restore a wild type phenotype. NMT1 and nmt1-181 sequences were fused to the very strong, galactose-inducible GAL1 promoter (Johnston and Davis, 1984), and introduced into haploid strains carrying *nmt1* null alleles. Growth of strains YB143 (GAL1-NMT1) and YB220 (GAL1-nmt1-181) was comparable on YPGAL (Fig. 6 A) at both 24°C and 36°C. Western blot analysis showed that the (elevated) steady-state level of the mutant 53-kD enzyme in YB220 after growth at 36°C on YPGAL was equivalent to that achieved in YB143 (data not shown). These results indicate that overexpression of nmt1-181 is able to overcome the growth arrest observed at 36°C with a single copy nmt1-181 allele (Fig. 6 A). YB143 (GAL1-NMT1) was able to grow on YP-glucose at a rate comparable to YM2061, which contains a single copy of NMT1 (data not shown). Glucose repression of the GAL1-NMT1 fusion gene is therefore not sufficient to elicit the inviable nmt1 null phenotype. However, YB220 (GAL1-nmt1-181) was not viable on YP-glucose at any temperature (data not shown), indicating that expression of GAL1-nmt1-181 under glucose-repressing conditions is not sufficient to support growth, even at the permissive temperature (24°C).

Curiously, growth of YB143 (GAL1-NMT1) and YB220 (GAL1-nmt1-181) was similar at 24°C and 36°C on YP-galactose supplemented with 0.03% (wt/vol) O6 (Fig. 6 B). YB220 was not viable on YP-glucose-O6 at either temperature (data not shown). Thus, overexpression of nmt1-181 is able to overcome the inhibitory effects of O6 observed at permissive and nonpermissive temperatures. While the underlying mechanism of this phenomenon remains uncertain, some possible explanations are described in the Discussion.

### Analysis of Intragenic Suppressors of nmt1-181 Suggests that the Net Charge of NMT May Be Functionally Important In Vivo

A selection was employed to obtain suppressors of the nmt1-181 mutant phenotype. MATa and MATa strains carrying the nmt1-181 allele were treated with EMS, grown to stationary phase at 24°C, and incubated at 36°C for several days on YPD plates. 120 single colony isolates were backcrossed to the original nmt1-181 strain of the opposite mating type. All diploids (except one) were able to grow on YPD at 36°C, indicating that the suppressors were dominant. A single isolate appeared to be partially recessive to nmt1-181 (i.e., the nmt1-181 suppressor allele diploid grew more slowly at 36°C than

*S. cerevisiae* and suggest that its growth inhibition of nmt1-181 strains may reflect interaction with components of *S. cerevisiae* other than NMT.

**nmt1-181 Contains a Single Nucleotide Alteration Resulting in a Gly<sup>451</sup>→Asp Substitution**

Retrieval of a mutant allele from *S. cerevisiae* can be accomplished by transformation and prototrophic marker selection using a linear plasmid in which a double strand gap has been created in DNA sequences homologous to a specific genomic locus. Frequently, the gap is repaired in vivo using genomic sequences as template, with retention of the plasmid as an episome (Orr-Weaver and Szostak, 1983). The now circular...
Figure 6. Overexpression of nmtl-181 suppresses the mutant phenotype. (A) Strains YM2061 (NMT1), YB218 (nmtl-181), YB207 X YM2061 (nmtl-181/NMT1), YB220 (GALI-nmtl-181), YB224 (NMT1/GALI-nmtl-181), and YB143 (GALI-NMT1) were streaked on YPGAL and YPGAL plus 0.03% (wt/vol) myristate and 1% (wt/vol) Brij 58 (YPGAL-MYR). (B) Strains YM2061, YB218, YB143, and YB220 were streaked onto YPGAL plus 0.03% (wt/vol) 6-oxatetradecanoic acid and 1% (wt/vol) Brij 58 (YPGAL-O6). All plates were grown at 24°C or 36°C for 4 d.

