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Transcription of *INO2* and *INO4* is regulated by the state of protein N-myristoylation in *Saccharomyces cerevisiae*

Steven J. Cok, Ciara G. Martin and Jeffrey I. Gordon*

Department of Molecular Biology and Pharmacology, Box 8103, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA

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ABSTRACT

Inositol regulates transcription of *Saccharomyces cerevisiae* genes required for *de novo* synthesis of acylCoAs and phospholipids. Removal of inositol results in transcriptional activation by heterodimeric complexes of two bHLH proteins, Ino2p and Ino4p. In the presence of inositol, transcription is repressed by Opi1p. MyristoylCoA:protein N-myristoyltransferase (Nmt1p) is an essential enzyme whose activity is influenced by cellular myristoylCoA pool size and availability. nmt451Dp contains a $Gly^{451}\rightarrow Asp$ substitution that produces temperature-dependent reductions in affinity for myristoylCoA and associated reductions in acylation of cellular N-myristoylproteins. The conditional lethality produced by *nmt1-451D* is rescued at temperatures up to 33°C by withdrawal of inositol. We tested the hypothesis that N-myristoyl-proteins function to regulate *INO2*, *INO4* and/or *OPI1* transcription, thereby affecting the expression of inositol-sensitive genes that influence myristoylCoA metabolism. The effect of *nmt1-451D* on *INO2*, *INO4* and *OPI1* promoter activities was examined by introducing episomes, containing their 5′ non-transcribed domains linked to reporters, into isogenic *NMT1* and *nmt1-451D* cells. The activity of *INO2* is significantly higher, *INO4* significantly lower and *OPI1* unaffected in *nmt1-451D* cells, both in the presence and absence of inositol. These changes are associated with a net increase in expression of some inositol target genes, including *FAS1*. *FAS1* encodes one of the subunits of the fatty acid synthase complex that catalyzes *de novo* acylCoA (including myristoylCoA) biosynthesis. Augmented expression of *FAS1* overcomes the kinetic defects in nmt451Dp. *FAS1* expression is Ino2p-dependent in *NMT1* cells at 24–33°C. In contrast, *FAS1* expression becomes Ino2p-independent in *nmt1-451D* cells at temperatures where efficient acylation of cellular N-myristoylproteins is jeopardized. The ability to maintain expression of *FAS1* in *nmt1-451Dino2*Δ cells suggests the existence of another transcription factor, or factors, whose expression/activity is inversely related to overall levels of cellular protein N-myristoylation. This factor is not functionally identical to Ino2p since other inositol-responsive genes (e.g. *CHO1*) maintain *INO2*-dependent expression in *nmt1-451D* cells.

INTRODUCTION

In *Saccharomyces cerevisiae*, transcription of genes required for *de novo* synthesis of acylCoAs and phospholipids is coordinately regulated by the phospholipid precursor molecules inositol and choline (1). Removal of inositol from the media results in activation of these genes by Ino2p and Ino4p. Ino2p and Ino4p are basic helix–loop–helix (bHLH) transcription factors that form heterodimeric complexes which bind to inositol-choline response elements (ICRE, also known as UAS_{INO}; 2–4). When inositol is available, genes required for *de novo* acylCoA and phospholipid biosynthesis are repressed. Repression requires Opi1p. The mechanism of Opi1p-mediated repression is not known (5,6).

Much remains to be defined about the cellular factors that influence expression of *INO2*, *INO4* and *OPI1*. N-myristoylproteins represent one potential set of regulators, given the sensitivity of myristoylCoA:protein N-myristoyltransferase (Nmt1p) to cellular acylCoA pool size and composition (7–9). Nmt1p catalyzes the transfer of myristate (C14:0) from CoA to the N-terminal glycine of nascent proteins (10,11). Sixty-four known or potential Nmt1p substrates have been identified among the 6220 conventional open reading frames represented in the yeast genome. [To identify these 64 sequences, the translation products of all standard open reading frames listed in the *S.cerevisiae* genome database (SGD; http://genome-www.stanford.edu/Saccharomyces/) were scanned for the presence of $M^1G^2X^3X^4X^5X^6X^7$ at their N-termini, using the PatMatch sequence analysis tool contained in the SGD website. Based on previous analyses of the peptide substrate specificity of Nmt1p (12–14), E, D, R, K, H, P, F, Y and W were not allowed at position 3 (X^3), all possible amino acids were allowed at X^4 and X^5, only S, T, A, G, C or N were permitted at X^6, and all residues except P were allowed at X^7.] Many of these proteins are involved in signal transduction pathways and include known or putative phosphatases, serine/threonine and tyrosine

*To whom correspondence should be addressed. Tel: +1 314 362 7243; Fax: +1 314 362 7047; Email: jgordon@pharmdec.wustl.edu

kinases, and the α-subunits of two heterotrimeric G proteins (Gpa1p and Gpa2p). Genetic analyses have established that *NMT1* is essential for vegetative growth (15–17). Studies using conditional lethal *nmt1* alleles encoding acyltransferases with reduced affinity for their myristoylCoA substrate have shown that regulation of myristoylCoA pool size has a great impact on the efficiency of protein N-myristoylation and that such pools are generated from two sources: *de novo* synthesis by fatty acid synthase (Fas) and activation of free C14:0 by several acylCoA synthetases (fatty acid activation proteins or Faas; 8,17). Removal of inositol from the media partially rescues the conditional lethality imparted by several *nmt1* mutants (7,17). Rescue was thought to be due, at least in part, to an induction of *FAS* gene transcription. This assumption was based on the observations of Chirala and co-workers that *FAS* is induced in *NMT1* strains when inositol is withdrawn from the medium (18,19).

