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 with single oxygen or sulfur for methylene group substitutions partially complement the phenotype, while others inhibit growth even at the permissive temperature (24°C). Cerulenin, a fatty acid synthetase inhibitor, also prevents growth of the mutant at 24°C. Complementation 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 Gly^{451}→Asp substitution. Analyses of several intragenic suppressors suggest that Gly^{451} is critically involved in NMT catalysis. In vitro kinetic studies with purified mutant enzyme revealed a 10-fold increase in the apparent K_m 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 (NMT;[1] EC 2.3.1.97) catalyzes the cotranslational (Wilcox et al., 1987; Deichaite et al., 1988) covalent linkage of myristate, a rare (Orme et al., 1972; Awaya et al., 1975) 14-carbon saturated fatty acid, to the NH_2-terminal glycine residue of a variety of proteins having diverse functions (reviewed in Towler et al., 1988; James and Olson, 1990). These include the catalytic subunit of mammalian cAMP-dependent serine/threonine kinase, members of the $p60^{src}$ family of tyrosine kinases, several retroviral gag polyprotein precursors such as the $Pr55^{gag}$ of HIV-1, capsid components of some picorna- and papovaviruses, and the α subunit of many signal transducing, heterotrimeric G proteins. Changing or deleting the Gly^2 residue of N-myristoyl proteins completely blocks acylation in vivo, providing a useful strategy for defining the functional role of this C14:0 fatty acid. Such studies have established that myristate is required for the plasma membrane association and oncogeneity of $p60^{v\text{-}src}$ (Cross et al., 1984; Kamps et al., 1985; Buss et al., 1986; Heuckeroth and Gordon, 1989), and the assembly and budding of HIV-1 and other retroviruses (Gottlinger et al., 1989; Bryant and Ratner, 1990; Rein et al., 1986; Rhee and Hunter, 1987).

Recent evidence suggests that in some cases the myristoyl moiety may not simply serve as a hydrophobic anchor in lipid bilayers, but rather provides a necessary determinant for specific protein–protein interactions. Binding of $p60^{v\text{-}src}$ to plasma membrane preparations is mediated through an interaction with a 32-kD membrane protein that requires the myristoyl moiety (Resh, 1989; Goddard et al., 1989; Resh and Ling, 1990). Myristate is also required for stable association of G_α proteins with the plasma membrane (Mumby et al., 1990; Jones et al., 1990). Using a coexpression system (Duronio et al., 1990; 1991) that permits production of non-myristoylated and N-myristoylated G_α proteins in *E. coli*, Linder et al. (1991) have shown that N-myristoylation increases the affinity of $G_{o\alpha}$ (an abundant G protein in brain) for β_γ subunits. The β_γ subunit complex can associate tightly with phospholipid vesicles, and therefore may represent a membrane anchor for G_α (Sternweiss, 1986). Refinement of the structure of the poliovirus virion to 2.9 Å resolution provided direct evidence of an interaction between the myristoyl moiety of the VP4 capsid protein and amino acid side chains of VP3 and VP4 (Chow et al., 1987).

NMT has been purified to homogeneity from *S. cerevisiae* (Towler et al., 1987*a*, *b*), and its gene isolated and sequenced (Duronio et al., 1989). Insertional mutagenesis of the single copy *NMT1* gene causes recessive lethality (Duronio et al., 1989), indicating that one or more essential cellular proteins of this yeast requires N-myristoylation for proper biological function. In vitro kinetic studies have revealed that this 455 residue protein contains two functionally distinguishable binding sites: one for its acyl-CoA ligand and one for its nascent protein substrate (Towler et al., 1987*b*; Heuckeroth et

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 (Heuckeroth 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., 1987*a*; 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 residue, 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 $\alpha_6\beta_6$ fatty acid synthetase complex, are encoded by *ACC1* (Mishina et al., 1980) and the *FAS1* (β) and *FAS2* (α) loci (Schweizer 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, C16:0, or C18:0 (Schweizer and Bolling, 1970). The majority of isolates contained *fas1* and *fas2* mutations. In these cases, exogenous lipids replete pools that are lost due to blockade of de novo fatty acid synthesis. Meyer and Schweizer (1974) also characterized a single mutation (designated LK181) that affected a locus other than *FAS1* or *FAS2*. LK181 cells are specifically auxotrophic for myristate at 36°C: other chain length fatty acids such as C16:0 or C12:0 cannot replace C14:0. Moreover, the fatty acid synthetase activity of LK181 is normal. At partially restrictive temperatures (e.g., 30°C), the relative rate of LK181 growth on media supplemented with different fatty acids parallels the acyl-CoA chain length specificity of NMT in vitro. We now show that the molecular lesion of LK181 arises from a single base substitution in codon 451 of *NMT1*, resulting in a Gly→Asp substitution. Genetic and biochemical analyses of this mutation have provided unexpected insights about the regulation of protein N-myristoylation in *S. cerevisiae*.

Materials and Methods

Yeast Strains and Media

The genotypes of all *Saccharomyces cerevisiae* strains used in this study are listed in Table I. YPD (1% yeast extract, 2% peptone, 2% dextrose) was used as the standard growth medium with 2% galactose replacing dextrose in YPGAL medium. Fatty acid concentration in YPD medium was 0.03% (wt/vol) except where indicated. Brij 58 (1% wt/vol) (Sigma Chemical Co., St. Louis, MO) was added to all media containing fatty acid and/or cerulenin (Sigma Chemical Co.) to help solubilize lipids. All saturated fatty acids (C10:0 through C16:0) were purchased from NuChek Prep, Inc. (Elysian, MN).

Methods used for the syntheses of single oxygen-containing and single sulfur-containing analogues of myristate, and their subsequent purification and characterization, are described in Heuckeroth et al. (1988, 1990) and Kishore et al. (1991).

NMT1 Genomic Alterations

Construction of the *nmt1::HIS3* allele, which disrupts the *NMT1* gene at codon 279, was described previously (Duronio et al., 1989). The genomic *nmt1Δ2.5::HIS3* deletion allele was made by transforming lithium acetate competent (Ito et al., 1983) YB100 with a linear restriction fragment of DNA containing the desired mutation flanked by sequences homologous to the *NMT1* locus, and selecting for histidine prototrophy. Individual His^+ transformants were screened for the intended alteration by Southern blot analysis of genomic DNA. To generate the *nmt1Δ2.5::HIS3* mutation, a 2.5-kb BamHI restriction fragment containing *NMT1* (Duronio et al., 1989) was replaced with a 1.7-kb BamHI *HIS3* fragment (Struhl, 1985). This construct (pBB141) contains 3.25 kb of DNA 5′ and 1.6 kb of DNA 3′ of the deletion end points that are homologous to the *NMT1* locus.

Complementation tests using the *nmt1Δ2.5::HIS3* allele were performed by transforming diploid strain YB152 with episomal plasmids, followed by sporulation and tetrad dissection. Complementation was scored by rescue of the 2:2 (wild type/lethal mutant) tetrads to 3:1 or 4:0, and by the presence of His^+, Ura^+ segregants (the *URA3* gene was present in all of the complementing plasmids).

