MOLECULAR AND CELLULAR BIOLOGY, Sept. 2001, p. 5710–5722 Vol. 21, No. 17
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.17.5710–5722.2001

Inhibition of Acetyl Coenzyme A Carboxylase Activity Restores Expression of the *INO1* Gene in a *snf1* Mutant Strain of *Saccharomyces cerevisiae*

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Received 31 January 2001/Returned for modification 14 March 2001/Accepted 5 June 2001

Mutations in the *Saccharomyces cerevisiae SNF1* gene affect a number of cellular processes, including the expression of genes involved in carbon source utilization and phospholipid biosynthesis. To identify targets of the Snf1 kinase that modulate expression of *INO1*, a gene required for an early, rate-limiting step in phospholipid biosynthesis, we performed a genetic selection for suppressors of the inositol auxotrophy of *snf1*Δ strains. We identified mutations in *ACC1* and *FAS1*, two genes important for fatty acid biosynthesis in yeast; *ACC1* encodes acetyl coenzyme A carboxylase (Acc1), and *FAS1* encodes the β subunit of fatty acid synthase. Acc1 was shown previously to be phosphorylated and inactivated by Snf1. Here we show that *snf1*Δ strains with increased Acc1 activity exhibit decreased *INO1* transcription. Strains carrying the *ACC1* suppressor mutation have reduced Acc1 activity in vitro and in vivo, as revealed by enzymatic assays and increased sensitivity to the Acc1-specific inhibitor soraphen A. Moreover, a reduction in Acc1 activity, caused by addition of soraphen A, provision of exogenous fatty acid, or conditional expression of *ACC1*, suppresses the inositol auxotrophy of *snf1*Δ strains. Together, these findings indicate that the inositol auxotrophy of *snf1*Δ strains arises in part from elevated Acc1 activity and that a reduction in this activity restores *INO1* expression in these strains. These results reveal a Snf1-dependent connection between fatty acid production and phospholipid biosynthesis, identify Acc1 as a Snf1 target important for *INO1* transcription, and suggest models in which metabolites that are generated or utilized during fatty acid biosynthesis can significantly influence gene expression in yeast.

Cellular responses to environmental signals are often mediated by protein kinases and phosphatases. In *Saccharomyces cerevisiae*, the Snf1 protein kinase plays a central role in the response to glucose availability. Together with Snf4 and one of three β subunits, Snf1 activates the transcription of glucose-repressed genes under conditions of glucose depletion via the glucose response signal transduction pathway (7, 63). The activity of the Snf1 kinase is itself regulated by the Glc7 phosphatase and its regulatory subunit, Reg1 (36, 48, 61, 77). Snf1 also regulates other events in yeast, including sporulation, glycogen accumulation, peroxisome proliferation, and phospholipid synthesis (8, 67, 69, 75). The mammalian homologue of Snf1, the AMP-activated protein kinase, is activated by environmental conditions that diminish the energy supplies of a cell and raise the AMP-to-ATP ratio (27). Therefore, both the AMP-activated protein kinase and Snf1 have been classified as environmental sensors for eukaryotic cells (26–28). The yeast and mammalian proteins have at least one common substrate, since both can directly phosphorylate and inactivate acetyl coenzyme A (acetyl-CoA) carboxylase (Acc1), the enzyme that catalyzes the rate-limiting step in fatty acid biosynthesis (51, 81).

Snf1 is thought to regulate gene expression by at least two mechanisms. First, Snf1 can directly phosphorylate and alter the activities of gene-specific transcriptional activators and repressors (7). In response to low glucose levels, Snf1 phosphorylates the Mig1 transcriptional repressor, a protein that binds specifically to the promoters of several glucose-repressed genes (70, 76). This event correlates with the translocation of Mig1 from the nucleus to the cytoplasm (16). Second, several observations suggest that Snf1 directly influences the activity of the RNA polymerase II holoenzyme. Genetic selections for extragenic suppressors of a *snf1* mutation identified six *SSN* (suppressors of *snf1*) genes that encode components of the Srb-mediator complex (9, 43, 72). The Srb-mediator complex is associated with the carboxy-terminal repeat domain (CTD) of RNA polymerase II and is involved in the response to transcriptional activators and repressors (6). More recently, Snf1 has been shown to interact physically with some members of the Srb-mediator complex (42). In addition, mutations in *SNF1* and mutations that truncate the CTD cause similar mutant phenotypes, including inositol auxotrophy and defects in galactose-regulated transcription (32, 38, 55, 62).

The inositol auxotrophy of RNA polymerase CTD mutants correlates with a failure to express the *INO1* gene (62). Certain mutants defective in the TATA binding protein (TBP), which is encoded by the *SPT15* gene, or in histone acetylation are also impaired in *INO1* transcription (3, 21, 59). The *INO1* gene encodes inositol 1-phosphate synthase, the enzyme that catalyzes the conversion of glucose 6-phosphate to inositol 1-phosphate (17). In yeast, this reaction is rate limiting for the syn-

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TABLE 1. *Saccharomyces cerevisiae* strains

Strain	Genotype	Source or reference
FY454	*MAT***a** *snf4Δ1 his4-912δ lys2-128δ leu2Δ1 ura3-52*	F. Winston
KY214	*MATα spt15-328 his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*	3
KY231	*MATα spt15-341 his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*	3
PY129	*MAT***a** *snf1Δ10 his3Δ200 leu2Δ1 ura3-52 ade8*	This study
PY130	*MAT***a** *snf1Δ10 ura3-52 ade8*	This study
PY131	*MATα snf1Δ10 his3Δ200 ura3-52 trp1Δ63*	This study
PY132	*MATα snf1Δ10 leu2Δ1 ura3-52*	This study
PY133	*MAT***a** *snf1Δ10 his3Δ200 ura3-52*	This study
PY165	*MAT***a** *his3Δ200 leu2Δ1 ura3-52*	This study
PY188	*MATα ino2Δ::TRP1 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*	This study
PY190	*MATα ino4Δ::LEU2 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*	This study
PY199	*MATα ACC1-794 leu2Δ1 ura3-52 trp1Δ63*	This study
PY731	*MAT***a** *snf1Δ10 sup731 ura3-52 ade8*	This study
PY794	*MAT***a** *snf1Δ10 ACC1-794 his3Δ200 ura3-52*	This study
PY802	*MAT***a** *snf1Δ10 opi1-802 his3Δ200 ura3-52*	This study
PY803	*MAT***a** *snf1Δ10 fas1-803 his3Δ200 ura3-52*	This study
YUG37	*MAT***a** *ura3-52 trp1-63 leu2Δ1::tTA-LEU2*	J. Hegemann
AUY009	*MAT***a** *tetO7-ACC1 ura3-52 trp1-63 leu2Δ1::tTA-LEU2*	This study
ENYFB73-4D	*MATα cat1(snf1)::HIS3 his3 leu2-3, 112 ura3-52 trp1-289 MAL3 SUC3 MAL2-8*	D. Entian
acc1-2150	*MAT***a** *acc1-2150 ade*	50
479-2A	*MATα* $acc1^{cs}$ *his3-11, 15 leu2-3, 112 ura3-1 trp1-1 ade2-1 can1-100*	64
YAXU008-3a	*MATα lys2 leu2 ura3 trp1*	This study
YAXU008-3b	*MATα acc1-2150 snf1::HIS3 his3 leu2 ura3 ade*	This study
YAXU008-3d	*MAT***a** *snf1::HIS3 his3 lys2 leu2 ura3 trp1 ade*	This study
YAXU009-6a	*MAT***a** $acc1^{cs}$ *(479-2A) snf1Δ10 ura3 his3 leu2 trp1 ade2*	This study
YAXU015-1a	*MAT***a** *tetO7-ACC1 snf1Δ10 ura3 his3 leu2Δ1::tTA-LEU2*	This study

thesis of inositol-containing phospholipids when inositol is absent from the growth medium. However, when inositol is present, transcription of the *INO1* gene is repressed more than 10-fold by a mechanism that requires the negative regulatory protein Opi1 (31). Expression of the *INO1* gene requires the Ino2 and Ino4 transcriptional activators (2, 31, 33, 54) that bind to a repeated element, UAS_{INO}, found in the promoters of *INO1* and other genes subject to regulation by inositol (10, 11, 23, 30).