In this report, we have addressed a related possibility—namely, that the state of acylation of one or more cellular N-myristoyl-proteins serves to influence expression of *INO2*, *INO4* or *OPI1*, and in so doing to regulate myristoylCoA metabolism through effects on *FAS* transcription. To test this possibility, we chose *nmt1-451D* as the 'provocative' allele. *nmt1-451D* encodes an enzyme with a Gly^{451} to Asp substitution that reduces its affinity for myristoylCoA and produces global defects in protein N-myristoylation. These defects worsen progressively as incubation temperatures are increased from 24 to 30°C (16,20). A shift to non-permissive temperatures (≥30°C) results in growth arrest within 1 h and subsequent loss of viability (7). The conditional lethality does not require components of the mating pathway and can be rescued at temperatures up to 37°C by adding myristate to the medium or by expressing Nmt1p (7). As described below, studies using isogenic strains containing various combinations of *NMT1* or *nmt1-451D* plus wild type or null alleles of *INO2*, *INO4* and *OPI1* allowed us to establish that a functional linkage does indeed exist between the state of protein N-myristoylation, the inositol response pathway and *FAS* transcription.

MATERIALS AND METHODS

Yeast strains

YB427 (*MAT***a**, *nmt1-451D*, *ura3*, *his3*, *ade2*, *ade3*, *leu2*, *trp1*) has been described previously (7). The *nmt1-451D* allele in this strain was replaced by *NMT1* using the integrating vector pBB395. pBB395 is a derivative of pRS305 (21) that contains a region of *NMT1* extending from its nucleotide 1031 (15) to 552 bases downstream of its stop codon. pBB395 was linearized by digestion with *Mlu*I and used to transform YB427, yielding YB553 (*NMT1* and a leucine prototroph). YB579 was constructed using a similar strategy except that the *Avr*II–*Hin*dIII fragment containing the *NMT1* domain described above was subcloned into pRS306 (21) and YB427 transformants were isolated based on their uracil prototrophy.

INO2, *INO4* or *OPI1* was deleted in YB427 and YB553 and replaced by *HIS3* (22). To do so, the isogenic strains were first transformed with pTSV31A (kindly supplied by Alan Bender, Indiana University). pTSV31A contains *URA3* and *ADE3*, and rescues the histidine auxotrophy produced by the *ade3* allele in YB427 and YB553. The *HIS3* gene in pBM2815 (obtained from Mark Johnston, Washington University) was amplified by PCR using (i) a primer encompassing 45 nt immediately upstream of the initiator Met codon of the target gene, followed by 19 bases from the region 196–177 nt upstream of the ATG of *HIS3* (5′-GGCCTC-CTCTAGTACACTC-3′) and (ii) a primer encompassing the 45 nt immediately downstream of the stop codon of the target gene followed by 19 bases from the region 154–173 nt downstream of the *HIS3* stop codon (5′-GCGCGCCTCGTTCAGAATG-3′). The PCR product was introduced into YB427 and YB553 and transformants were isolated based on their histidine prototropy. Proper integration of *HIS3* at the locus of interest was confirmed by PCR using genomic DNA as template (23) plus primer pairs that flanked the junction between the promoter region of the deleted gene and the inserted *HIS3* DNA. Cells with proper integrations of *HIS3* were plated on synthetic media containing 5-fluoro-orotic acid (PCR, Inc.) to expel pTSV31A. These manipulations yielded the following panel of isogenic strains: YB582 (*nmt1-451D,ino2::HIS3*), YB583 (*NMT1,ino2::HIS3*), YB585 (*nmt1-451D,opi1::HIS3*), YB586 (*NMT1,opi1::HIS3*), YB588 (*nmt1-451D,ino4::HIS3*), YB589 (*NMT1,ino4::HIS3*).

Promoter activity assays

The promoter activities of seven genes were assessed in these isogenic strains. Given the relatively large number of combinations of strains and genes to be assayed, we elected to define promoter activities using episomes containing portions of the 5′ non-transcribed regions of these genes linked to one of two reporters.

Plasmids containing the chloramphenicol acetyltransferase gene (*cat*) fused to 5′-transcriptional regulatory regions from *INO2*, *INO4*, *OPI1* and *INO1* were generously supplied by John Lopes (Wayne State University) and are described in Ashburner and Lopes (24). Their inserts were removed by digestion with *Kpn*I and *Sac*I in the case of *INO2-cat* and *OPI1-cat*, or *Ksp*I and *Cla*I in the case of *INO4-cat* and *INO1-cat*. These restriction fragments were then inserted into *Kpn*I/*Sac*I- or *Ksp*I/*Cla*I-digested pRS316 (21). The resulting recombinant plasmids were used to transform the yeast strains listed above.

Plasmids containing *Escherichia coli* β-galactosidase (*lacZ*) fused to the 5′ transcriptional regulatory regions of *FAS1* and *FAS2* (pSCFAS1 and pSCFAS2, respectively; 18) were kindly supplied by Subrahmanyam Chirala (Baylor College of Medicine). An *ACC1-lacZ* fusion gene was constructed by PCR amplification of the region of *ACC1* bounded by its *Sal*I and *Hin*dIII sites and then subcloning the product into YEp365 (American Type Culture Collection). The result was pBB405 which contained the 1700 nt of *ACC1* located upstream of its initiator Met codon plus the first four codons of its open reading frame (ORF), fused in-frame to the *lacZ* ORF. The *FAS1-lacZ*, *FAS2-lacZ* and *ACC1-lacZ* plasmids were each introduced into isogenic strains containing *nmt1-451D* (YB427) or *NMT1* (YB553, YB579).

A YEp-based plasmid containing *FAS1*, previously isolated as a high-copy suppressor of *nmt1-451D* (7), plus a YEp plasmid containing *FAS2* (YEPFAS2; from Subrahmanyam Chirala), were also each introduced into YB427 and YB579.