Meiotic Mapping of the NMT1 Locus

An *NMT1::HIS3* allele was constructed by the method of Orr-Weaver et al. (1981) in order to score the *NMT1* locus during tetrad analysis. YB131 is a His^+ transformant that was identified by DNA blot hybridization as having a single copy of plasmid pBB118 integrated between two tandem copies of *NMT1*. pBB118 is a pUC13 derivative containing a 1.7-kb BamHI *HIS3* restriction fragment placed next to a 2.1-kb BamHI-HindIII *NMT1* fragment. The meiotic products of *NMT1/NMT1::HIS3* heterozygotes segregated in the predicted 2 His^+:2 His^- manner in 94–99% of the tetrads examined, depending on the particular strains used to generate the diploid. Linkage of markers to the *NMT1*, *CDC25*, and *RDN1* loci was evaluated by scoring the prototrophic segregation of (*a*) *NMT1::HIS3* in a *his3/his3* background; (*b*) *CDC25::URA3* (Robinson et al., 1987; Johnson et al., 1987) in a *ura3/ura3* background; and (*c*) *RDN1::LEU2* (Petes, 1980) in a *leu2/leu2* background. Strains with the temperature-sensitive *cdc3-1* or *cdc42-1* alleles (Johnson et al., 1987; Johnson and Pringle, 1990) were scored by replica plating to YPD at 36°C. Tetrads with three and four viable spores were used to determine the numbers of parental ditype (PD), nonparental ditype (NPD), and tetratype (TT) asci, assuming 2:2 segregation of heterozygous loci in three spore tetrads. (This assumption should not affect the estimation of map distances since the average non-Mendelian segregation for all markers examined was 2–3% of total meioses.) Map distances were determined using Perkins' (1949) equation ($Xp = 50(TT + 6NPD)/(PD + NPD + TT)$) and the normogram presented in Fig. 2 of Mortimer and Schild (1980).

Metabolic Labeling Studies

Strains YM2061 and YB218 were grown to an A_{600} of 0.95 in YPD broth at 24°C. A 2-ml aliquot of each culture was metabolically labeled with either [9,10(n)^{3}H]myristic acid (39.3 Ci/mmol; NEN Research Products, Dupont, Boston, MA; 50 μCi/ml of culture) or [^{35}S]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

of lysate protein from each sample (100 μg) was subjected to SDS-PAGE (Laemmli, 1970). Fluorography was performed by treating gels with EN^{3}HANCE (NEN Research Products, Dupont).

Western Blotting

Unlabeled whole cell lysates were prepared from *S. cerevisiae* exactly 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 antiserum designated 53N2, and in *E. coli* lysates with a rabbit anti-*S. cerevisiae* NMT antiserum 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 Cα was detected with rabbit antiserum 307-1 (Duronio et al., 1990). Antigen-antibody complexes were visualized with [^{125}I]protein A (Burnette, 1981).

Accumulation of [^{3}H]Fatty Acids in S. cerevisiae

Incorporation of radiolabeled fatty acids into whole yeast cells was quantitated using the method of Kamiryo et al. (1976) with minor modifications. Strain YM2061 was grown at 30°C in YPD to an A_{600} = 1.9. Duplicate 1-ml aliquots were then incubated with 50 μCi of either [9,10(n)^{3}H]myristate, [9,10(n)^{3}H]palmitate (NEN Research Products, Dupont), [9,10(n)^{3}H]6-oxatetradecanoate acid, or [10,11(n)^{3}H]13-oxatetradecanoate (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 at 4°C. After the final wash, the cell pellet was resuspended in 100 μl of 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 3A70 scintillation cocktail (Research Products, Inc., Mount Prospect, IL). Each assay was performed in duplicate.

Cloning of NMT1 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 subcloned 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 leaving 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 lithium acetate–treated cells to Ura$^+$ (Orr-Weaver and Szostak, 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, 1,077–1,116, 1,300–1,316, 1,503–1,519, and 1,703–1,719 of the minus strand (see Duronio et al., 1989). Derivatives of pBB170 with *nmt1* mutations were designated as follows: pBB171 (*nmt1-181*, Asp^{451}), pBB176 (*nmt1-81*, Asn^{451}), pBB181 (*nmt1-181,61*, Lys^{293}/Asp^{451}), and pBB190 (*nmt1-181,65*, Lys^{167}/Asp^{451}).

Whole Cell Mutagenesis

Approximately 10^8 YB218 and 10^7 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 24°C. Cells were then diluted twenty-fold in 5% sodium thiosulfate to inactivate the EMS, and washed once with an equal volume of water. EMS treatment killed ~50% of the cells. Viable cells were grown to stationary phase in YPD at 24°C, and then spread onto YPD plates at roughly 1×10^7 cells/plate for YB218 and 2×10^6 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 $\sim 5 \times 10^{-5}$, while the frequency for EMS-treated YB219 was $\sim 5 \times 10^{-6}$. 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 BclI-HindIII *NMT1* fragment into BamHI-HindIII–digested pRS316 (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′-GGCGTAGCAATGTC*AAG*GTTGTTATGTTGTAG-3′ and *E. coli* strain BW313 (*dut*$^-$ung$^-$). DNA sequencing of the entire *NMT1* open reading frame confirmed that only the desired GGT (Gly^{451}) to AAG (Lys^{451}) change was present. The resulting plasmid was designated pBB182.

A *GAL1-NMT1* fusion construct was made by a different method. A 779-bp region (representing nucleotides 214–993) of the 2.1-kb BamHI-HindIII genomic *NMT1* fragment (Duronio et al., 1989) was amplified using the polymerase chain reaction (Saiki et al., 1988) and a mutagenic oligonucleotide (5′-*GGGGATCC*ATAGAATGTCAGAAGAGGATAAAGCGAAA-AAATT-3′). This procedure introduced a BamHI restriction enzyme site just upstream of the initiator ATG codon of *NMT1*. The BamHI site allowed us to link the *NMT1* gene to the *GAL1* 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 Val^{54} codon (GTC) was also detected, presumably resulting from misincorporation by the Taq polymerase during amplification. A *GAL1-nmt1-181* fusion (pBB173) was constructed by subcloning a 730-bp MluI-HindIII fragment containing the Asp^{451} codon into a derivative of pBB128 lacking the BamHI site (pBB139).

Genomic Library Transformation of nmt1-181

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 Cα in E. coli

NMT1 alleles containing mutations at codon 451 were subcloned into the *E. coli* NMT-expression vector pBB131 as 730-bp MluI-HindIII restriction fragments. The Asp^{451}, Asn^{451}, and Lys^{451} derivatives of pBB131 were designated pBB172, pBB185, and pBB186, respectively. These plasmids were each cotransformed into *E. coli* strain JM101 with pBB132, a mouse Cα 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 Cα were co-induced and labeled with [9,10(n)^{3}H]-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 Cα. 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 nmt-181 from E. coli

Four one-liter cultures of *E. coli* strain JM101 containing the *nmt1-181* expression vector pBB172 were grown in Luria broth plus 100 μg/ml kanamycin sulfate at 24°C to an A_{600} of 0.9. Expression of nmt-181 was induced by the addition of isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co.) to a final concentration of 1 mM. After a 2-h incubation at 24°C, nmt-181 was purified to apparent homogeneity from these cells exactly as described for wild type *S. cerevisiae* NMT (Rudnick et al., 1990), except that the second P11 phosphocellulose chromatographic step was omitted. Purified enzyme was stored at 1 mg/ml in 50 mM MES, pH 6.5, 200 mM NaCl at 4°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 [^{3}H]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-Ala-Arg-Arg-NH_2 are then added. The resulting [^{3}H]acyl-peptide is separated from the reaction mix-

ture by C18 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 μM myristoyl-CoA and 180 μM 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 μM [^{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 μM), and 180 μM peptide. Peptide K_m and V_{max} values were determined at 24°C and 36°C using 0.23 μM [^{3}H]myristoyl-CoA, and varying amounts of peptide (from 20 to 200 μM). Kinetic data represent the results of 4–6 experiments. Note that the critical micelle concentration of myristoyl-CoA is 210 μM (Smith and Powell, 1986).