Previous studies involving the isolation of suppressors of the inositol auxotrophy conferred by specific *ino4* and *spt15* alleles resulted in the identification of recessive *reg1* mutations and a dominant allele of *SNF4*, establishing a connection between *INO1* expression and members of the glucose response pathway (57, 67). To identify potential targets of Snf1 that are important for *INO1* transcription, we performed a genetic selection for mutations that suppress the inositol auxotrophy of *snf1*Δ strains. This work uncovered a connection between genes involved in fatty acid synthesis, notably *ACC1* and *FAS1*, and the regulation of *INO1* transcription by Snf1.

MATERIALS AND METHODS

Genetic methods and media. Media used for the experiment depicted in Fig. 1 were essentially as described by Shirra and Arndt (67). All other experiments were performed in defined synthetic media containing (+I) or lacking (−I) 75 μM inositol, prepared as described previously (24). In some cases 10 μM inositol was used (+I10). Where noted, media also contained 0.5 mM palmitoleic acid (C16:1) dispersed in 1% Brij 58 (final concentrations). Thus, the synthetic media used for these studies contained various combinations of inositol (I) and palmitoleic acid (C16:1) and are abbreviated as follows: (i) −I −C16:1, (ii) +I −C16:1, (iii) −I +C16:1, (iv) +I +C16:1, (v) +I10 −C16:1, and (vi) +I10 +C16:1. Soraphen A, a gift of A. Freund (BASF, Ludwigshafen, Germany), was added to the media from a 10-mg/ml stock solution in methanol. The FY, KY, and PY strains, described in Table 1, are congenic with FY2, a derivative of S288C (80).

Isolation of extragenic suppressors of *snf1Δ10*. Five parental strains, PY129 to PY133, of both mating types and with complementing auxotrophies, were used for the selection of *snf1Δ10* suppressors. For each strain, 28 individual colonies were patched to yeast extract-peptone-dextrose (YPD) solid medium and replica plated to medium lacking inositol. Patches were mutagenized with UV radiation of 0 to 1,500 μJoules/cm^2 in a Stratalinker (Stratagene). No more than one Ino^+ colony was purified from each patch to ensure that all suppressor candidates were independently derived. Following purification, 97 Ino^+ suppressor strains were obtained. These strains were mated to *snf1Δ10* parental strains of the opposite mating type to determine if the suppressor mutations were dominant or recessive. Of the 97 suppressor strains, 79 were found to harbor dominant mutations that suppressed the *snf1Δ10* inositol auxotrophy. Genetic crosses followed by tetrad analysis showed that the dominant mutations in three of these strains were tightly linked, and tetrad analysis of crosses with *snf1Δ10* parental strains showed that the suppressor mutations were in a single gene (data not shown). One of these dominant suppressor strains, PY794, was selected for further study (see below). The remaining 18 suppressor strains contained recessive or partially recessive mutations. For 16 of these strains, the Ino^+ phenotype segregated 2:2 in backcrosses with *snf1Δ10* parental strains, indicating that the suppressor mutations affected a single gene. Complementation analysis was complicated by the partially recessive Ino^+ phenotype of many of these strains; however, three strains were found to contain clearly noncomplementing suppressor mutations: PY731, PY802, and PY803.

Cloning of suppressor genes. Because previous work had shown that a mutation in *OPI1* can suppress the inositol auxotrophy of a *snf1Δ10* strain (67), we tested whether a plasmid containing wild-type *OPI1*, pPS31 (67), would complement the suppressor mutations in PY731, PY802, or PY803. pPS31 fully reversed the Ino^+ phenotype of PY802, suggesting that PY802 contains a mutation in *OPI1*. This assignment was confirmed by linkage analysis of a cross between PY802 and an *opi1Δ::HIS3* strain. The Ino^+ phenotype of PY731 was partially reversed by pPS31, and this strain was not selected for further study. pPS31 did not alter the Ino^+ phenotype of PY803.

To facilitate identification of the suppressor mutation in PY803, we tested this strain for additional mutant phenotypes. We found that PY803 was unable to grow on YPD medium containing 15 mM caffeine, and this caffeine sensitivity cosegregated with suppression of *snf1Δ10*. Using a plasmid-based yeast genomic library (67), constructed with the pRS316 vector (68), we cloned the PY803 suppressor gene by complementation of the caffeine and inositol phenotypes. Three complementing plasmids, which contained overlapping sequences from chromosome XI, were isolated. The only open reading frame included on all plasmids was the *FAS1* gene, which encodes one of two subunits of fatty acid synthase. Linkage of the PY803 suppressor mutation to *FAS1* was confirmed by

a cross between PY803 and a strain which contained *URA3* integrated at the *FAS1* locus.

To identify the dominant suppressor in PY794, a plasmid library of PY794 genomic DNA was constructed in pRS316 (68) using a protocol provided by Craig Thompson (74). Upon transformation into a *snf1Δ10* strain, PY133, one plasmid was isolated that conferred an Ino^+ phenotype to the strain. pPS65 contains yeast genomic sequences from chromosome XIV, 666087 to 654165 (numbering is per the *Saccharomyces* Genome Database [http://genome-www.stanford.edu/Saccharomyces]), which includes the gene encoding acetyl-CoA carboxylase, *ACC1*. A plasmid subclone of pPS65, containing *ACC1* sequences as the only complete open reading frame, also suppressed the Ino^- phenotype of PY133 (data not shown). In addition, the presence of a mutation in *ACC1* was supported by results of biochemical assays of Acc1 activity (see Results) and by linkage of the PY794 suppressor mutation to the chromosomal *ACC1* locus (data not shown).

Construction of a conditional *ACC1* allele. A conditional allele of *ACC1* under the control of the doxycycline-regulatable *tetO7* promoter (22) was constructed as follows. A 2,731-bp integration cassette carrying the *tetO7-CYC1* promoter and the *kanMX4* marker was generated by PCR using a plasmid template, which carries the *tetO7-CYC1* hybrid promoter linked to a *kanMX4* marker (J. Hegemann et al., unpublished data). The hybrid primers contained 20 nucleotides homologous to the *loxP-kanMX4-loxP-tetO7* region of the template. In addition, the hybrid primers contained 50 nucleotide extensions homologous either to the region 50 bp upstream of the start codon or to the first 50 bp of the coding region of the *ACC1* gene. The PCR fragment was transformed into strain YUG37 harboring the tetracycline-controlled transactivator gene (tTA) integrated into the *LEU2* locus (Hegemann et al., unpublished data). Transformants were selected based on the kanamycin resistance gene, *kanMX*, on YPD medium containing 200 μg of G418 (Calbiochem)/ml. Correct integration of the *tetO7* promoter was verified by colony PCR. *ACC1* expression was reduced by addition of 2 to 100 μg of doxycycline/ml.

Northern hybridization analysis. Cells were grown at 30°C to a density of 1×10^7 to 2×10^7 cells/ml in the appropriate media and harvested or induced as described in the figure legends (see also Results). Isolation of RNA and Northern analyses were performed as described previously (3). Hybridization probes for *INO1*, *TUB2*, *SPT15*, and *ACC1* were prepared from pJH310 (31), pYST138 (71), pDE32-1 (18), and YEp352-ACC1 (66), respectively, using a nick translation kit (Roche) or PCR.

Phospholipid analysis. Wild-type and *snf1Δ10* cells grown to mid-logarithmic phase in synthetic medium containing 1% Brij 58 and 75 μM inositol were harvested and washed with sterile water. Each strain was used to inoculate four cultures at an optical density at 600 nm (OD_{600}) of ≈0.5 in the following media: −I −C16:1, +I −C16:1, −I +C16:1, and +I +C16:1. The strains were grown for 1 h at 30°C, at which time 10 μCi of $[^{32}P]H_3PO_4$/ml was added to the medium. Following 20 min of labeling, the cells were harvested, suspended in 5% trichloroacetic acid, and placed on ice for 30 min. Lipids were extracted (4), individual phospholipid species were resolved by two-dimensional paper chromatography (73), and phospholipids were quantified by PhosphorImager analysis.

β-galactosidase assays. Strains were transformed to uracil prototrophy with a plasmid (pJH359) bearing an *INO1-CYC1-lacZ* fusion (47). Wild-type and *snf1Δ10* cells grown to mid-logarithmic phase in synthetic medium containing 1% Brij 58 and 75 μM inositol were harvested and washed with sterile water. Each strain was used to inoculate two cultures at an OD_{600} of ≈0.2 in −I −C16:1 medium and −I +C16:1 medium. At various times, aliquots of the cultures were removed and assayed for β-galactosidase activity using the Pierce Chemical Company yeast β-galactosidase assay kit. Units of β-galactosidase activity were calculated with the formula $A_{420} \times 1{,}000/(\text{min} \times \text{ml} \times OD_{600})$.