YB427, YB553 and YB579 plus their derivatives, with and without these episomes, were grown at 24°C to mid-log phase (OD_{600} = 0.5–1.0) in synthetic medium (Bio101) containing 2% (w/v) glucose plus 75 μM inositol and lacking either leucine or uracil. For some experiments, the medium was supplemented with 500 μM myristate (NuCheck-Prep) and/or 1% (w/v) Brij 58. Mid-log phase cells were collected from 50 ml cultures by centrifugation at 1600 *g* for 10 min, washed twice in 1 M sorbitol and resuspended in the same medium with no inositol or 75 μM

inositol. Equivalent numbers of cells (2–5 × 10^7/ml) were incubated at 24, 30 or 33°C for an additional 4 h. At the end of this period, cells were pelleted and resuspended in lysis buffer [100 mM Tris–HCl (pH 8), 1 mM DTT, 20% glycerol for β-galactosidase assays; 0.25 M Tris–HCl (pH 7.5) for CAT assays; 250 µl of buffer/5 ml of culture]. Cell suspensions were frozen, thawed on ice and 4-(2-aminoethyl)-benzensulfonyl fluoride hydrochloride (Boehringer Mannheim) was added to a final concentration of 200 µM. Cells were then disrupted by vortexing with an equal volume of 425–600 µm glass beads (Sigma; vortexing = six cycles of 15 s each). Cellular debris was removed by centrifugation at 16 000 *g* for 5 min. The protein concentration of cleared lysates was measured using the BCA assay kit (Pierce). β-Galactosidase activity was measured using chlorophenol red β-galactopyranoside (Boehringer Mannheim) as the substrate (25). Assays were performed using 5–50 µl of cleared lysate (2.5–150 µg protein). Units of β-galactosidase activity were defined as the change in absorbance at 570 nm/min/µg of protein. CAT activity was determined by the phase extraction method (26). Units of CAT activity were defined as c.p.m. in the organic phase and expressed as a percentage of total c.p.m./µg protein/h of incubation. All strains were assayed in duplicate on at least three different occasions.

RNA analysis

Mid-log phase cells from 5 ml cultures were harvested by centrifugation at 1600 *g* for 5 min at 4°C and washed twice with phosphate-buffered saline (PBS). Spheroplasts were prepared by first resuspending the cell pellet in 0.6 ml of cell wall digestion buffer [1 M sorbitol, 0.1 M EDTA, pH 7.4, 50 U of Zymolyase 100-T (ICN) per ml of culture (N.B. Zymolyase 100-T was activated with addition of 10 µl of 2-mercaptoethanol per ml of cell wall digestion solution)]. This mixture was then incubated at 24°C with shaking for 15 min. Spheroplasts were lysed and RNA was recovered using the Purescript RNA Isolation kit (Gentra Systems, Inc.). For northern blot analysis, RNA samples (10 µg) were fractionated by denaturing formaldehyde–agarose gel electrophoresis and transferred to GeneScreen Plus hybridization membrane (NEN Life Sciences). Blots were probed with pAS103 (*CHO1*; 24) and pBM659 (*ACT1*; obtained from Mark Johnston, Washington University). These DNAs were labeled with ^{32}P using the Random Primed DNA Labeling Kit (Boehringer Mannheim). Following hybridization (GeneScreen *Plus* protocol) and washing, the amount of bound probe was quantitated using a phosphorimaging system (Molecular Dynamics).

Statistical analysis

Comparison of means were performed using Students *t*-Test (Microsoft Excel version 5.0).

RESULTS

nmt1-451D affects *INO2* and *INO4* promoter activity

To determine whether expression of *INO2*, *INO4* and/or *OPI1* are affected by *nmt1-451D*, 506, 495 and 439 bp of their 5′ non-transcribed domains (respectively) were linked to a *cat* reporter and the fusion genes were introduced into isogenic *NMT1* and *nmt1-451D* strains as *CEN*-containing episomes. Transformants were grown at 24°C to mid-log phase in synthetic medium, harvested, and equal numbers of cells incubated for 4 h at 24, 30 and 33°C in 75 µM inositol or in the absence of inositol.