Results

The Lesion of S. cerevisiae Strain LK181 Is Allelic with NMT1

We previously probed DNA blots of intact *S. cerevisiae* chromosomes separated by pulse-field gel electrophoresis to determine that *NMT1* 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 *NMT1* (Duronio et al., 1989). We mapped the *NMT1* locus by standard tetrad analysis (Mortimer and Hawthorne, 1975) to determine if any of these loci are allelic with *NMT1*. A single copy of the *HIS3* gene was integrated at the *NMT1* locus in a nondisruptive manner (Orr-Weaver et al., 1981). The resulting *NMT1::HIS3* allele was reliably scored as histidine prototrophy in a *his3* background. A cross of strain YB137 to YB142 (Table I) revealed no linkage of *NMT1::HIS3* to either the *ilv5* or the *ura4* loci located on the distal right arm of chromosome XII (data not shown). Significant linkage was detected between *NMT1::HIS3* and the *cdc42* locus. No nonparental ditype asci 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 *NMT1::HIS3* and *cdc42* relative to the centromere. The cross of YB198 to YB201 revealed significant linkage between *NMT1::HIS3* and the *RDN1::LEU2* and *CDC25::URA3* markers (Table II, cross III). *NMT1::HIS3* also showed significant linkage to *cdc3* in the cross of YB195 to YB137 (Table II, cross I). In all crosses, the *NMT1::HIS3* allele was scored relative to auxotrophic markers on chromosomes other than XII. As expected, no linkage was detected between *NMT1::HIS3* and any of these loci (Table II). *NMT1* 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 *CEN12-RDN1-NMT1-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 *NMT1* since it does not display a similar recombination frequency with *CDC42* and *RDN1* (Mortimer et al., 1989). Together the data demonstrate that *NMT1* is not allelic with a previously mapped locus.

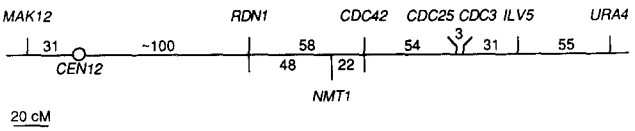

Figure 1. Genetic map of the region of chromosome XII containing *NMT1*. Loci and map distances (in centimorgans) appearing above the line were taken from Johnson et al. (1987). The location of the *NMT1* locus and corresponding map distances shown below the line were determined by standard tetrad analysis as described in the text.

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 C14:0. 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 *NMT1::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 *NMT1* coding sequences was able to fully complement the ts phenotype (data not shown). A plasmid that lacked *NMT1* sequences did not complement, indicating that LK181 contains a defective *NMT1* gene. The mutant allele was therefore designated *nmt1-181*.

Metabolic Labeling of a nmt1-181 Strain with [^{3}H]myristate Reveals a Temperature-sensitive Defect in Protein N-myristoylation

Metabolic labeling studies using [^{3}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 [^{3}H]myristate into proteins was affected at the restrictive temperature in cells containing the *nmt1-181* allele. Strains YM2061 (*NMT1*) and YB218 (*nmt1-181*) 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 [^{3}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 [^{3}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 [^{3}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 polypeptides. Several proteins

Table I. Yeast Strains

Strain	Genotype	Source
C82-1857	*MATa ilv5-1 ura4 asp5 met1 arg1 gal2*	Yeast Genetic Stock Center*
104BD4-3D	*MATα cdc3-1*	Johnson et al. (1987)
DJMD2-7C	*MATα cdc42-1 ura3 his4 leu2 gal2 RDN1::LEU2*	Johnson et al. (1987)
DJMD12-41A	*MATa cdc3-1 ura3 leu2 CDC25::URA3 RDN1::LEU2*	J. Pringle‡
YB137	*MATa 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	*MATa cdc42-1 his3Δ200 ade2-101 ura3 leu2 RDN1::LEU2*	This work
YB201	*MATα cdc3-1 his3Δ200 ade2-101 lys2-801 ura3 leu2 NMT1::HIS3 CDC25::URA3*	This work
YM2061	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ LEU2::pRY181 can1*	Flick and Johnston (1990)
YB100	*MATa/MATα ura3-52/ura3-52 his3Δ200/his3Δ200 ade2-101/ade2-101 lys2-801/lys2-801 met⁻/+ +/tyr1-501 LEU2::pRY181/LEU2::pRY181 can1/can1*	Duronio et al. (1989)
YB102	Isogenic with YB100 except *nmt1::HIS3/+*	Duronio et al. (1989)
YB152	Isogenic with YB100 except *nmt1Δ2.5::HIS3/+*	This work
YB143	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1::HIS3 LEU2::pRY181 can1* pBB128 (*GAL1-NMT1*)	This work
LK181	*MATa nmt1-181*	Meyer and Schweizer (1974)
YB191	*MATa nmt1-181 his3Δ200 ade2-101 lys2-801 LEU2::pRY181*	This work
YB206	*MATa nmt1-181 ura3-52 his3Δ200 ade2-101 lys2-801 LEU2::pRY181*	This work
YB216	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1Δ2.5::HIS3 LEU2::pRY181 can1* pBB171 (*nmt1-181*)	This work
YB218	*MATa 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 tyr1-501 LEU2::pRY181*	This work
YB220	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1Δ2.5::HIS3 LEU2::pRY181* pBB173 (*GAL1-nmt1-181*)	This work
YB224	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ LEU2::pRY181 can1* pBB173	This work
A2017	*MATa faa1*‖	T. Kamiryo¶
YB241	*MATa faa1 ura3-52 his3Δ200 ade2-101 lys2-801 met⁻*	This work
YB253	*MATa faa1 nmt1-181 ura3-52 his3Δ200 ade2-101 lys2-801 met⁻*	This work
YB256	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1-81 LEU2::pRY181*	This work
YB258	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1-181,61 LEU2::pRY181*	This work
YB268	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1-181,65 LEU2::pRY181*	This work
YB260	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1Δ2.5::HIS3 LEU2::pRY181* pBB176 (*nmt1-81*)	This work
YB261	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1Δ2.5::HIS3 LEU2::pRY181* pBB181 (*nmt1-181,61*)	This work
YB276	*MATa ura3-52 his3Δ200 ade2-101 lys2-801 met⁻ nmt1Δ2.5::HIS3 LEU2::pRY181* pBB190 (*nmt1-181,65*)	This work

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‡ Department of Biology, The University of Michigan, Ann Arbor, MI.
§ 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.
¶ Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima, Japan.

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*	50	14	118	66
	lys2-801 vs. *NMT1::HIS3*	31	30	124	unlinked‡
	ade2-101 vs. *NMT1::HIS3*	40	27	114	unlinked
II. YB197 × YB137	*cdc42-1* vs. *NMT1::HIS3*	132	0	102	22
	ura3 vs. *NMT1::HIS3*	29	38	173	unlinked
	ade2-101 vs. *NMT1::HIS3*	44	39	154	unlinked
III. YB198 × YB201	*RDN1::LEU2* vs. *NMT1::HIS3*	77	11	150	48
	CDC25::URA3 vs. *NMT1::HIS3*	65	15	172	59
	RDN1::LEU2 vs. *CDC25::URA3*	44	29	133	unlinked
	lys2-801 vs. *NMT1::HIS3*	44	51	154	unlinked
IV. YB191 × YB140	*nmt1-181* vs. *NMT1::HIS3*	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 [^{3}H]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 [^{3}H]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