Acc1 activity determination. Due to the significant background signal in the standard Acc1 activity assay (44), the enzyme was purified from cytosolic fractions by means of biotin-avidin affinity chromatography as follows. Cells were harvested at $4{,}000 \times g$ for 10 min, washed with 0.1 M K-PO_4 buffer (pH 6.5), mixed with breaking buffer (50 mM Tris-HCl, 100 mM NaF, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride [pH 7.5]) and glass beads (0.30-mm diameter) in a ratio of 1:1:1 (wt/vol/wt) and disrupted by four 1-min bursts in a Braun-Melsungen homogenizer under CO_2 cooling. Afterwards, the homogenate was centrifuged at $20{,}000 \times g$ for 20 min. The supernatant (equal amounts of total protein for the various preparations) was loaded onto a preconditioned avidin column (200 μl; Pierce, Inc.), and unbound protein was eluted with 10 ml of phosphate-buffered saline buffer (0.1 M K-PO_4 [pH 7.2], 0.15 M NaCl). Acc1 and other biotin enzymes (e.g., pyruvate carboxylase) were eluted with 4 ml of phosphate-buffered saline buffer containing 2 mM biotin (Pierce). All steps were carried out at 4°C. Activity of enriched acetyl-CoA carboxylase was determined using a photometric assay in a coupled enzymatic reaction as described previously (49) immediately after chromatography. All enzyme measurements were carried out at 24°C.

RESULTS

Isolation of suppressors of the inositol auxotrophy of *snf1Δ10* strains. Previously, in two independent studies, we uncovered a role for members of the glucose response pathway and the Snf1-Snf4 kinase complex in the regulation of *INO1* transcription (57, 67). However, no substrate was identified for the kinase. The study by Shirra and Arndt (67) suggested that the Opi1 regulatory factor might be a target. However, mutant analyses showed that Opi1 could not be the only target relevant to inositol regulation (67). To further analyze the connection between the Snf1 kinase and *INO1* transcription, we performed a genetic selection to identify extragenic suppressors of the inositol auxotrophy of *snf1Δ10* mutant strains (see Materials and Methods for details). We reasoned that we might isolate mutations in other negative regulators of *INO1* transcription, which might be direct targets of the kinase. Standard cloning procedures, followed by linkage tests, indicated that we isolated recessive suppressor mutations in the *OPI1* and *FAS1* genes and a dominant mutation in the *ACC1*gene.

Isolation of an *opi1* mutant in a screen for *snf1Δ* suppressors was expected, as *opi1* mutations had previously been shown to suppress the inositol auxotrophy of *snf1Δ* strains (67). The identification of mutations in *FAS1* and *ACC1* was more surprising. *FAS1* encodes the β subunit of the heteromeric fatty acid synthase enzyme, and *ACC1* encodes acetyl-CoA carboxylase (79). Both enzymes are involved in the synthesis of long-chain fatty acids from acetyl-CoA. Importantly, Acc1, in both yeast and mammals, is known to be a direct target of the Snf1 kinase, and phosphorylation by Snf1 inactivates purified Acc1 in vitro (26, 27, 51, 81).

The *snf1Δ* suppressors restore *INO1* transcription. To determine whether the suppressor mutations act at the level of *INO1* transcription, Northern analysis on the parental and double-mutant strains was performed (Fig. 1). Compared to a wild-type strain, the *snf1Δ10* strain showed a 3.5-fold-lower level of *INO1* transcription under the derepressing conditions used here (Fig. 1, compare lanes 2 and 4). The level of *INO1* mRNA in the wild-type strain is probably underestimated, because wild-type strains reach growth saturation during the induction and *INO1* is repressed in stationary phase (37). All three suppressor mutations, *ACC1-794*, *opi1-802*, and *fas1-803*, conferred a high level of *INO1* transcription in strains containing the *snf1Δ* mutation (Fig. 1, lanes 6, 8, and 10). As expected from previous studies on *OPI1*, the *opi1-802* strain transcribed *INO1* even in the presence of high levels of inositol (Fig. 1, lane 7). Therefore, the suppressor mutations restore the ability of *snf1Δ10* strains to grow on medium lacking inositol by increasing *INO1* transcription.

Suppression by *ACC1-794* is specific to mutations in *SNF1* and *SNF4*. To determine if the suppression of *snf1Δ10* by a mutation in *ACC1* is specific to the Snf1 kinase pathway, we investigated whether *ACC1-794* could also suppress the inositol auxotrophy caused by other mutations. We chose to examine two mutations in the *SPT15* gene, which encodes the general transcription factor TBP. The inositol auxotrophy conferred by these mutations, *spt15-328* and *spt15-341*, was previ-

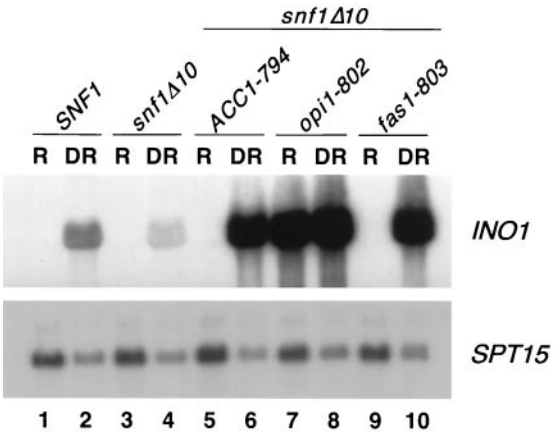

FIG. 1. Suppressor mutations significantly increase transcription of *INO1* in strains containing *snf1Δ10.* Northern analysis of *INO1* transcription is shown. Repressed RNA samples (R) were obtained from cells grown in −I media supplemented with 200 μM inositol. Derepressed RNA samples (DR) were obtained from cells that were grown in 200 μM inositol media, washed, resuspended in −I media supplemented with 10 μM inositol, and harvested after incubation at 30°C for an additional 10 h. Strains used were as follows: PY165 (lanes 1 and 2), PY133 (lanes 3 and 4), PY794 (lanes 5 and 6), PY802 (lanes 7 and 8), and PY803 (lanes 9 and 10). The filter from the upper panel was reprobed for *SPT15* mRNA as a control. A representative experiment is shown.

ously shown to be suppressed by a dominant mutation in *SNF4*, *SNF4-204,* which enhances the physical interaction between Snf1 and Snf4 (67). We also examined null mutations that remove the transcriptional activators of *INO1*, Ino2, and Ino4 (2, 31, 33, 54). We transformed the strains with a plasmid containing the dominant *ACC1* suppressor mutation. As a control, we also tested suppression by the dominant mutation, *SNF4-204*. Table 2 shows that *ACC1-794* only suppressed mutations in the Snf1-Snf4 pathway, suggesting that the suppression mechanism is specific to Snf1 and requires additional signals supplied by TBP and the Ino2 and Ino4 transcription factors. Furthermore, the strong suppression of the TBP mutants by *SNF4-204* suggests an additional role for the Snf1 kinase in *INO1* transcription that is independent of its function as an inhibitor of Acc1.

Suppression of the *snf1Δ* Ino$^-$ phenotype by *ACC1* mutations is allele specific. Mutants with defects in Acc1 have been isolated in genetic screens involving diverse phenotypes. While the *ACC1-794* allele reported here was isolated as a suppressor of the Ino$^-$ phenotype of a *snf1Δ* mutant, the recessive, cold-sensitive allele, *acc1*^{cs}, was identified in a screen for mutations that are synthetically lethal with the hyperrecombination mutant, *hpr1* (64). In addition, a temperature-sensitive allele of *ACC1*, *mtr7*, was isolated as a mutation affecting mRNA transport out of the nucleus (66), and *acc1-2150* is a conditional fatty acid auxotroph (50).

To test the allele specificity of *snf1Δ* suppression by *acc1* alleles, double mutants were constructed by standard genetic crosses. As shown in Fig. 2, the cold-sensitive, recessive *acc1*^{cs} allele, like the dominant *ACC1-794* allele, suppressed the inositol auxotrophy of *snf1Δ* mutants. However, the *acc1-2150* (Fig. 2) and *mtr7* alleles (data not shown) did not suppress the *snf1Δ* phenotype. Therefore, *acc1* mutations suppress the inositol auxotrophy of *snf1Δ* mutants in an allele-specific fashion.