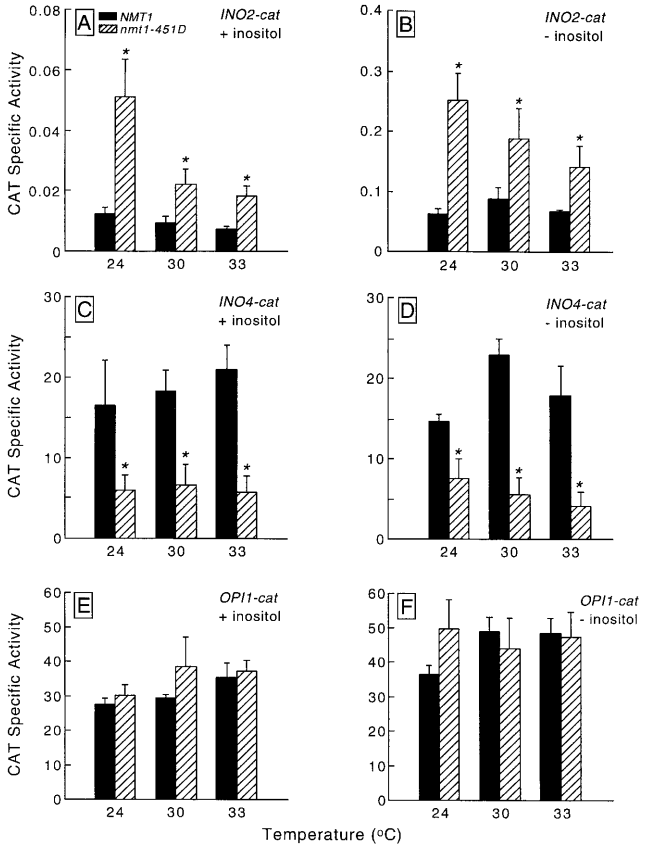

Figure 1. nmt451Dp-dependent changes in *INO2*, *INO4* and *OPI1* promoter activities. Isogenic *NMT1* and *nmt1-451D* cells with *INO2-cat*, *INO4-cat* or *OPI1-cat* episomes were grown at 24°C to mid-log phase in synthetic media containing 75 µM inositol. Cells were resuspended in synthetic media containing 0 or 75 µM inositol, and incubated for 4 h prior to determination of cellular CAT activity. Results are plotted as the mean ± S.E.M. of three to four independent experiments, each done in duplicate. An asterisk indicates a significant difference compared to *NMT1* cells ($P < 0.05$).

Removing inositol resulted in a 5–10-fold increase in *INO2-cat* expression in both *NMT1* and *nmt1-451D* cells at 24–33°C (Fig. 1A and B). However, at each temperature, the specific activity of CAT was 2–4-fold higher in *nmt1-451D* cells ($P < 0.05$; Fig. 1A and B). In contrast, expression of *INO4-cat* was not significantly affected by withdrawal of inositol and was 2–4-fold lower in *nmt1-451D* cells ($P < 0.05$; Fig. 1C and D). *OPI1-cat* expression was not significantly altered by *nmt1-451D* at any of the temperatures tested (Fig. 1E and F).

The alterations in *INO2-cat* promoter activity produced by *nmt1-451D* are corrected by exogenous myristate

The growth defect of *nmt1-451D* cells can be rescued by supplementing the medium with myristate (7,16). Previous studies have shown that the exogenous myristate is imported, activated by *FAA1* and *FAA4* to myristoyl-CoA and then utilized by nmt451Dp to increase the level of acylation of cellular N-myristoylproteins such as ADP ribosylation factors 1 and 2 (Arf1p and Arf2p; 17,20).

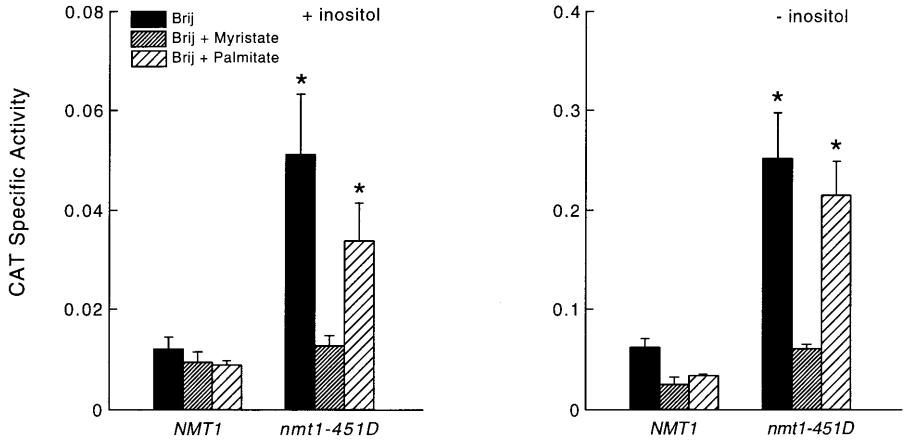

Figure 2. Exposure of *nmt1-451D* cells to myristate returns *INO2-cat* expression to levels encountered in isogenic *NMT1* cells. Cells were grown at 24°C to mid-log phase in synthetic media containing 75 µM inositol. Cells were resuspended in media containing either 0 or 75 µM and either 1% Brij alone, 1% Brij plus 500 µM myristate or 1% Brij plus 500 µM palmitate. Following a 4 h incubation at 24°C, CAT activity was measured in cell lysates. Mean values ± S.E.M. of three independent experiments, each done in duplicate, are plotted. An asterisk indicates a significant difference ($P < 0.05$) compared to *NMT1* cells.

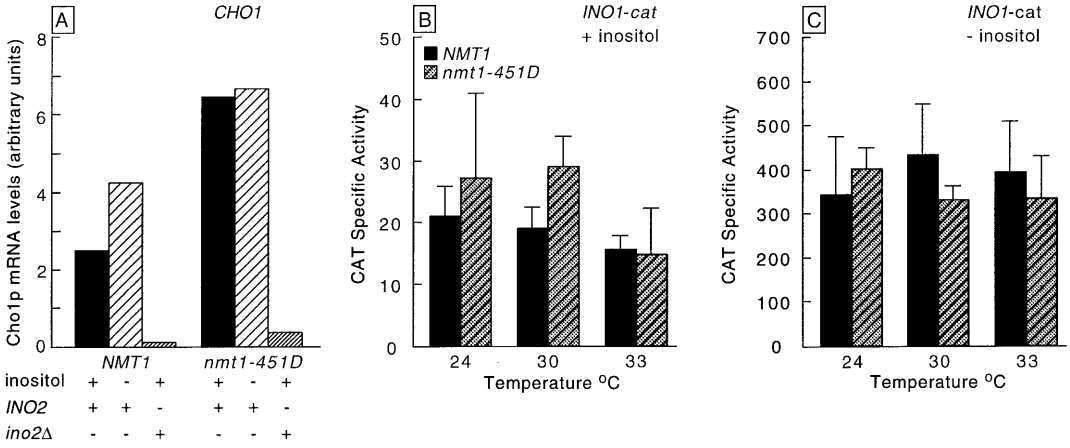

Figure 3. Effects of *nmt1-451D* on expression of known inositol-responsive genes, *CHO1* and *INO1*. Isogenic strains were grown at 24°C to mid-log phase in the presence of 75 µM inositol. Cells were resuspended in media containing 0 or 75 µM inositol, incubated for 4 h at 30°C and total RNA was isolated. (**A**) Cho1p mRNA levels were defined by northern blot analysis and normalized to actin (Act1p) mRNA. (**B** and **C**) A separate series of experiments where mid-log phase cells containing an *INO1-cat* episome were incubated for 4 h at 24–33°C prior to assaying CAT activity. Mean values ± S.E.M. obtained from three to four independent experiments, each done in duplicate, are plotted. There are no significant differences in CAT activity between *NMT1* and *nmt1-451D* cells.