13 isolates (including the recessive one) were crossed to an appropriate NMT1::HIS3 marker strain and the resulting diploids sporulated. Tetrad analysis indicated that the mutation responsible for suppression was tightly linked to the NMT1 locus, and therefore probably represented some type of mutation at the nmtl-181 locus. To test this hypothesis, the NMT1 locus of several suppressors was cloned by rescue of a gapped plasmid (as described above) and sequenced. Two classes of revertants resulting from G→A transitions were found: (a) two independent isolates with an Asp451 (GAT) to Asn451 (AAT) change (designated nmtl-81); and (b) isolates that retained Asp451 but also had a Glu to Lys substitution elsewhere in the molecule. Of this latter class, two independent isolates had a Glu167 (GAA) to Lys167 (AAA) change (nmtl-181,65). Also, the single nmtl-181-recessive isolate had a mutation of codon 293 (Glu, GAA) to Lys (AAA) (nmtl-181,61). All three types of pseudo-reversion alleles were shown to be responsible for suppression of the nmtl-181 growth defect by constructing haploid strains with an nmtl deletion and a centromere plasmid containing either nmtl-81, nmtl-181,65, or nmtl-181,61 (Table I). Strains YB260 (nmtl-81, Asn451), YB261 (nmtl-181,61, Lys293/Asp451), and YB276 (nmtl-181,65, Lys167/Asp451) were able to grow at 36°C, while YB216 (nmtl-181, Asp451) was not (data not shown).

Fig. 3 shows the phenotype of strains YB256 and YB258, which contain a single genomic copy of the nmtl-81 (Asn451) and nmtl-181,61 (Lys293/Asp451) alleles, respectively. Neither grows as competently as wild type at 36°C, and YB258 (nmtl-181,61, Lys293/Asp451) grows somewhat poorer than YB256.
transformants that were tested for growth at the restrictive

We recently described a coexpression system that allows

Residue 451 Is Critically Involved in NMT Catalysis

that has no endogenous NMT activity (Duronio et al., 1990). S. cerevisiae NMT and the catalytic subunit (Ca) of mouse cAMP–dependent protein kinase (PK-A), a known N-myristoylprotein, were simultaneously produced in E. coli using separate plasmids containing (a) individually inducible transcriptional promoters; (b) different, but compatible, origins of replication; and (c) different antibiotic resistance genes (ampicillin and kanamycin). N-myristoylation in E. coli is specific for C14:0, and absolutely requires the Gly2 of Ca. Since the system provides a rapid and sensitive means for monitoring NMT activity with a specific protein substrate in vivo, we examined the extent of Ca acylation using the Asp451, Asn451, and Lys451 mutants. A portion of the NMTI coding region containing each mutation was subcloned into pBB131, a P15A-KAN® plasmid (Chang and Cohen, 1978) that efficiently expresses the wild type acyltransferase after IPTG induction of its tac promoter (Amann et al., 1983; Duronio et al., 1990). These plasmids were cotransformed into E. coli strain JM101 with pBB132, a ColEl-AMP® plasmid that directs production of Ca after nalidixic acid induction of its recA promoter (Feinstein et al., 1983; Duronio et al., 1990). Ampicillin/kanamycin–resistant double transformants were induced and labeled at 24°C or 36°C with [3H]myristic acid as described in Materials and Methods. Cellular proteins were subjected to SDS-PAGE and fluorography. Co-expression of wild type NMT and Ca resulted in the labeling of a 40-kD band at each temperature (Fig. 7, lanes 2 and 8). The appearance of this band was dependent upon the presence of plasmids containing NMTI (lanes 6 and 12) and Ca cDNA (lanes 7 and 11). Immunological and chemical studies have established that labeling of this 40-kD band represents N-myristoylation of Ca (Duronio et al., 1990).

The Asp451 and Asn451 mutant NMTs were each capable of acylating Ca at 24°C (Fig. 7, lanes 3 and 4, respectively). At 36°C, the Asp451 mutant enzyme became much less active (lane 9) than both wild type NMT (lane 8) and the Asn451 mutant (lane 10). The Lys451 mutant was not able to N-myristoylate Ca at either temperature (lanes 5 and 11), consistent with its inability to rescue the nmtlΔ2.5::HIS3 allele in S. cerevisiae. The differences in labeling of Ca cannot simply be attributed to differences in the steady-state level of these mutant acyltransferases. Western blot analysis of unlabeled E. coli lysates revealed no significant differences in the accumulation of wild type and mutant NMT species at 36°C (Fig. 7 B) or at 24°C (data not shown). In addition, the steady-state level of Ca is essentially the same among all the 36°C lysates surveyed (Fig. 7 C).