Suppression of the inositol auxotrophy of the *snf1Δ10* mutant results from inactivation of acetyl-CoA carboxylase. The observation that a recessive, loss-of-function mutation such as *acc1*^{cs} could suppress the *snf1Δ10* mutation suggested that the mechanism of suppression might involve inactivation of acetyl-CoA carboxylase activity, although the *ACC1-794* suppression phenotype is dominant. To determine whether inactivation of Acc1 correlates with suppression of the inositol auxotrophy of the *snf1Δ* mutant, we employed soraphen A, a potent inhibitor of Acc1 activity (78). Cells with elevated Acc1 activity are more resistant to this compound than wild-type cells, while cells with lowered Acc1 activity are more sensitive (64, 65). Relative to a wild-type strain, a *snf1Δ* strain was significantly more resistant to soraphen A, suggesting an increase in Acc1 activity in the mutant strain (Fig. 3A). The *ACC1-794* strain was even more sensitive to soraphen A than the wild type, indicating reduced Acc1 activity, while growth of the *fas1-803* mutant was comparable to that of the wild type. The *ACC1-794 snf1Δ* double-mutant strain exhibited a sensitivity to soraphen A that was intermediate to those of strains containing either single mutation. The *fas1-803 snf1Δ10* strain, on the other hand, exhibited a sensitivity to the drug that was comparable to that of the *snf1Δ* parent.

Addition of soraphen A to plates lacking inositol partially reversed the Ino$^-$ phenotype of *snf1Δ* strains (Fig. 3B). Since soraphen A is highly specific for Acc1, these findings suggest that a reduction in Acc1 activity suppresses the inositol auxotrophy of *snf1Δ* strains. On plates lacking inositol, the presence of the *fas1* mutation presumably results in reduced production of fatty acids, rendering the cells unable to grow if the flux through the fatty acid synthesis pathway is further reduced by inhibiting Acc1 (Fig. 3B).

To further test the correlation between acetyl-CoA carboxylase activity and *snf1Δ* suppression, a conditional allele of *ACC1* was constructed by replacing the endogenous promoter with the regulatable *tetO7* promoter (see Materials and Methods) (22). In this construct, *ACC1* expression levels can be modulated by the addition of doxycycline, which interacts with the expression-activation system and results in repression. Acc1 is an essential enzyme, and addition of 50 μg of doxycycline/ml to YPD media completely abolished growth of haploid

TABLE 2. Specificity of suppression by *ACC1-794*

Relevant genotype[a]	Growth with[b]:		
	Vector	*ACC1-794*[c]	*SNF4-204*[d]
snf1Δ10	−	+	−
snf4Δ1	−	+	+
spt15-328	−	−/+	+
spt15-341	−	−−/+	+
ino2Δ	−	−	−
ino4Δ	−	−	−

[a] The following yeast strains were tested: PY133, FY454, KY214, KY231, PY188, and PY190.
[b] Symbols indicate relative growth on solid media lacking inositol after 3 days at 30°C. Strong, weak, very weak, and no growth are indicated by +, −/+, −−/+, and −, respectively.
[c] pPS65 contains *ACC1-794* sequences (see Materials and Methods).
[d] pPS47 contains *SNF4-204* sequences (67).

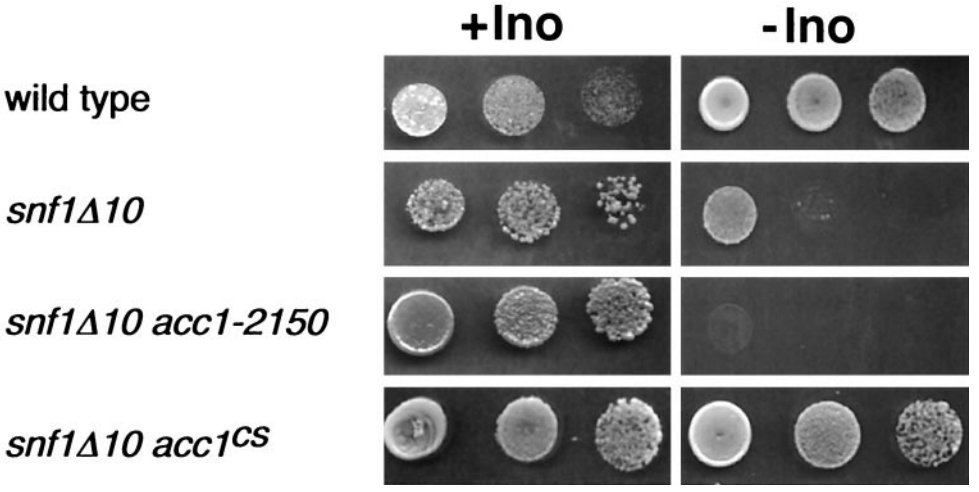

FIG. 2. Suppression of the *snf1Δ10* Ino$^-$ phenotype by *ACC1* mutations is allele specific. Strains grown overnight in inositol-containing medium were harvested, washed, normalized by OD_{600}, and spotted onto plates in a series of three 10-fold dilutions. All media contained 1% Brij 58 and either contained (+Ino) or lacked (−Ino) 75 μM inositol. Plates were allowed to grow for 3 days at 30°C. Strains used were as follows: YAXU008-3a, YAXU008-3d, YAXU008-3b, and YAXU009-6a.

strains harboring the *tetO7-ACC1* allele (data not shown). By addition of limiting amounts of doxycycline (2 μg/ml), the Ino$^-$ phenotype conferred by *snf1Δ* was suppressed on plates lacking inositol (Fig. 3C). Taken together, these in vivo results strongly suggest that the inositol auxotrophy of *snf1Δ* strains arises, in part, from an elevation in Acc1 enzyme activity and that a reduction in Acc1 activity, caused by mutation, drug inactivation, or conditional expression, suppresses this phenotype.

Supplementation with fatty acid suppresses the inositol auxotrophy and *INO1* transcriptional defect of *snf1Δ* strains. In mammalian cells, fatty acyl-CoAs are known to inhibit Acc1 activity (53, 56), and in *S. cerevisiae* the addition of exogenous fatty acids to the medium inhibits both Acc1 and fatty acid synthase activity (12) through a mechanism that appears to require acyl-CoA synthase activity (19, 39). Therefore, we asked whether addition of exogenous fatty acids to medium lacking inositol would restore growth of a *snf1Δ10* strain. As shown in Fig. 4, supplementation of medium with 0.5 mM palmitoleic acid (C16:1) allowed growth of the *snf1Δ* mutant strain (lower left panel). In medium that contained C16:1 and lacked inositol, the *snf1Δ10* strain grew similarly to the *snf1Δ10 ACC1-794* strain in the absence of inositol (Fig. 4, lower panels). However, the *snf1Δ* strain exhibited a slightly longer lag time and did not grow to as high a density in −I +C16:1 medium as in +I −C16:1 medium (Fig. 4, lower left panel).

To test whether the growth effect of fatty acid supplementation correlated with an increase in *INO1* transcription in *snf1Δ* strains, Northern analysis was performed. As shown in Fig. 5, addition of palmitoleic acid (C16:1) to medium lacking inositol restored *INO1* transcription in *snf1Δ10* strains (lane 8). Addition of C16:1 to the growth medium of the *snf1Δ10 ACC1-794* strain also increased *INO1* transcription (Fig. 5, lanes 10 and 11). However, this higher level of *INO1* transcription was no greater than that seen in strains containing *ACC1-794* alone, in the presence or absence of exogenous C16:1 (Fig. 5, lanes 12 and 15).

To analyze the kinetics of *INO1* derepression in *snf1Δ* and wild-type strains under conditions of fatty acid supplementation, we employed a strain carrying an *INO1-CYC1-lacZ* fusion. Cells carrying this fusion were grown in inositol-containing medium (+I −C16:1), shifted to inositol-free medium, and harvested for β-galactosidase assays. As expected, the *snf1Δ10* mutant strain was unable to derepress *INO1-CYC1-lacZ* in inositol-free medium, while the wild-type strain exhibited rapid derepression, as previously reported (58). The *snf1Δ10* mutant, however, was able to induce *INO1* expression in inositol-free medium when palmitoleic acid was supplied (−I +C16:1). However, the kinetics of induction were not as rapid as those observed for the wild-type strain shifted to −I medium, with or without fatty acid. The increase in β-galactosidase expression driven by the *INO1* promoter with the *snf1Δ* strain grown in −I +C16:1 medium paralleled the increase in optical density of the culture (Fig. 6, lower panel). The wild-type strain also exhibited greater *lacZ* induction when transferred to −I +C16:1 medium than when transferred to −I −C16:1 medium. Thus, addition of C16:1 fatty acid appears to result in an increase in *INO1* transcription in both wild-type and *snf1Δ10* cells.