We compared *INO2-cat* expression in mid-log phase *nmt1-451D* cells after incubation at 24°C for 4 h in medium with or without myristic acid supplementation, with or without inositol. A supplemental dose of 500 µM myristate was chosen because earlier studies had shown that it was sufficient to correct the reduced levels of Arfp N-myristoylation at temperatures ranging from 24 to 37°C (20). Addition of myristate had no statistically significant effects on CAT activity in *NMT1* cells, whether they were incubated at 24°C in the presence or absence of inositol (Fig. 2). In contrast, myristate decreased *INO2-cat* expression in *nmt1-451D* cells, grown with or without inositol, to levels that were equivalent to those in isogenic *NMT1* cells (Fig. 2).

The decrease in *INO2-cat* expression was specific for myristate: palmitate had no significant effect (Fig. 2). PalmitoylCoA is not a substrate for Nmt1p or nmt451Dp (9,17,27). Moreover, addition of palmitate to *nmt1* cells does not correct the reduced acylation of N-myristoylproteins such as Arf1p or Arf2p (17).

These findings indicate that the increased level of *INO2-cat* expression observed in *nmt1-451D* cells is likely due to reduced acylation of one or more cellular N-myristoylproteins. Ino2p, Ino4p or Opi1p are not among these proteins since none have N-terminal sequences that would allow them to be recognized as Nmt1p (or nmt451Dp) substrates.

The effects of altered *INO2* and *INO4* expression in *nmt1-451D* cells on inositol-responsive genes

The change in *INO2* and *INO4* promoter activities were in opposite directions. Therefore, we examined the net effect of these changes on expression of several inositol-responsive target genes.

CHO1 encodes phosphatidylserine synthase and is normally induced when inositol is absent (28). RNA blot hybridization studies revealed that the steady-state level of Cho1p mRNA was ~2-fold higher in mid-log phase *nmt1-451D* cells compared to *NMT1* cells after they had been incubated for 4 h at 24°C in the presence or absence of inositol (Fig. 3A). Deletion of *INO2* results in a marked (17–20-fold) reduction in Cho1p mRNA levels in mid-log phase *nmt1-451D* (and *NMT1*) cells after a 4 h incubation with or without inositol (Fig. 3A plus data not shown), thereby confirming that *CHO1* transcription is greatly dependent upon Ino2p. Thus, the net effect of increased *INO2* expression and decreased *INO4* expression in *nmt1-451D* cells is augmented expression of one of their target genes—*CHO1*.

The changes in *INO2* and *INO4* promoter activities observed in *nmt1-451D* cells is not associated with an increase in expression of all inositol-responsive genes. *INO1* encodes inositol-1-phosphate synthase, is sensitive to inositol and is tightly regulated by Ino2p and Ino4p (5,6,29). *INO1-cat* expression in *NMT1* and *nmt1-451D* cells was assayed using the same conditions described above for *INO2-cat*. The *nmt1-451D* allele had no significant effect on *INO1* promoter activity whether cells were incubated with or without inositol (Fig. 3B and C).

FAS1 and *FAS2* encode the β- and α-subunits of the $\alpha_6\beta_6$ Fas complex. *ACC1* encodes acetylCoA carboxylase which produces the malonyl-CoA substrate used by Fas. All three genes have ICREs in their 5′ non-transcribed domains (18,19,30,31). MyristoylCoA is one of the products of Fas and is normally a rare cellular acylCoA species (32). Changes in *FAS1* expression in *NMT1* cells are known to affect expression of *FAS2* but not vice versa: i.e. deletion of *FAS1* results in a decrease in Fas2p mRNA levels while deletion of *FAS2* has no detectable effect on steady state Fas1p mRNA concentrations (33). We found that transformation of *nmt1-451D* cells with a high-copy YEp plasmid containing *FAS1* rescues growth at 30 and 33°C. In contrast, transformation of the same cells with a YEp plasmid containing *FAS2* does not rescue growth (Fig. 4A). Control experiments using a *NMT1* strain with a well characterized mutant *FAS2* allele (*fas2-38*; 34), demonstrated that the *FAS2* episome rescues the fatty acid auxotrophy (data not shown), confirming that the plasmid produced a functional product. Together, these observations suggest that regulation of *FAS1* expression is a critical determinant of overall Fas activity in *nmt1-451D* cells and augmented Fas expression can rescue the kinetic defects of nmt451Dp at elevated temperatures.

To determine the net effect of the altered *INO2* and *INO4* promoter activities in *nmt1-451D* cells on *FAS* and *ACC1* expression, *FAS1-lacZ*, *FAS2-lacZ* and *ACC1-lacZ* episomes were introduced into the isogenic *NMT1* and *nmt1-451D* strains. β-Galactosidase activity was measured in mid-log phase cells harvested after a 4 h incubation at 24, 30 or 33°C in the presence or absence of inositol. *FAS1-lacZ* expression was significantly higher in *nmt1-451D* cells at all temperatures tested, both in the presence or absence of inositol (Fig. 4B and C). In contrast, *FAS2-lacZ* expression did not differ significantly between strains at any temperature, with or without exposure to inositol (Fig. 4D and E). *ACC1-lacZ* expression was also similar in both strains with one exception: a slight but statistically significant increase in reporter activity was noted in *nmt1-451D* compared to *NMT1* cells at 30°C in the absence of inositol (Fig. 4F and G). Thus, augmented *INO2* and reduced *INO4* expression are associated with a net increase in *FAS1* promoter activity.