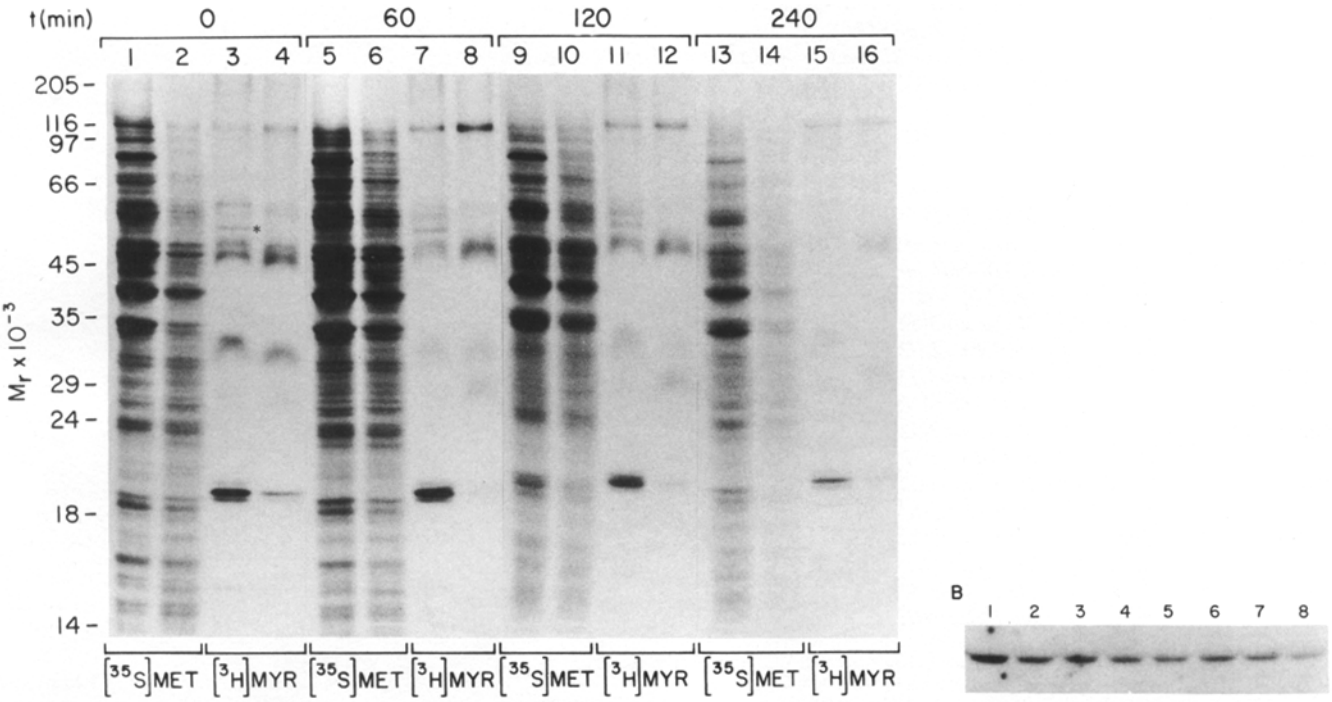

Figure 2. Metabolic labeling of *nmt1-181* mutant cells. (*A*) Strains YM2061 (*NMT1, odd numbered lanes*) and YB218 (*nmt1-181, even numbered lanes*) were grown to an A_{600} = 0.95 in YPD at 24°C. Aliquots were removed at the indicated times after shifting the cultures to 36°C and incubated for an additional 30 min with either 1.0 μCi/ml [^{35}S]methionine (lanes *1, 2; 5, 6; 9, 10*; and *13, 14*) or 50 μCi/ml [^{3}H]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.

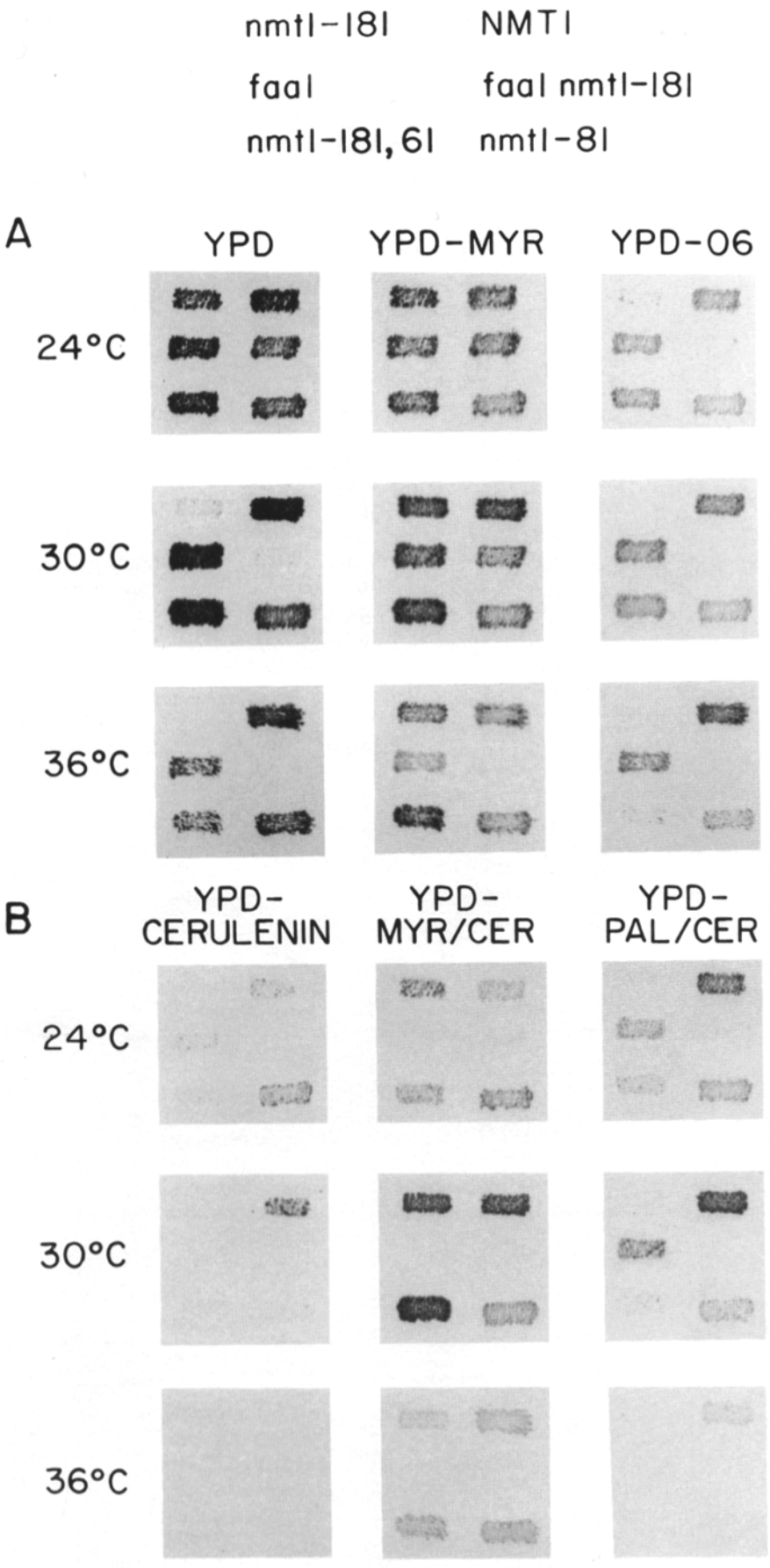

Figure 3. Phenotype caused by *nmt1-181*. Strains YB218 (*FAA1 nmt1-181*, Asp^{451}), YM2061 (*FAA1 NMT1*, Gly^{451}), YB241 (*faa1 NMT1*), YB253 (*faa1 nmt1-181*), YB258 (*FAA1 nmt1-181,61*, Lys^{293}/Asp^{451}), and YB256 (*FAA1 nmt1-81*, Asn^{451}) were incubated for 2 d at the indicated temperatures 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 μM cerulenin (YPD-CERULENIN), YPD plus 25 μM cerulenin and 0.03% (wt/vol) myristic acid (YPD-MYR/CER), and YPD plus 25 μM 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.