Purified nmt-181 Has Altered Kinetic Properties

nmt-181 was purified from E. coli using P11 phosphocellulose and FPLC Mono S cation exchange chromatography (Rudnick et al., 1990). SDS-PAGE and silver staining of the purified protein preparation revealed a single band of ~53 kDa whose mobility was indistinguishable from that of purified, wild type E. coli–derived NMT (data not shown). The IEF point of apo-nmt-181 is 6.9, compared to a value of 8.15 for the wild type apoenzyme (Table III A). This finding is consistent with the Gly451→Asp substitution, which is predicted to result in an acidic shift in pI. Interestingly, while addition of myristoyl-CoA to the wild type apoenzyme results in a change in pI to 6.7, reflecting formation of a high affinity, possibly covalent acyl-enzyme intermediate (Rud-
Figure 7. Coexpression of wild type and mutant yeast NMTs with mouse Ca in E. coli. (A) E. coli strain JM101 containing NMTI alone (lanes 1 and 7), NMTI + Ca (lanes 2 and 8), nmtl-181 (Asp451) + Ca (lanes 3 and 9), nmtl-81 (Asp451) + Ca (lanes 4 and 10), nmtl-K451 + Ca (lanes 5 and 11), or Ca alone (lanes 6 and 12) were induced, incubated with [3H]myristate at the indicated temperatures, and whole cell lysates prepared as described in Materials and Methods. Labeled proteins were subsequently analyzed by SDS-PAGE and fluorography. The gel was exposed for 9 d. The arrow indicates the position of migration of myristoyl-Ca. Identical, unlabeled lysates were prepared after incubation at 36°C and analyzed by Western blotting using rabbit anti-NMT serum (B) or a rabbit anti-Ca serum (C). Antigen-antibody complexes were visualized with [125I]protein A. Results identical to those shown in B and C were obtained with lysates prepared from cells induced at 24°C (data not shown).
Table III. Physical and Kinetic Properties of nmt-181

| A. Physical characterization | nmt-181 | wild type |
|-----------------------------|---------|-----------|
| mass                        | 53 kD   | 53 kD     |
| pl (apo enzyme)             | 6.9     | 8.2       |
| apo-enzyme + myristoyl-CoA   | 6.9     | 6.7       |

| B. Specific activity*        | nmt-181 | wild type |
|-----------------------------|---------|-----------|
| 24°C                        | 5.4%    | 100%†     |
| 30°C                        | 4.8%    | 185%      |
| 36°C                        | 0.8%    | 458%      |

| C. Myristoyl-CoA Km#         | nmt-181 | wild type |
|-----------------------------|---------|-----------|
| Km(µM) 24°C                 | 1.1 ± 0.1 | 1.6 ± 0.3 |
| 36°C                        | 6.1 ± 1.6 | 0.6 ± 0.2 |

| D. Peptide Km (GNAAAARR-NH3)¶ | nmt-181 | wild type |
|-------------------------------|---------|-----------|
| Km(µM) 24°C                   | 484 ± 11 | 38 ± 17   |
| 36°C                         | 1550 ± 450 | 92 ± 37   |

* Assay conditions: [myristoyl-CoA] = 0.23 µM, [GNAAAARR-NH3] = 180 µM.
† 4.8 × 10⁵ pmol acylpeptide/minute/mg NMT.
‡ Assay conditions: [GNAAAARR-NH3] = 180 µM.
§ Assay conditions [myristoyl CoA] = 0.23 µM.
¶ Assay conditions [myristoyl CoA] = 0.23 µM.
** 3.5 × 10⁶ ± 9.4 × 10⁴ pmol acylpeptide/min/mg NMT.

Discussion

We have shown that a previously unidentified mutation of *S. cerevisiae* causing temperature-sensitive, myristic acid auxotrophy results from a Gly⁴⁵→Asp missense mutation of the NMTI gene. Our studies indicate that the mutant gene product is a sensitive reporter of myristoyl-CoA pools in this lower eukaryote, and can be used to examine metabolic regulation of acyl-CoA metabolism in vivo. Studies with heterotatom-containing analogues of myristate suggest that some can be utilized by cellular acyl:CoA synthetase and NMT to overcome the growth defect, while others block growth at the permissive temperature, possibly through disruption of critical functions prior to the interaction of acyl-CoAs with NMT. Finally, genetic and biochemical studies have confirmed that Gly⁴⁵ is critical for acyl-CoA acquisition and productive catalysis.