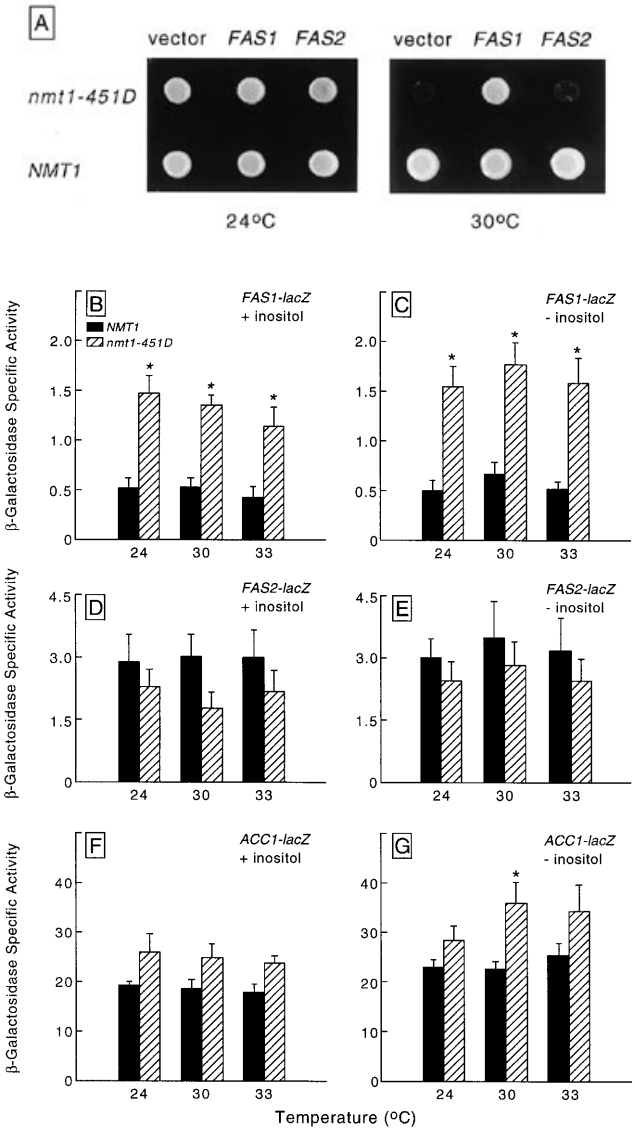

Figure 4. *FAS1-lacZ* expression is increased in *nmt1-451D* cells. (**A**) Rescue of growth by *FAS1* but not *FAS2*. *NMT1* and *nmt1-451D* strains, containing high copy YEp plasmids with either *FAS1* or *FAS2* inserts, were grown overnight at 24°C in synthetic medium lacking leucine and containing 75 μM inositol. An equal number of cells were then plated on the same medium and incubated at 24 or 30°C for 3 days. (**B**–**G**) Isogenic *NMT1* and *nmt1-451D* cells containing *FAS1-lacZ*, *FAS2-lacZ* or *ACC1-lacZ* episomes were grown at 24°C to mid-log phase in synthetic media containing 75 μM inositol, resuspended in synthetic media containing 0 or 75 μM inositol, incubated for 4 h at the indicated temperature and then assayed for cellular β-galactosidase activity. Results are plotted as the mean ± S.E.M. of three to nine independent experiments, each done in duplicate. The asterisk indicates a significant difference compared to *NMT1* cells ($P < 0.05$).

Evidence that *FAS* expression in *nmt1-451D* cells is regulated by factors other than *INO2* and *INO4*

FAS1-lacZ and *FAS2-lacZ* activities were compared in isogenic *NMT1* and *nmt1-451D* strains with wild type and null alleles of *INO2*, *INO4* or *OPI1*. Cells were grown at 24°C in synthetic

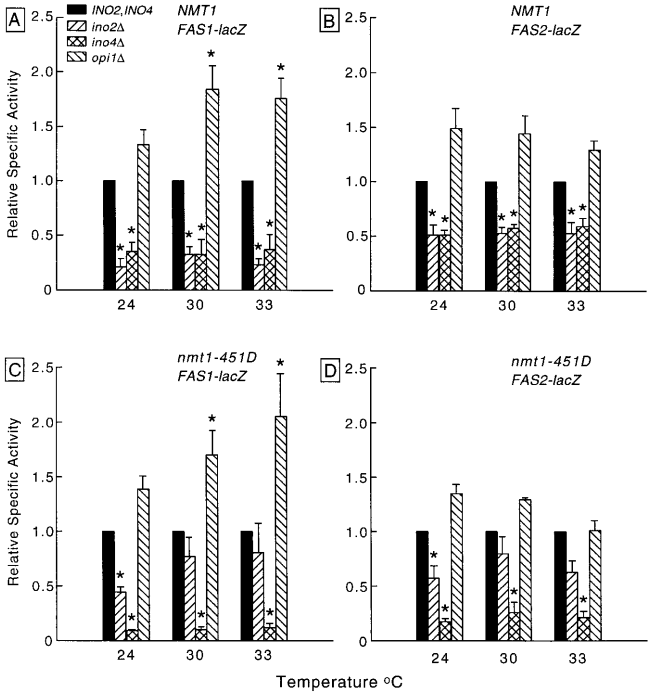

Figure 5. *FAS1* and *FAS2* promoter activities in *NMT1* and *nmt1-451D* cells in the presence or absence of Ino2p, Ino4p or Opi1p. Expression of *FAS1-lacZ* and *FAS2-lacZ* was measured as described in Figure 4. Results are presented as the change in reporter expression relative to the *NMT1* or *nmt1-451D* parental strain lacking the gene deletion. Values represent the mean ± S.E.M. of three independent experiments each performed in duplicate. An asterisk indicates a significant difference compared to the parental strain ($P < 0.05$).