[^{35}S]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 nmt1-181 Cells by Exogenous Myristate Requires acyl:CoA Synthetase

Fig. 3 *A* further illustrates the temperature sensitivity of *nmt1-181* strains in the absence of exogenous fatty acid. Absolutely no growth was detected on YPD medium after a 2-d incubation of strain YB218 (*nmt1-181*) 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 (*faa1*, Kamiryo et al., 1976, 1977*a*) 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 faa1 enzyme has reduced activity at 24°C compared to wild type, and that this difference becomes more marked at 36°C (Kamiryo et al., 1977*a*). Yeast strains carrying only the *faa1* or *nmt1-181* alleles grew on YPD-MYR at all temperatures surveyed. However, a strain (YB253) with both the *faa1* and *nmt1-181* mutations failed to grow on this medium at 36°C (Fig. 3 *A*).

nmt1-181 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 *nmt1-181* allele could be used to report changes in these pools and define the mechanisms that regulate myristic acid metabolism in this lower eukaryote. Cerulenin (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 cerulenin 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

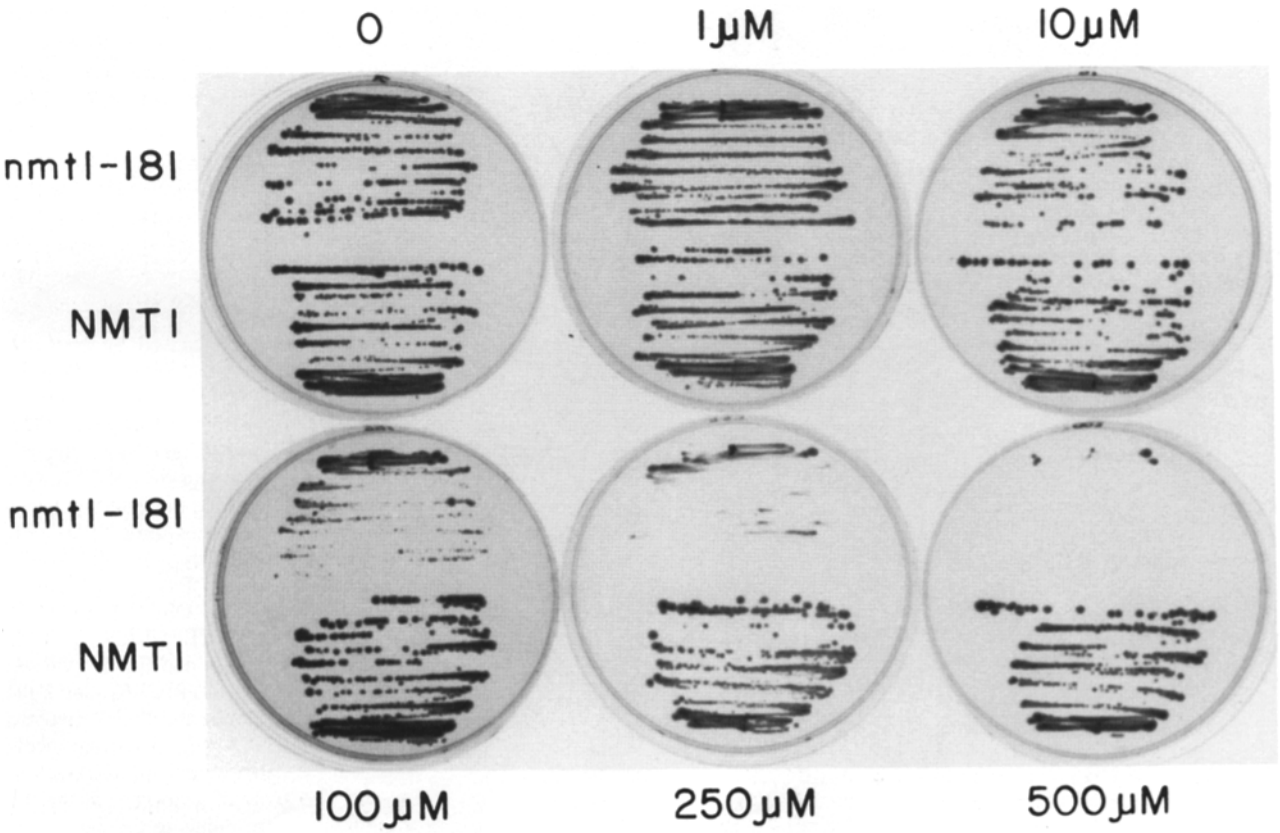

Figure 4. Dose-response of *nmt1-181* growth inhibition by 6-oxatetradecanoate. Strains YM2061 (*NMT1*) and YB218 (*nmt1-181*) were streaked onto YPD (0) or YPD supplemented with the indicated amounts of 6-oxatetradecanoic acid and grown for 4 d at 24°C.

synthesis without inhibiting elongation or desaturation systems (Kamiryo et al., 1976; Awaya 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 (Kamiryo et al., 1976, 1977*a*). 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.

Heteroatom-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 C1) have shown that such substitutions along the length of the hydrocarbon chain are well accommodated by purified *S. cerevisiae* NMT (Kishore et al., 1991).[2] These compounds are selectively incorporated into subsets of cellular N-myristoylproteins, 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-myristoylprotein 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 plating 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 substitution at positions 3 through 13 were surveyed. YPD, YPD-MYR, and YPD-ANALOG plates were incubated for 2 d at 24 or 36°C. None of the analogues had any effect on the growth of the wild type strain (YM2061) at either temperature. However, the effects of the various analogues on YB218 growth could be grouped into two general categories: those that partially supported growth at 36°C, and those that inhibited growth at 24°C. For example, 9-thiatetradecanoate (S9) was able to rescue the ts auxotrophy to a degree that was comparable to that achieved with 500 μM C14:0 at 36°C (data not shown). This complementation required a functional acyl:CoA synthetase, since strain YB253 (*nmt1-181, faa1*) did not grow on YPD medium supplemented with S9 at 36°C. A different compound, 6-*oxa*tetradecanoate (O6), inhibited growth of YB218 (*nmt1-181, FAA1*) at the permissive temperature without having any discernible effect on the wild type strain, YM2061 (Fig. 3 *A*).

6-Oxatetradecanoic Acid Does Not Efficiently Accumulate in S. cerevisiae

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 [^{3}H]myristate or [^{3}H]6-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-myristoylproteins. 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 [^{3}H]myristate, [^{3}H]palmitate, [^{3}H]O6, or [^{3}H]O13, 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*). [^{3}H]O13 produced a 10-fold higher level of accumulation of radioactivity than [^{3}H]O6. Addition of 100 or 500 μM [^{3}H]myristate to YM2061 resulted in a large increase in cellular radioactivity compared to levels achieved with 1.6 μM [^{3}H]myristate alone (Fig. 5 *B*). 100 and 500 μM [^{3}H]O6 produced a level of accumulation of label that was 26- and 28-fold less than the amount observed with 100 and 500 μm [^{3}H]myristate. The respective values obtained with [^{3}H]O13 were 8- and 13-fold less than [^{3}H]-myristate. Control experiments performed under identical conditions revealed that the level of accumulation of [^{3}H]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

2. Substitution of an oxygen or sulfur atom for methylene in C14:0 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 *oxa*tetradecanoic acids relative to C14:0 is therefore greater than that obtained with *thia*tetradecanoic acids.