**nmt1-181 Is a Sensitive Indicator of Fatty Acyl-CoA Pools in *S. cerevisiae***

Examination of the phenotype of strain YB218 (nmt1-181, PAI) in the presence of the fatty acid synthetase inhibitor cerulenin produced a number of observations about regulation of fatty acid metabolism in *S. cerevisiae*. Because YB218 is sensitive to cerulenin, we could conclude that NMT can access myristoyl-CoA synthesized de novo by the FAS complex located in the cytosol of *S. cerevisiae* (Schweizer et al., 1978). Since exogenous myristate rescues the mutant phenotypes, NMT also appears to have access to C14:0 derived from outside of the cell. Whether these two sources of fatty acid comprise a single pool or distinct pools of myristoyl-CoA in *S. cerevisiae* cannot be discerned at present. By utilizing mutations of two genes encoding different acyl-CoA synthetases, Kamiryo et al. (1979) were able to demonstrate the existence of independent pools of acyl-CoA in *Candida lipolytica*. The acyl-CoA synthetase I of this yeast (Kamiryo et al., 1977b) supplies long chain acyl-CoAs for phospholipid biosynthesis, but not for β-oxidation, while acyl-CoA synthetase II provides fatty acyl-CoAs exclusively for catabolic use via β-oxidation. Given that exogenous C14:0 can complement the mutant phenotype, and that nmt1-181 has decreased affinity for myristoyl-CoA, our data indicate that the intracellular pool(s) of myristoyl-CoA accessi-
Alternative acyl-CoAs as Substrates for nmt-181

The phenotype of nmt1-181 strains suggests that odd chain length acyl-CoAs can be utilized by the mutant enzyme to overcome the growth defect at 36°C. We observed a slight but significant complementation of strain YB206 (nmt1-181, FAAI) at 36°C with tridecanoate (C13:0) and pentadecanoate (C15:0). Moreover, at 30°C C13:0 was able to sustain growth of YB218 (nmt1-181, FAAI) at nearly wild type levels. The rescue of growth by C13:0 and C15:0, as well as heteroatom-substituted analogues of myristate, required a wild type FAAI gene, and hence conversion to CoA thioesters in vivo. Failure of these fatty acids to completely rescue the mutant phenotype could be explained by a number of possible mechanisms: (a) certain fatty acids may not complement because they are selectively incorporated into only a subset of N-myristoylproteins; (b) incorporation is as efficient as C14:0 into all critical N-myristoyl proteins but causes slow growth due to decreased biological activity; and/or (c) incorporation has a minimal effect on protein function but retarded growth results from inefficient utilization of the alternate fatty acids by nmt1-181 or inefficient uptake from the medium. In case (c), a sub-optimal fraction of a critical N-myristoyl protein(s) would be acylated, causing the mutant phenotype. The ability of C13:0 to rescue the ts phenotype is unlikely due to any conversion to C14:0 since odd chain length fatty acids are elongated by two carbon units in S. cerevisiae (Orme et al., 1972). The efficiency of utilization of an alternative acyl-CoA by NMT depends on the sequence of the peptide substrate (Heuckeroth et al., 1988). Therefore, in vitro studies of the transfer of odd chain length and heteroatom-substituted fatty acids to peptides derived from known yeast N-myristoyl proteins, plus in vivo metabolic labeling studies employing radiolabeled fatty acids, should help to distinguish among these proposed mechanisms. Also, the use of cerulenin to deplete endogenous pools of myristoyl-CoA may provide a way to increase the proportion of analogue-substituted proteins in strains containing the nmt1-181 allele, and in this way to probe structure/function relationships in the acyl chain of specific N-myristoylproteins.

6-oxatetradecanoic Acid Affects the Growth of nmt1-181 Strains at 24°C

Several analogues prevented growth of nmt1-181 strains at the permissive temperature. This effect was most pronounced, and dose-dependent, with 6-oxatetradecanoic (O6). [3H]O6 does not accumulate to appreciable levels within S. cerevisiae (compared to C14:0), nor does it produce detectable labeling of cellular N-myristoyl proteins in this yeast. In vitro studies using purified S. cerevisiae NMT and a variety of octapeptide substrates indicate that O6-CoA and myristoyl-CoA are comparable substrates (Heuckeroth et al., 1988; Bryant et al., 1991). These observations suggest that O6 may affect a cellular function prior to any interaction with NMT. For example, O6 could inhibit S. cerevisiae acyl-CoA synthetase or fatty acid synthetase, thereby reducing intracellular levels of myristoyl-CoA needed for growth of nmt1-181 cells. S. cerevisiae may possess a cell surface fatty acid receptor/translocator involved in the uptake of exogenous fatty acids that is specifically inhibited by analogues such as O6. In E. coli, cellular uptake of long chain fatty acids requires both an outer membrane translocator protein (fadL) and the acyl-CoA synthetase (fadD) associated with the cytoplasmic side of the inner membrane (Kameda and Nunn, 1981; Black et al., 1987). Accumulation of [3H]O6 in this prokaryote is ~100-fold less efficient than C14:0 (Duronio et al., 1991; Bryant et al., 1991). Single cell microorganisms having outer surfaces that are impermeable to hydrophobic molecules may need specific transporters to efficiently retrieve long chain fatty acids from the environment (Black et al., 1987). Disruption of such a proposed import apparatus may explain why overexpression of nmt1-181 is sufficient to overcome the growth inhibition of O6 at 24°C: reductions in the import of exogenous myristate and/or lowering available intracellular pools of myristoyl-CoA may require a compensatory increase in mutant enzyme to achieve a necessary level of acylation of critical N-myristoylproteins. Suppressors of the phenotypes elicited by analogues such as O6 in strains containing nmt1-181 may encode components involved in the import and metabolic processing of C14 fatty acids prior to their interaction with NMT.