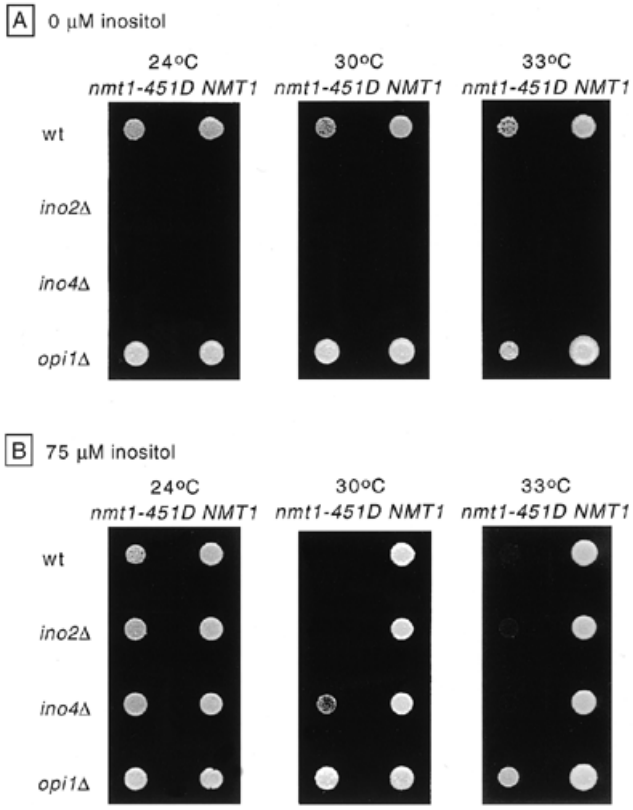

Figure 6. Effect of deleting *INO2, INO4* or *OPI1* on growth of *NMT1* and *nmt1-451D* cells. Strains were grown to mid-log phase at 24°C in synthetic media containing 75 μM inositol. An equal number of cells were transferred to plates of synthetic media either lacking inositol or containing 75 μM inositol and incubated at the indicated temperature for 3 days.

media containing 75 μM inositol to mid-log phase, transferred to fresh media (with inositol) and then incubated at 24, 30 or 33°C for an additional 4 h before reporter activity was measured in cell lysates.

In *NMT1* cells, deletion of *INO2* results in a significant decrease in the expression of *FAS1-lacZ* (and *FAS2-lacZ*) at 24, 30 and 33°C (Fig. 5A and B). Remarkably, although deletion of *INO2* results in a decrease in *FAS1-lacZ* (and *FAS2-lacZ*) expression in *nmt1-451D* cells at 24°C, when the temperature is increased to 30 or 33°C this diminished expression disappears (Fig. 5C and D). Thus, it appears that *nmt1-451Dino2*Δ cells are able to compensate for the loss of Ino2p and maintain *FAS1* promoter activity at augmented levels in response to deficiencies in protein N-myristoylation.

This compensation is not due to a loss of dependency on Ino4p in *nmt1-451D* cells. In *NMT1ino4*Δ cells, loss of Ino4p produces a decrease in *FAS1-lacZ* and *FAS2-lacZ* expression similar to that observed with loss of Ino2p (Fig. 5A and B). Moreover, deletion of *INO4* in *nmt1-451D* cells results in an even greater decrease in *FAS1* and *FAS2* promoter activity than in *NMT1* cells (Fig. 5A–D). The compensation is also not due to a loss of sensitivity to Opi1p repression. Deletion of *OPI1* causes a significant increase in *FAS1-lacZ* (but not *FAS2-lacZ*) expression at 30 and 33°C. The increase is similar in *NMT1* and *nmt1-451D* cells (Fig. 5A–D).

Together, these results suggest that *FAS1* expression is augmented in *nmt1-451D* cells in response to undermyristoylation of one or more cellular N-myristoylproteins through increased expression of *INO2* and increased activity of an unknown transcriptional activator (or activators) that can substitute for Ino2p and/or compensate for reduced expression of *INO4*.

To further assess the relative importance of Ino2p, Ino4p, Opi1p and the as yet unknown myristoylation-sensitive transcription factor(s) (MSTF) to *nmt1-451D* cells, we compared the growth of *NMT1* and *nmt1-451D* strains containing a wild type or null allele of *INO2*, *INO4* or *OPI1*. Each of the eight isogenic strains was allowed to grow at 24°C to mid-log phase on synthetic medium containing 75 μM inositol, after which time equal numbers of cells were plated on synthetic medium with or without inositol. *NMT1* and *nmt451D* cells containing either *ino2*Δ or *ino4*Δ should be inositol auxotrophs and as expected, they fail to grow without inositol (Fig. 6A). In addition, removal of the Opi1p repressor produces no further benefit to the rescue of *nmt1-451D* cells produced by inositol withdrawal. In medium containing 75 μM inositol, removal of the Opi1p repressor rescues growth of *nmt1-451D* cells at 30 and 33°C (Fig. 6B)—a result consistent with the finding that *opi1*Δ causes a significant increase *FAS1* promoter activity under these conditions (see above). Removal of Ino2p does not rescue growth of *nmt1-451D* cells at 30 or 33°C in medium containing inositol (Fig. 6B). This result suggests that the unknown MSTF(s) cannot fully substitute for Ino2p and maintain the viability of this strain under these conditions. Finally, growth of *nmt1-451D* cells at 30°C is rescued by *ino4*Δ (Fig. 6B). This finding indicates that further reductions in the