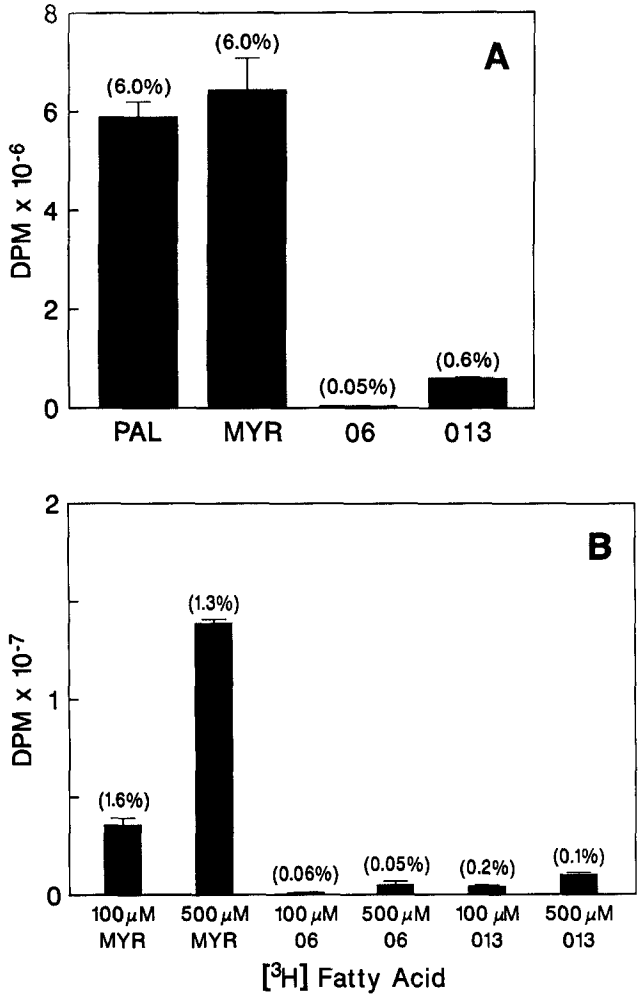

Figure 5. 6-Oxatetradecanoate does not efficiently accumulate in *S. cerevisiae*. (*A*) YM2061 was grown to an A_{600} = 1.9 in YPD at 30°C and incubated with 1.6 μM [^{3}H]myristate, 1.6 μM [^{3}H]palmitate, 1.6 μM [^{3}H]O6, or 1.6 μM [^{3}H]O13 (all at 30 Ci/mmol) for 30 min at 30°C. (*B*) YM2061 was grown to an A_{600} = 1.7 in YPD at 30°C and incubated for 30 min at 30°C with either (*a*) 100 or 500 μM [^{3}H]myristate; (*b*) 100 or 500 μM [^{3}H]O6; or (*c*) 100 or 500 μM [^{3}H]O13. The specific activity of all fatty acids was adjusted to 1 Ci/mmol. The concentration of unlabeled myristate in the medium was 1.6 μM. The amount of radiolabel associated with washed whole cells was quantitated as described in Materials and Methods. Shown in parentheses are these values expressed as a percentage of the total labeled fatty acid added to the media at t = 0.

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^{451}→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 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^{451}→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 *NMT1* 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 nmt-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 *MATα* 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

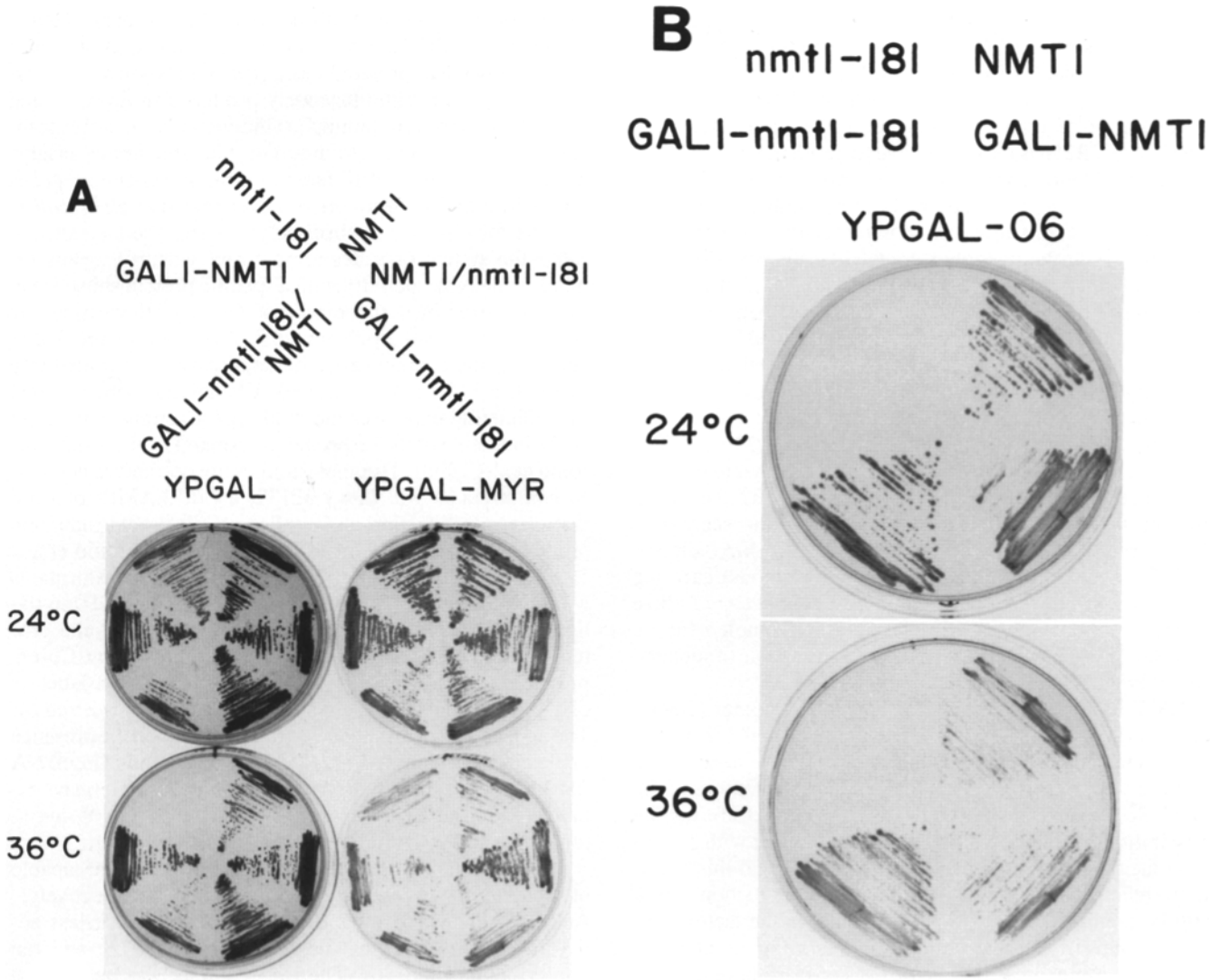

Figure 6. Overexpression of *nmtl-181* suppresses the mutant phenotype. (*A*) Strains YM2061 (*NMT1*), YB218 (*nmtl-181*), YB207 × YM2061 (*nmtl-181/NMT1*), YB220 (*GALl-nmtl-181*), YB224 (*NMT1/GALl-nmtl-181*), and YB143 (*GALl-NMT1*) were streaked on YPGAL and YPGAL plus 0.03% (wt/vol) myristate and 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.

the corresponding *suppressor allele* haploid, but slightly better than the original *nmtl-181* strain). Tetrad analysis of several of these diploids revealed 2:2 Mendelian segregation for growth at 36°C, consistent with mutation of a single gene in each case.

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 Asp^{451} (GAT) to Asn^{451} (AAT) change (designated *nmtl-81*); and (*b*) isolates that retained Asp^{451} but also had a Glu to Lys substitution elsewhere in the molecule. Of this latter class, two independent isolates had a Glu^{167} (GAA) to Lys^{167} (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*, Asn^{451}), YB261 (*nmtl-181,61*, Lys^{293}/Asp^{451}), and YB276 (*nmtl-181,65*, Lys^{167}/Asp^{451}) were able to grow at 36°C, while YB216 (*nmtl-181*, Asp^{451}) 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* (Asn^{451}) and *nmtl-181,61* (Lys^{293}/Asp^{451}) alleles, respectively. Neither grows as competently as wild type at 36°C, and YB258 (*nmtl-181,61*, Lys^{293}/Asp^{451}) grows somewhat poorer than YB256

(*nmt1-81*, Asn[451]). Unlike YB218 (*nmt1-181*, Asp[451]), neither strain is sensitive to O6 at 24°C, although YPD plus 0.03% (wt/vol) O6 blocks growth of YB258 (*nmt1-181,61*, Lys[293]/Asp[451]) at 36°C and reduces growth of YB256 (*nmt1-81*, Asn[451]) at 36°C relative to that on YPD alone (Fig. 3 *A*). Similarly, YB256 is more sensitive to cerulenin than YB258, and both are more sensitive than the wild type strain, YM2061 (Fig. 3 *B*). Thus, these strains bearing intragenic suppressors have an intermediate phenotype relative to *NMT1* and *nmt1-181*, with the phenotype caused by the *nmt1-81* allele (Asn[451]) being more similar to that of the *NMT1* strain than that caused by the *nmt1-181,61* allele (Lys[293]/Asp[451]).