Inhibition of Acc1 enzyme activity correlates with suppression of the *snf1Δ* inositol auxotrophy. To confirm data obtained from our in vivo studies, Acc1 activity and protein levels, as well as *ACC1* steady state mRNA levels, were determined. Total activity of Acc1 isolated from a *snf1Δ10* strain was elevated approximately threefold relative to that of Acc1 isolated from a *SNF1*$^+$ strain (Fig. 7A), and this activity from *snf1Δ10* cells was more resistant to soraphen A in vitro (data not shown). However, *ACC1* mRNA and protein levels (Fig. 7C and data not shown) were lower in the *snf1Δ* mutant strain, suggesting significantly increased specific activity (5- to 7-fold) of acetyl-CoA carboxylase if it remains unphosphorylated by the Snf1 kinase. The level of acetyl-CoA carboxylase activity was lower in the *ACC1-794* mutant (Fig. 7A) despite increased *ACC1* expression (Fig. 7C) and about fourfold-higher levels of Acc1 protein (data not shown). These data suggest that Acc1 activity controls an autoregulatory loop, leading to reduced expression of *ACC1* in a *snf1Δ* strain where Acc1 activity is

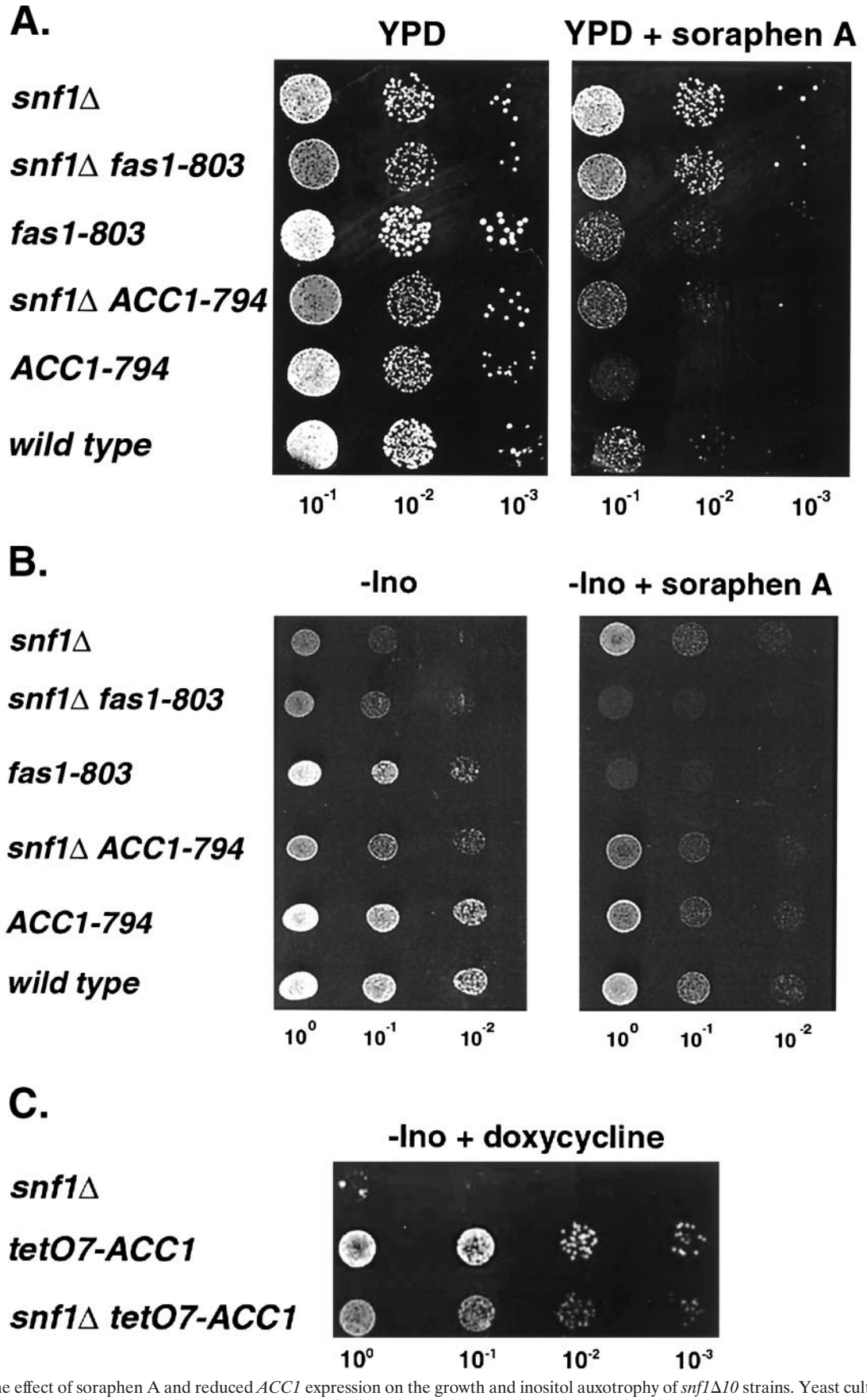

FIG. 3. The effect of soraphen A and reduced *ACC1* expression on the growth and inositol auxotrophy of *snf1Δ10* strains. Yeast cultures, grown overnight in YPD, were diluted in sterile water to the OD_{600} indicated at the bottom of each lane, and 5-μl samples were spotted onto the following media: YPD and YPD plus 0.25 μg of soraphen A/ml (A); -Ino and -Ino plus 0.25 μg of soraphen A/ml (B); and -Ino + 2 μg of doxycycline/ml (C). The following yeast strains were tested: PY133, PY803, PY170, PY794, PY199, PY165, AUY009, and YAXU015-1a.

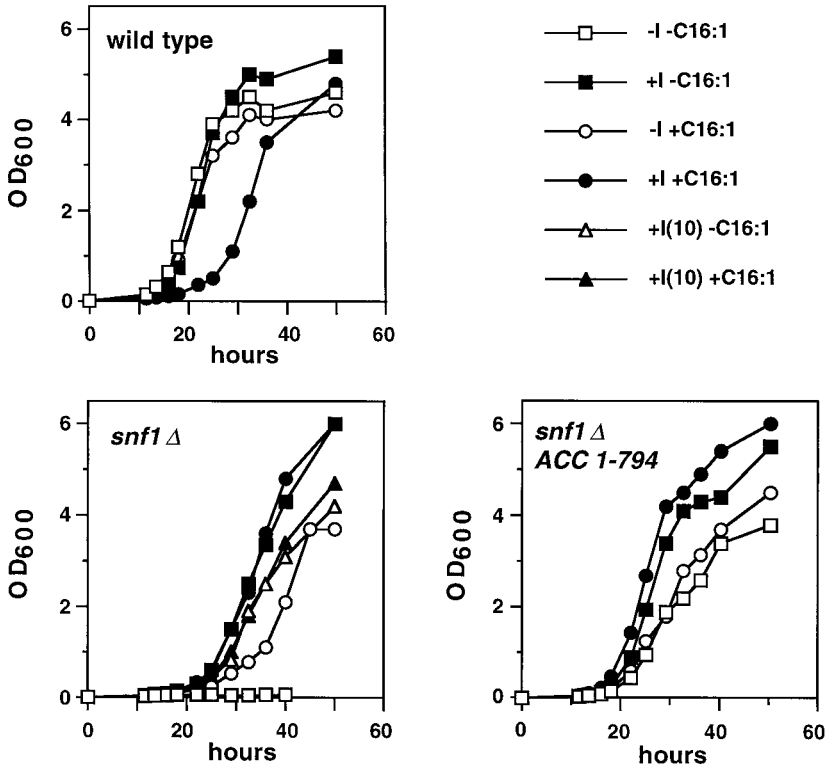

FIG. 4. Palmitoleic acid suppresses the inositol auxotrophy of *snf1Δ10* strains. Strains grown overnight in inositol-containing medium (+I −C16:1) were harvested, washed, and used to start liquid cultures at an OD_{600} of 0.01 for growth at 30°C. All media contained 1% Brij 58 and the indicated combinations of inositol (+I, 75 μM; −I, 0 μM) and palmitoleic acid (+C16:1, 0.5 mM; −C16:1, 0 mM). In the case of the *snf1Δ10* mutant, the media contained 10 μM inositol instead of 0 μM inositol. Strains used were as follows: *SNF1* (PY165), *snf1Δ10* (PY133), *fas1-803* (PY170), *ACC1-794* (PY199), *snf1Δ10 fas1-803* (PY803), and *snf1Δ10 ACC1-794* (PY794).

stimulated by a lack of phosphorylation. Conversely, increased *ACC1* expression is observed in the *ACC1-794* strain, which has impaired enzymatic activity.