Gly451 Critically Affects NMT Catalysis

The sequence of the cloned nmt1-181 allele revealed a missense mutation encoding an Asp for Gly substitution at position 451 of the 455 residue protein. EMS-induced intragenic suppressors indicated that changes in the physicochemical properties of several residues could overcome the effects of the Gly451→Asp substitution. Strains with an Asp451-substituted NMT, or a Glu→Lys substitution at residue 167 or 293 that retains Asp451, could each grow at 36°C, albeit not quite as well as a wild type strain. Cells containing a Lys451 substitution of NMT were not viable. These data suggest that the overall charge of NMT, or at least the charge of residue 451, is important for catalysis (and possibly access to myristoyl-CoA pools, see Rudnick et al., 1990). The pl of the purified Asp451 protein was acid shifted relative to wild type, consistent with this hypothesis. Co-expression of the position 451 mutants with Caβ in E. coli allowed us to
assess the ability of each NMT species to incorporate exogenous \([\text{H}]\)myristate into the protein substrate. The NMT activity of the Asp\(^{451}\) mutant was temperature sensitive, the Lys\(^{451}\) inactive, while the Asn\(^{451}\) form had an activity similar to wild type in this coexpression system.

The coexpression assay not only provided a way to survey the activities of mutant NMTs against a given protein substrate, but also provided a way of obtaining large quantities of the Asp\(^{451}\) species for further analysis. Using a discontinuous assay of NMT activity, we demonstrated that the specific activity and the myristoyl-CoA and peptide kinetic parameters of the mutant enzyme were altered relative to wild type at 24°C. This is consistent with the various phenotypic abnormalities observed even at the permissive temperature (slower growth, decreased \([\text{H}]\)myristate incorporation into proteins, and inhibition of growth by glucose repression of \(\text{GALI-nmtl-181}\)). Raising the reaction temperature to 36°C revealed a 10-fold increase in the myristoyl-CoA \(K_m\) of nmt-181, such that the catalytic efficiency \((V_{\text{max}}/K_m)\) of the mutant enzyme was over 200-fold lower than wild type.

We conclude from these observations that at the restrictive temperature \(nmt1-181\) cells are deficient in the acylation of some or all essential N-myristoylated proteins. The increase in the proportion of nonmyristoylated proteins becomes rate limiting for growth in the mutant. The critical NMT substrates may include the GTP-binding GAP1 and ARFI proteins (Miyajima et al., 1987; Stearns et al., 1990; Duronio et al., 1991). Overexpression of the mutant gene product overcome the \(K_m\) defect, as does increasing the intracellular concentration of acyl-CoA substrate with exogenous myristic acid. Underexpression of \(nmt1-181\) or depletion of myristoyl-CoA pools below a critical level also results in growth inhibition.

Conditional lethality in the absence of exogenous myristate may be a feature of many NMT mutants. We have preliminary evidence that other independently isolated alleles of \(NMT1\) also demonstrate growth complementation with exogenous myristate (Duronio, R. J., and J. I. Gordon, unpublished observations; Reed et al., 1988). Elevated intracellular concentrations of myristoyl-CoA may be required to stabilize domain(s) of NMT critical for catalysis. Such structural features could be compromised in temperature-sensitive mutants under standard growth conditions. In the case of nmt-181, this hypothesis suggests that Gly\(^{451}\) is normally positioned so as to influence the acyl-CoA binding properties of NMT. Alternatively, increases in intracellular levels of myristoyl-CoA may not affect the conformation of the mutant enzyme, but rather drive the reaction to acylation of critical substrates to a degree that is sufficient for vegetative growth. NMT may normally exist in vivo complexed with myristoyl-CoA: the steady-state levels of holoprotein and apoenzyme may be altered in the mutant reflecting the decreased affinity of nmt-181 for this ligand and/or an inability to interact with intracellular compartments that generate and store myristoyl-CoA. In this sense, mutations like \(nmt1-181\) highlight the need to understand how and where this acyltransferase acquires its acyl-CoA substrates in vivo. Such acquisition may represent a critical step in the regulation of cell growth and division.

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