The nature of the amino acid changes specified by the *nmt1-181,61*, *nmt1-181,65*, and *nmt1-81* alleles suggest that the overall charge of NMT, or at least the charge of residue 451, is very important for activity in vivo. Site-directed mutagenesis was therefore used to change codon 451 of *NMT1* to Lys. The resulting centromere plasmid (pBB182) was unable to complement the temperature sensitivity of strain YB218 containing the *nmt1-181* allele. No Ura$^+$ His$^+$ meiotic segregants from over 30 tetrads of a *NMT1/nmt1Δ2.5::HIS3* diploid (YB152, see Table I and Materials and Methods) carrying plasmid pBB182 were observed, indicating that a *nmt1* allele encoding an enzyme with Lys[451] could not complement the *nmt1Δ2.5::HIS3* null allele and thus was incapable of supporting growth.

NMT is apparently active in vitro as a monomer (Towler et al., 1987*b*). As stated above, the *nmt1-181/nmt1-181,61* diploid remained temperature sensitive, while the *nmt1-181,61* haploid was not. The phenotypic dominance caused by the *nmt1-181* allele relative to the *nmt1-181,61* allele raises the possibility that these mutant NMTs can interact with a common factor in a diploid cell. nmt1-181 may bind this factor more efficiently than nmt-181,61, but the interaction would still be nonfunctional. NMT itself could be the factor, i.e., nmt-181/nmt-181,61 heterodimers may form but be inactive. Similarly, overexpression of nmt-181 seems to have a dominant negative effect on wild type NMT. Strain YB224 (*GAL1-nmt1-181/NMT1*) grew more poorly on YPGAL than did YB220 (*GAL1-nmt1-181*) (Fig. 5 A); this effect was not apparent on YPD medium (data not shown).

The possibility of extragenic suppression of *nmt1-181* by high gene dosage was also tested by transforming a mutant strain with a *S. cerevisiae* genomic library cloned in a 2 μm circle vector (see Materials and Methods). This vector is maintained in high copy number in yeast cells. No new genes were identified among the ~12,000 independent Ura$^+$ transformants that were tested for growth at the restrictive temperature. Two independent transformants containing overlapping pieces of the *NMT1* locus were obtained, suggesting that a significant portion of the yeast genome had been surveyed. This result implies that there is more than one critical N-myristoylprotein necessary for vegetative growth of *S. cerevisiae*, if overexpression of one of NMT's protein substrates could overcome a catalytic defect of nmt-181 (see below). Alternatively, overexpression of such critical N-myristoylprotein(s) may not be sufficient to complement a defect of this sort.

Residue 451 Is Critically Involved in NMT Catalysis

We recently described a coexpression system that allows N-myristoylation to be reconstituted in *E. coli*, a bacterium that has no endogenous NMT activity (Duronio et al., 1990). *S. cerevisiae* NMT and the catalytic subunit (Cα) 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 Gly[2] of Cα. 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 Cα acylation using the Asp[451], Asn[451], and Lys[451] mutants. A portion of the *NMT1* coding region containing each mutation was subcloned into pBB131, a P15A-KAN^R 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 ColE1-AMP^R plasmid that directs production of Cα 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 [^{3}H]myristic acid as described in Materials and Methods. Cellular proteins were subjected to SDS-PAGE and fluorography. Co-expression of wild type NMT and Cα 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 *NMT1* (lanes *6* and *12*) and Cα cDNA (lanes *1* and *7*). Immunological and chemical studies have established that labeling of this 40-kD band represents N-myristoylation of Cα (Duronio et al., 1990).

The Asp[451] and Asn[451] mutant NMTs were each capable of acylating Cα at 24°C (Fig. 7, lanes *3* and *4*, respectively). At 36°C, the Asp[451] mutant enzyme became much less active (lane *9*) than both wild type NMT (lane *8*) and the Asn[451] mutant (lane *10*). The Lys[451] mutant was not able to N-myristoylate Cα at either temperature (lanes *5* and *11*), consistent with its inability to rescue the *nmt1Δ2.5::HIS3* allele in *S. cerevisiae*. The differences in labeling of Cα 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 Cα 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 kD 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 Gly[451]→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-

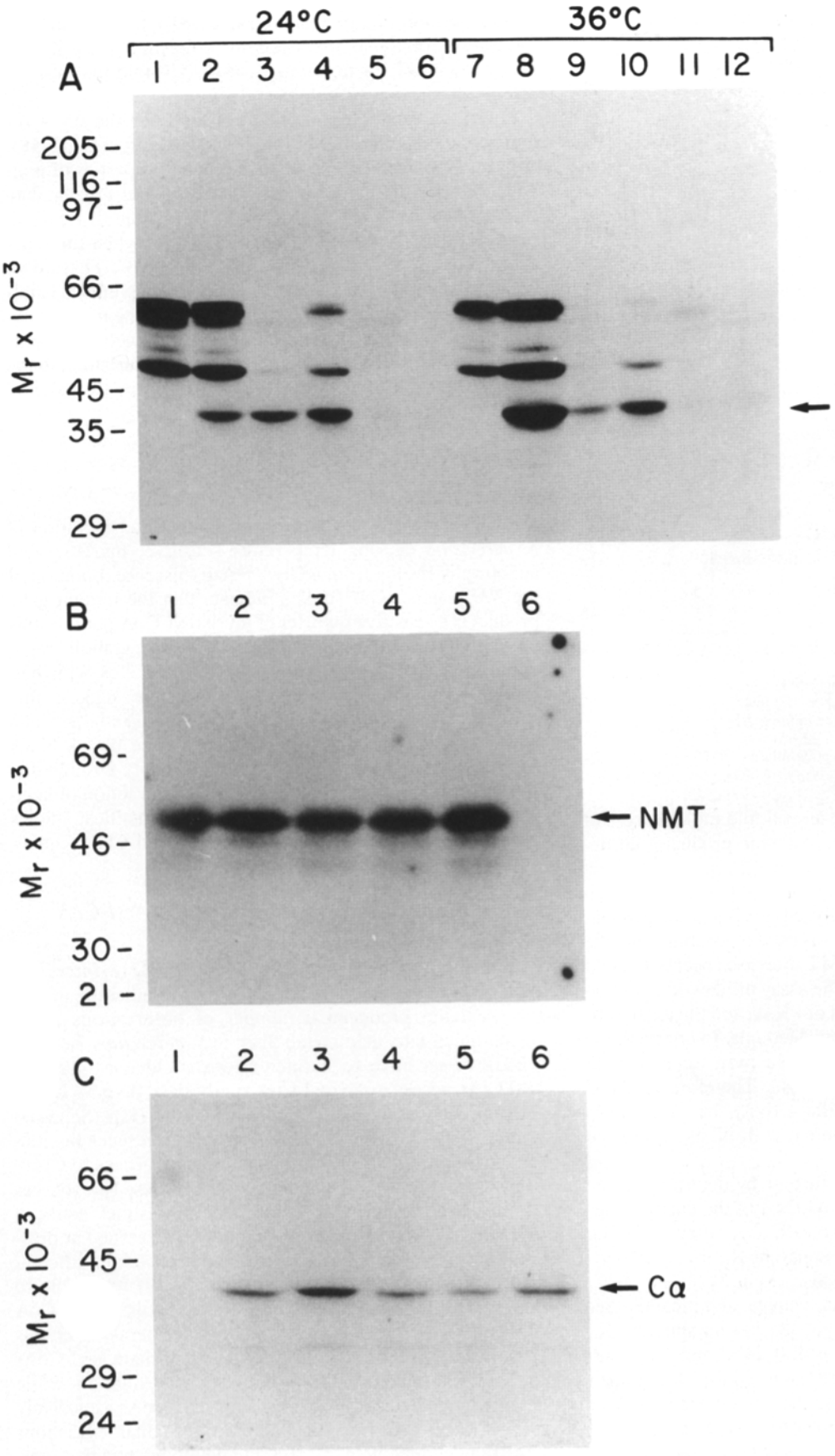

Figure 7. Coexpression of wild type and mutant yeast NMTs with mouse Cα in *E. coli*. (*A*) *E. coli* strain JM101 containing *NMT1* alone (lanes *1* and *7*), *NMT1* + Cα (lanes *2* and *8*), *nmt1-181* (Asp^{451}) + Cα (lanes *3* and *9*), *nmt1-81* (Asn^{451}) + Cα (lanes *4* and *10*), *nmt1-K451* + Cα (lanes *5* and *11*), or Cα alone (lanes *6* and *12*) were induced, incubated with [^{3}H]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-Cα. 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-Cα serum (*C*). Antigen-antibody complexes were visualized with [^{125}I]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
pI (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 K_m§