Supplementation of the growth medium of the *snf1*Δ strain with fatty acid (C16:1; 100 μM without detergent) resulted in a reduced level of Acc1 activity, from a level threefold higher than that of the wild type to a level 1.4-fold higher than that of the wild type (Fig. 7B). This reduction in Acc1 activity by more than 50% in the *snf1*Δ and wild-type strains grown in the presence of fatty acid was comparable to the relative drop in *ACC1* expression under these conditions (Fig. 7C).

The phospholipid composition of *snf1*Δ cells does not reflect the state of *INO1* expression. Changes in the pattern of phospholipid synthesis have been implicated in the mechanism of *INO1* derepression (30). Wild-type cells grown in media lacking inositol exhibit increased synthesis of phosphatidic acid (PtdOH) and CDP diacylglycerol (CDP-DAG) and decreased synthesis of phosphatidylinositol (PtdIns) compared to the same cells grown in media containing inositol (5, 40). By pulse labeling phospholipids with ^{32}P, we found that both wild-type and *snf1Δ10* strains displayed elevated synthesis of PtdOH and CDP-DAG following transfer to −I media, whether or not C16:1 was present (data not shown). Thus, neither the inositol auxotrophy of *snf1Δ10* strains nor the suppression of this phenotype by C16:1 appears to be correlated to alterations in phospholipid synthesis. These data suggest that Snf1 may act downstream of or independently of the signal produced through phospholipid metabolism to affect *INO1* transcription.

DISCUSSION

Previous selections for extragenic suppressors of *snf1* mutations relied on the inability of *snf1* mutants to derepress glucose-repressible genes such as *SUC2* (9, 43, 72). These studies resulted in the isolation of mutations in components of the

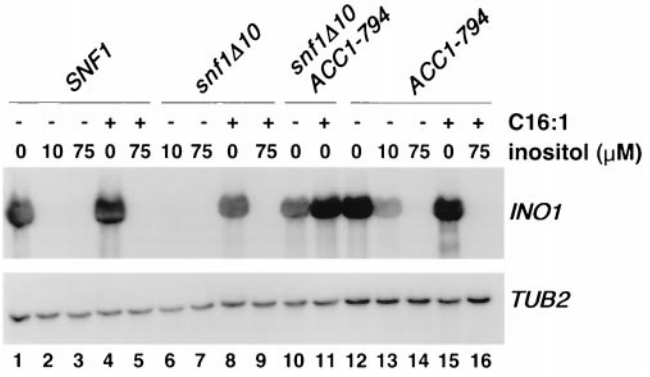

FIG. 5. Fatty acid supplementation supports a high level of *INO1* transcription in *snf1Δ10* strains. Northern analysis of *INO1* transcription is shown. Cells were grown in media containing 1% Brij 58 detergent in the presence or absence of 0.5 mM palmitoleic acid (C16:1) and the indicated concentrations of inositol. Cells were harvested at a cell density of 1×10^7 to 2×10^7 cells/ml. Strains used were as follows: PY165 (lanes 1 to 5), PY133 (lanes 6 to 9), PY794 (lanes 10 to 11), and PY199 (lanes 12 to 16). The filter from the upper panel was reprobed for *TUB2* mRNA as a control. A representative experiment is shown.

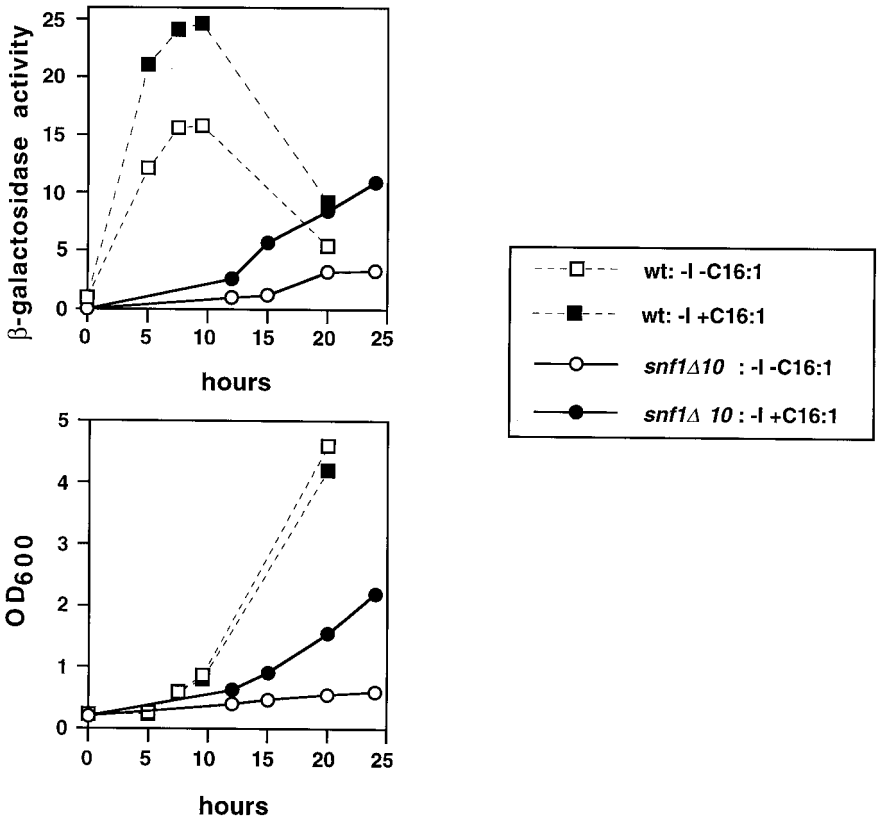

FIG. 6. Effect of palmitoleic acid on the kinetics of *INO1* derepression. Strains bearing the plasmid pJH359 (*INO1-CYC1-lacZ*) (47) were grown to mid-logarithmic phase in synthetic medium containing 1% Brij 58 and 75 μM inositol. Following harvesting and washing, each strain was used to inoculate two different media (−I −C16:1 and −I +C16:1) at an OD_{600} of ≈0.2. At various times, aliquots of the cultures were removed and assayed for β-galactosidase activity. Data represent the averages of results of two independent experiments. Strains used were as follows: *SNF1* (PY165), *snf1Δ10* (PY133), and *snf1Δ10 ACC1-794* (PY794). The apparent decrease in β-galactosidase activity [$A_{420} \times 1{,}000/(\text{min} \times \text{ml} \times OD_{600})$] in the wild-type culture at the 20-h time point is a reflection of the strain's continued growth, once its β-galactosidase activity has reached a plateau level.

Srb-mediator complex associated with RNA polymerase II. In contrast, we selected for suppressors of the inositol auxotrophy conferred by a *snf1*Δ mutation and identified components of the fatty acid biosynthetic pathway. We have shown that the inositol auxotrophy of *snf1*Δ cells, which correlates with decreased expression of the *INO1* gene, is suppressed by mutations in genes encoding acetyl-CoA carboxylase (*ACC1*) and a subunit of fatty acid synthase (*FAS1*) as well as by provision of exogenous fatty acid. The mutants isolated in the present study define a role in yeast for fatty acid biosynthesis in metabolic signaling and a role for the Snf1 kinase in controlling lipid metabolism. Moreover, we have identified acetyl-CoA carboxylase as a target of the Snf1 kinase that is relevant to transcriptional regulation of phospholipid biosynthesis.

Snf1 is necessary for expression but not regulation of *INO1*. Analysis of the pattern of *INO1* expression in diverse genetic backgrounds including mutants with defects in phospholipid metabolism supports the hypothesis that PtdOH, or a closely related lipid, generates a signal that results in derepression of UAS_{INO}-containing genes such as *INO1* (10, 30). Since fatty acids are immediate precursors of PtdOH (Fig. 8) and we have identified a role for fatty acid metabolism as a target for Snf1 signaling, we considered the possibility that Snf1 might transmit the inositol-sensitive signal controlling *INO1* expression. However, two lines of evidence, presented here, suggest that this is not the case. First, the response to inositol deprivation is believed to be initiated by a shift in the pattern of phospholipid metabolism that includes increased accumulation of PtdOH and CDP-DAG and decreased synthesis of PtdIns (40). However, the *snf1*Δ mutant exhibited a pattern of phospholipid synthesis comparable to that of wild-type cells when shifted to inositol-free medium, and this pattern was unaffected by the addition of fatty acid. Second, when *INO1* expression is restored in *snf1*Δ cells by provision of fatty acid (16:1) or by introduction of the *ACC1-794* or *fas1-803* mutations, regulation in response to inositol is also restored. Thus, an active Snf1 kinase is not needed to transmit the inositol-sensitive signal, and, furthermore, the presence or absence of an active *SNF1* gene product does not seem to influence the pattern of phospholipid synthesis that is believed to be involved in the signaling. Thus, the Snf1 kinase appears to affect the overall level of *INO1* expression rather than its regulation in response to inositol or phospholipid metabolism.