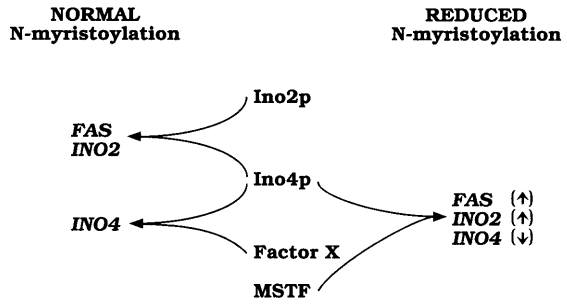

Figure 7. Working model of the relationship between protein N-myristoylation and regulation of Ino4p-dependent gene transcription. In wild type cells, Ino4p is known to partner with Ino2p to help maintain *FAS* expression and to autoregulate *INO2* transcription. Ino4p does not form a homodimer and apparently cannot act as a transcriptional activator without a partner (4). Autoregulation of *INO4* in wild type cells is *INO2* independent, suggesting the existence of another as yet unknown protein partner (24), termed factor X in the Figure. Conditions leading to a reduction in N-myristoylation of one or more cellular N-myristoylproteins result in an increase in *INO2* and *FAS* transcription. We hypothesize that Ino4p forms a heterodimer with MSTFs and that this Ino4p:MSTF heterodimer has limited functional overlap with the Ino2p:Ino4p heterodimer. The overlap includes the ability to support *FAS* transcription (even in *ino2*Δcells) and activate *INO2* transcription. Undermyristoylation of cellular proteins is also associated with a reduction in *INO4* promoter activity. MSTF:Ino4p heterodimers may have reduced ability to activate *INO4* or MSTF may block the ability of Factor X to interact with Ino4p and autoregulate *INO4*. Alternatively, MSTF(s) may be N-myristoylated and its (their) function regulated by the presence or absence of a myristoyl moiety—raising the possibility that MSTF is Factor X.

levels of *INO4* expression below those already manifested by *nmt1-451D* cells may be beneficial.

DISCUSSION

We have found that the promoter activities of *INO2* and *INO4* are sensitive to the state of protein N-myristoylation. The activity of *INO2* is significantly higher, and *INO4* significantly lower, in *nmt1-451D* compared to *NMT1* cells. The result is a net increase in expression of some inositol target genes, including *FAS1*. Augmented expression of *FAS1* is likely to help overcome the kinetic defects in nmt451Dp by providing more myristoylCoA substrate. *FAS1* expression is Ino2p-dependent in *NMT1* cells. Remarkably, *FAS1* expression is not reduced by deletion of *INO2* in *nmt1-451D* cells as incubation temperatures are increased to 30–33°C and efficient acylation of cellular N-myristoylproteins is threatened. The ability to maintain expression of *FAS1* in *nmt1-451D* cells that lack Ino2p suggests the existence of another transcription factor, or factors, whose expression/activity is (are) inversely related to the overall levels of cellular protein N-myristoylation.

We hypothesize that reduced acylation of one or more cellular N-myristoylproteins results in a compensatory increase in activity of this postulated transcription factor, or factors, which can substitute for Ino2p and maintain relatively high levels of *FAS1* expression. Expression of *INO2* is autoregulated: Ino2p is required to maintain *INO2* transcription through a mechanism that requires *INO4*. *INO4* is also autoregulated but through a mechanism that is *INO2*-independent (24). We do not know whether the myristoylation-sensitive transcription factor(s) (MSTF) is only expressed in the absence of Ino2p, whether it is capable of functioning together with Ino4p, whether it contributes to the increased *INO2* and decreased *INO4* promoter activities observed in *nmt1-451D* cells, whether its expression is directly regulated through an inositol-responsive pathway, and whether it acts through an UAS_{INO}.

We do know that while MSTF appears to be able to fulfill the role of *INO2* in regulating *FAS* expression in *nmt1-451D* cells, they must not be functionally equivalent since other inositol-responsive genes maintain *INO2*-dependent expression at 30–33°C. For example, deletion of *INO2* causes a loss in *CHO1* expression as acylation of N-myristoylproteins in *nmt1-451D* cells is diminished. Moreover, *nmt1-451D* cells devoid of Ino2p exhibit inositol auxotrophy at 24–33°C, indicating that *INO1* is not expressed at sufficient levels.

The identity of this factor or factors remain(s) unclear. Our findings suggest a working model where Ino4p may have more than one partner (Ino2p and MSTF; Fig. 7). Attempts to demonstrate interactions between each of the known *S.cerevisiae* bHLH proteins and Ino4p have failed to date, except in the case of Ino2p (John Lopes, personal communication). A number of regulatory proteins that do not belong to the bHLH family are known to affect transcriptional activation and repression of inositol-regulated genes. *RAP1*, *ABF1* and *REB1* are required for maximal expression of *FAS1* and *FAS2* (35). *SIN3* is required for maximal repression of *INO1* (36). *UME6* and *DEP1* affect repression and activation of phospholipid biosynthetic genes (37,38). The effect of *nmt1-451D* on their expression has not been defined. None of their protein products has an N-terminal Gly and therefore they cannot be Nmt1p substrates. These factors are unlikely to be the unknown transcriptional activator suggested by our studies: their mutational inactivation generally results in pleiotropic deficiencies in gene activation and repression, rather than the selective effects observed in *nmt1-451D* cells. Identification of the myristoylation-sensitive transcription factor or factors should provide molecular details about how the state of protein N-myristoylation in cells serves to influence expression of inositol-responsive genes, including *INO2* and *INO4*.

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