		nmt-181	wild type
$K_m(\mu M)$	24°C	1.1 ± 0.1	1.6 ± 0.3
	36°C	6.1 ± 1.6	0.6 ± 0.2
$V_m(\%)$	24°C	5.3 ± 1.1	100‖
	36°C	5.5 ± 1.9	107 ± 13

D. Peptide K_m (GNAAAARR-NH_2)¶

		nmt-181	wild type
$K_m(\mu M)$	24°C	484 ± 11	38 ± 17
	36°C	1550 ± 450	92 ± 37
$V_m(\%)$	24°C	10.9 ± 3.5	100**
	36°C	2.7 ± 0.5	352 ± 157

* Assay conditions: [myristoyl-CoA] = 0.23 μM, [GNAAAARR-NH_2] = 180 μM.
‡ 4.8×10^4 pmol acylpeptide/minute/mg NMT.
§ Assay conditions: [GNAAAARR-NH_2] = 180 μM.
‖ $3.8 \times 10^5 \pm 8 \times 10^4$ pmol acylpeptide/min/mg NMT.
¶ Assay conditions [myristoyl CoA] = 0.23 μM.
** $3.5 \times 10^4 \pm 9.4 \times 10^3$ pmol acylpeptide/min/mg NMT.

nick et al., 1990), addition of a several fold molar excess of myristoyl-CoA to the mutant apoenzyme produced no detectable alteration in IEF point (Table III *A*).

A discontinuous in vitro assay of NMT activity, which directly measures enzyme-catalyzed acyl-peptide production (Towler and Glaser, 1986), was used to establish that the specific activity of wild type NMT increases nearly fivefold at 36°C compared with 24°C. The assay utilizes an octapeptide derived from the Cα subunit of PK-A, and the assay conditions described in Materials and Methods. In contrast, the specific activity of nmt-181 decreases more than fivefold when measured at 36°C (Table III *B*). Therefore, at 24°C wild type NMT exhibits a specific activity that is 20 times greater than that of nmt-181, whereas at 36°C the difference becomes 600-fold.

This difference was explored further by determining the apparent K_m and V_{max} for myristoyl-CoA of the purified mutant and wild type enzymes (Table III *C*). Analysis of the wild type enzyme indicated an apparent K_m for myristoyl-CoA of 1.6 ± 0.3 μM and 0.6 ± 0.2 μM at 24°C and 36°C, respectively. The V_{max} does not change significantly between these two temperatures. nmt-181 has an apparent K_m for myristoyl-CoA of 1.1 ± 0.1 μM at 24°C and 6.1 ± 1.6 μM at 36°C. The V_{max} of nmt-181 was 20-fold lower than NMT at each temperature. Thus, the apparent affinities of wild type and mutant NMT for myristoyl-CoA (as indicated by K_m) are comparable at 24°C, but suggest a much diminished affinity of the mutant enzyme at 36°C. Perhaps this accounts for the failure of nmt-181 to show a pI shift upon ligand addition (the pI gels are run at 40°C). These in vitro analyses reveal that the catalytic efficiency (V_{max}/K_m) of wild type NMT for myristoyl-CoA is >10-fold that of nmt-181 at 24°C, and >200-fold at 36°C.

Given the cooperative interactions between the acyl-CoA and peptide binding sites of NMT (Heuckeroth et al., 1988), we next characterized the peptide kinetic properties of nmt-181. The peptide K_m of nmt-181 is 10–15-fold greater than that of wild type NMT at 36°C and 24°C (Table III *D*). Both enzymes display a threefold increase in K_m when the reaction temperature is raised from 24°C to 36°C. These data are consistent with the notion that Gly^{451}→Asp affects catalysis even at the permissive temperature. At the nonpermissive temperature, an even more profound reduction in catalytic efficiency occurs, reflecting in part a perturbation in the interactions of the enzyme with myristoyl-CoA (Rudnick et al., 1990).

Discussion

We have shown that a previously unidentified mutation of *S. cerevisiae* causing temperature-sensitive, myristic acid auxotrophy results from a Gly^{451}→Asp missense mutation of the *NMT1* 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 heteroatom-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 Gly^{451} 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, FAA1*) 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., 1977*b*) 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 nmt-181 has decreased affinity for myristoyl-CoA, our data indicate that the intracellular pool(s) of myristoyl-CoA accessi-

ble by NMT is not maintained at a fixed concentration, but can vary depending on the extracellular environment.

S. cerevisiae contains enzymes capable of elongating exogenous fatty acids (Orme et al., 1972). A single long chain fatty acid like myristate can therefore supply all lipid requirements in a *fas* mutant (Schweizer and Bolling, 1970). However, YB218 (*nmt1-181, FAA1*) cannot survive at 36°C on YPD supplemented with C10:0 and C12:0. Thus, these fatty acids are not elongated at a sufficient rate or extent to supply a pool of C14:0 that is required by nmt-181 for N-myristoylation of critical protein(s). Conversely, since YB218 is not viable on YPD media containing cerulenin and palmitate at any temperature, exogenous C16:0 is not converted to myristate in amounts required to support growth of this mutant. YM2061 (*NMT1, FAA1*) can grow on YPD-CER/PAL, indicating that there is either sufficient myristate in YPD or sufficient conversion of C16:0 to supply the wild type enzyme with a pool of C14:0 necessary to sustain an essential level of acylation of its substrates. Furthermore, C16:0 does not interfere with the supply of exogenous C14:0 to NMT, since YB218 is viable at 36°C on YPD-CER/MYR/PAL (data not shown).

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, FAA1*) 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, FAA1*) 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 *FAA1* 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 nmt-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 in part 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-oxatetradecanoate (O6). [^{3}H]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 [^{3}H]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 nmt-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.

Gly^{451} 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 Gly^{451}→Asp substitution. Strains with an Asn^{451}-substituted NMT, or a Glu→Lys substitution at residue 167 or 293 that retains Asp^{451}, could each grow at 36°C, albeit not quite as well as a wild type strain. Cells containing a Lys^{451} 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 pI of the purified Asp^{451} protein was acid shifted relative to wild type, consistent with this hypothesis. Co-expression of the position 451 mutants with Cα in *E. coli* allowed us to

assess the ability of each NMT species to incorporate exogenous [^{3}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 [^{3}H]myristate incorporation into proteins, and inhibition of growth by glucose repression of *GAL1-nmt1-181*). 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_{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 *nmt-181* cells are deficient in the acylation of some or all essential N-myristoylproteins. 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 GPA1 and ARF1 proteins (Miyajima et al., 1987; Stearns et al., 1990; Duronio et al., 1991). Overexpression of the mutant gene product overcomes the K_m defect, as does increasing the intracellular concentration of acyl-CoA substrate with exogenous myristic acid. Underexpression of nmt-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 ts 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 holo- 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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