Since enzymes involved in fatty acid biosynthesis clearly play a role in Snf1-dependent transcription of *INO1*, we considered whether *INO1* expression might correlate with fatty acid composition. The *snf1*Δ mutant had a slightly lower proportion of C16:0 fatty acids than the wild type, but so did the *fas1* mutant, whether or not the *snf1* mutation was present (unpublished observations). Cells carrying the *ACC1-794* mutation, like pre-

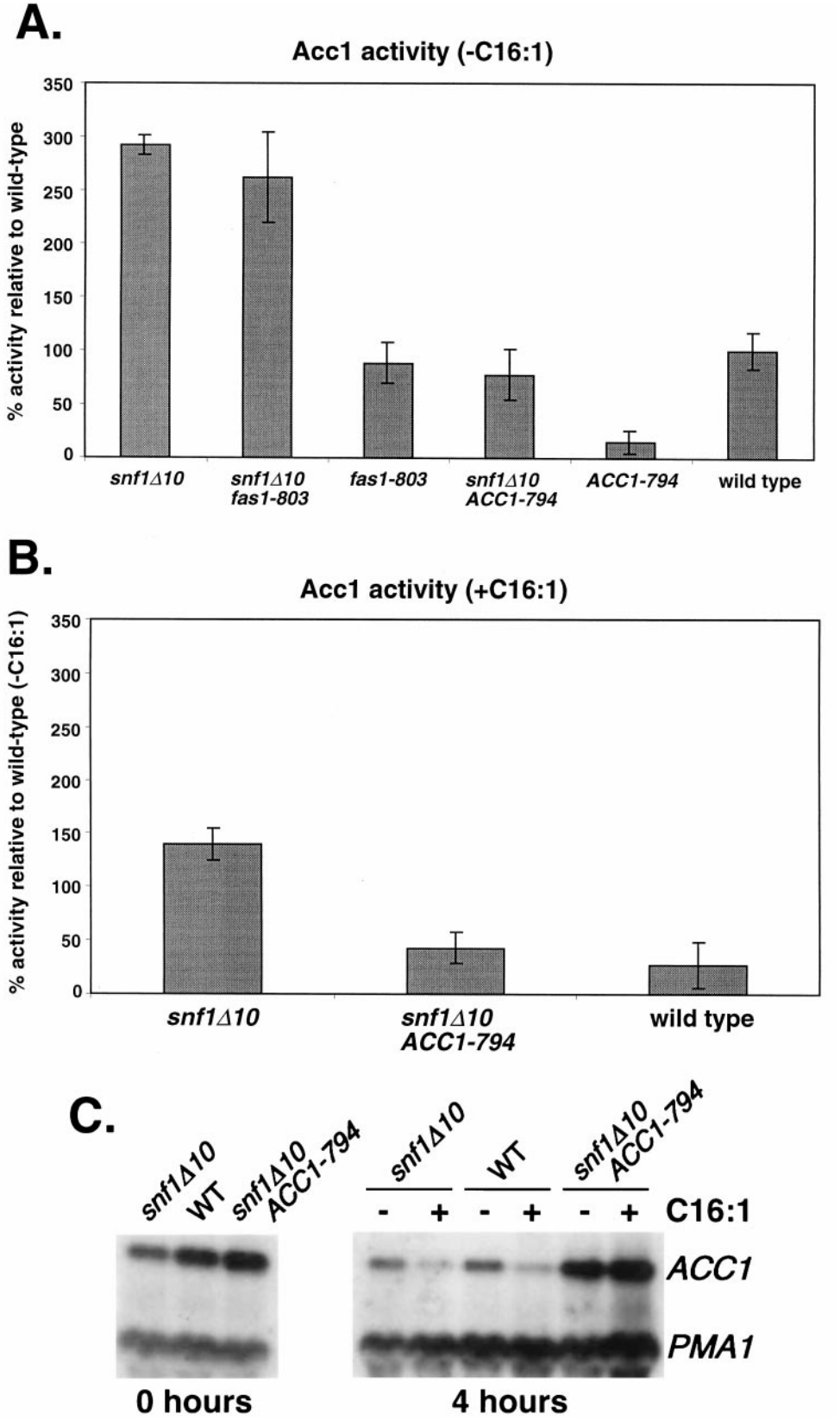

FIG. 7. Acc1 enzyme activity and *ACC1* expression in the absence and presence of exogenous palmitoleic acid. (A and B) Acc1 enzyme activity was determined as described in Materials and Methods. The activity was determined three to four times and normalized to the protein concentration in the homogenate, and it is depicted as specific activity relative to activity of a wild-type strain grown in the absence of exogenous C16:1 (set at 100%). For the experiment depicted in panel B, C16:1 was added to the growth media to a final concentration of 100 μM without detergent (detergent was found to interfere with the enzyme preparation and resulted in a loss of Acc1 activity). (C) Northern analysis of *ACC1* expression. Total RNA was prepared 0 and 4 h after addition of C16:1 (100 μM, where indicated), separated on denaturing agarose gels, blotted, and hybridized with digoxigenin-labeled *ACC1* and *PMA1* probes. Strains used were as follows: PY133, PY803, PY170, PY794, PY199, and PY165.

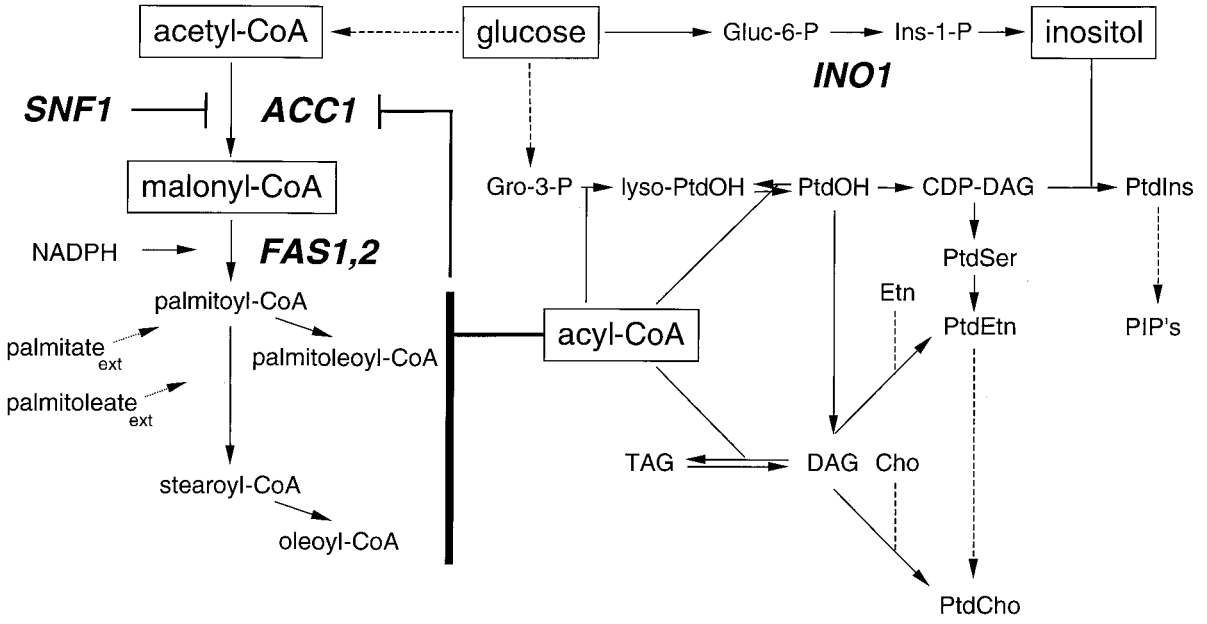

FIG. 8. Schematic diagram of phospholipid biosynthesis in *S. cerevisiae.* Solid arrows indicate direct enzymatic conversions. Dashed arrows indicate conversions that require more than one enzymatic step. Gene designations are in bold italics. Phosphorylation of Acc1 by the *SNF1* gene product inhibits Acc1 activity. Acyl-CoAs, including malonyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA, and oleoyl-CoA, inhibit Acc1 activity. Externally added palmitate (palmitate$_{ext}$) and palmitoleate (palmitoleate$_{ext}$) are converted to their respective CoA derivatives in the cell. Lyso-PtdOH, lysophosphatidic acid; Gro-3-P, glycerol-3-phosphate; Gluc-6-P, glucose-6-phosphate; Ins-1-P, inositol-1-phosphate; PtdOH, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; Etn, ethanolamine; Cho, choline; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PIP's, polyphosphoinositides.

viously described *acc1* mutants (65, 66), exhibited an increased proportion of 16 carbon fatty acids, whether or not the *snf1* mutation was also present. Thus, there was no clear correlation between the cellular fatty acid composition and the ability to express *INO1* in the *snf1* genetic background, which, however, does not exclude potential effects of specifically localized altered lipid species.

Identification of Acc1 as a Snf1 substrate important for *INO1* expression. Initially, it seemed paradoxical that mutations that presumably reduce the rate of fatty acid biosynthesis and provision of exogenous fatty acid had similar effects: namely, suppression of the inositol auxotrophy of *snf1*Δ cells. However, this apparent paradox was resolved with the recognition of a correlation between *INO1* expression and total cellular activity of acetyl-CoA carboxylase. Our first indication of such a relationship came from the growth properties of various yeast strains in the presence of soraphen A, a drug known to inhibit Acc1 specifically (78). As expected, *snf1*Δ cells, previously reported to have high levels of Acc1 activity (81), were more resistant to soraphen A than wild-type cells. The *ACC1-794* mutation increased the soraphen A sensitivity of both *SNF1*$^+$ and *snf1*Δ cells. Acc1 activity levels predicted by these phenotypic results were confirmed by enzyme assays.

In yeast, Acc1 is known to be inactivated by the Snf1 kinase (51, 81), and a similar regulatory relationship exists in mammalian cells (15, 52). In the *snf1*Δ *ACC1-794* double mutant strain, the absence of Snf1-dependent phosphorylation of Acc1 increases the activity of the Acc1-794 mutant enzyme to a level comparable to that of the wild type. The *ACC1-794* allele clearly reduces Acc1 function despite its apparent dominance as a suppressor of the *snf1*Δ Ino$^-$ phenotype. We propose, therefore, that suppression of the *snf1*Δ inositol auxotrophy is due to a partial loss of Acc1 function. In support of this conclusion, we have shown that the *acc1*^{cs} allele, another partial loss-of-function mutation, and reduced expression of *ACC1* from a doxycycline-repressible promoter also suppress the *snf1*Δ inositol auxotrophy.

The provision of exogenous fatty acids also lowers Acc1 activity in wild-type and *snf1*Δ cells, which is at least in part due to repression of *ACC1* expression. Acyl-CoA, the end product of fatty acid synthesis, is known to inhibit mammalian acetyl-CoA carboxylase activity in vitro (53, 56). Kamiryo et al. reported that exogenous fatty acid caused a reduction of Acc1 activity in yeast and, furthermore, that activation of exogenous fatty acid to acyl-CoA was necessary for the reduction in Acc1 activity (39). We found that the level of Acc1 activity in *snf1*Δ cells grown in the presence of exogenous 16:1 fatty acid is reduced to a level almost comparable to that for the wild-type strain grown in the absence of added fatty acid. Under these conditions, *INO1* is expressed. Indeed, in each case (the presence of the *ACC1-794* mutation or the provision of fatty acid), the ability of cells to express *INO1* is correlated with a reduction of Acc1 activity to a level comparable to or lower than that found in wild-type cells grown in the absence of exogenous fatty acid.

The nature of the Snf1-dependent signal controlling *INO1* expression. Our analysis of the *fas1-803* suppressor mutation presents a potential contradiction to the hypothesis that the ability to express *INO1* is correlated with Acc1 activity. The *fas1-803* strain exhibited Acc1 activity levels comparable to wild-type levels in vitro, despite the ability of the *fas1-803* mutation to suppress the Ino$^-$ phenotype conferred by *snf1*Δ*10*.

Consistent with this observation, the *fas1* mutant does not appear to have increased soraphen A sensitivity. The *fas1-803 snf1Δ10* mutant exhibits resistance to the drug that is, at best, only slightly reduced compared to that of the *snf1Δ10* strain.

These observations raise the possibility that the actual basis of suppression may not be reduction of Acc1 enzyme activity, per se, but rather may be related to the overall flux of metabolites through the fatty acid biosynthetic pathway. The step catalyzed by Acc1 is rate limiting for fatty acid biosynthesis in wild-type yeast cells (50, 60) and in mammalian cells (25). In *snf1Δ* cells which exhibit elevated Acc1 activity, a mutated Fas1 subunit might cause the step catalyzed by fatty acid synthase to become rate limiting. Overall, our results support the hypothesis that Acc1 either is directly involved in the mechanism by which Snf1 controls *INO1* expression or exerts its influence by affecting the flux of metabolites through the fatty acid biosynthetic pathway. Interestingly, *ACC1* expression itself is repressed by inositol in the growth medium through a regulatory circuit that involves the Ino2 and Ino4 transcription factors as well as Opi1, all of which also control *INO1* regulation (13, 29).

We favor the idea that the level of a metabolite(s) produced or utilized in fatty acid biosynthesis, an energy-demanding process, is responsible for generating a signal which affects *INO1* transcription. High levels of energy-rich metabolites may favor *INO1* transcription, while low levels may tend to repress *INO1* expression. Recent reports demonstrating that certain histone deacetylases, including those encoded by *SIR2* and its homologues (35, 45), require NAD^+ suggest the possibility that metabolic factors affecting the NAD^+ levels may globally affect patterns of gene expression through influencing chromatin structure. Malonyl-CoA levels might also serve as a metabolic sensor, as they have been postulated to do in mammalian cells, possibly through inhibition of Acc1 or by triggering other metabolic signals that may influence cellular energy levels and affect chromatin modification. Interestingly, a link between Acc1 activity and expression of another gene, *PHO5*, has been reported (46). In this case, constitutive *PHO5* expression was observed in several *acc1* mutant strains, and the authors also concluded that a metabolite(s) of fatty acid biosynthesis might serve as a signaling molecule for transcriptional regulation of this gene (46).

Acetyl-CoA, another metabolite that could potentially affect *INO1* expression, serves as a substrate for both fatty acid biosynthesis and histone acetylation. Since acetyl-CoA carboxylase uses acetyl-CoA directly as a substrate, high levels of Acc1 activity might deplete the pools of acetyl-CoA normally reserved for histone acetylation. Transcription of the *INO1* gene is known to be sensitive to mutations that affect histone acetyltransferase and histone deacetylase complexes. Mutations in *SIN3*, which encodes a component of the Sin3-Rpd3 histone deacetylase complex, lead to high levels of *INO1* expression (34). In contrast, mutations that remove certain components of the SAGA histone acetyltransferase cause inositol auxotrophy and a severe defect in *INO1* activation (21, 59). Because histone acetylation is required for the recruitment of certain transcriptional activators (14, 41) and ultimately for the recruitment of TBP (1) to promoters, metabolic changes that influence this process could lead to dramatic effects on gene regulation. Recently, acetyl-CoA has been shown to stimulate promoter binding by TFIID in vitro, suggesting another mechanism by which Acc1 may regulate transcription (20). Significantly, we initially uncovered a role for the Snf1 kinase pathway in *INO1* transcription by searching for suppressors of a mutant Ino4 activator protein and a DNA binding-defective TBP (57, 67). Continued genetic and biochemical studies will help elucidate how the Snf1 kinase pathway and additional signal transduction cascades control chromatin modification or other events that culminate in activation of *INO1* transcription.

ACKNOWLEDGMENTS

The first three authors contributed equally to this work.

We thank A. Tartakoff, H. Klein, E. Schweizer, J. Hegemann, F. Winston, and D. Entian for strains and plasmids; A. Jandrositz and G. Gogg for constructing the *tetO-ACC1* strain and G. Gogg for fatty acid analyses and support with enzyme preparations; A. Freund (BASF) for the gift of soraphen A; and K. Roinick for technical assistance.

This work was supported by grants from the National Institutes of Health to K.M.A. (GM52593 and AI01816) and S.A.H. (GM19629) and the Austrian Science Fund, FWF (F706), to S.D.K.

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