In *Saccharomyces cerevisiae* the transition between the fermentative and the oxidative metabolism, called the diauxic shift, is associated with major changes in gene expression. In this study, we characterized a novel family of five genes whose expression is induced during the diauxic shift. These genes, *FET3*, *FTR1*, *TIS11*, *SIT1*, and *FIT2*, are involved in the iron uptake pathway. We showed that their induction at the diauxic shift is positively controlled by the Snf1/Snf4 kinase pathway. The transcriptional factor Aft1p, which is known to control their induction in response to iron limitation, is also required for their induction during the diauxic shift. The increase of the extracellular iron concentration does not affect this induction, indicating that glucose exhaustion by itself would be the signal. The possibility that the Snf1/Snf4 pathway was also involved in the induction of the same set of genes in response to iron starvation was considered. We demonstrate here that this is not the case. Thus, the two signals, glucose exhaustion and iron starvation, use two independent pathways to activate the same set of genes through the Aft1p transcriptional factor.

Eukaryotic cells develop many processes to adapt to environmental changes. The yeast *Saccharomyces cerevisiae* is able to grow upon several carbon sources, among which glucose remains the favorite one. The use of glucose by fermentation leads to the production of ethanol in the medium. Then ethanol can be used after an adaptive process known as the diauxic shift, which is a transition from fermentative growth to respiratory growth. At this stage cells stop to divide, and the enzymatic equipment is modified to allow assimilation of carbon and production of energy from ethanol. This process involves a global change of gene expression. These changes have been monitored at the levels of proteome (1–3) and transcriptome (4). As glucose is depleted, transcription of genes involved in gluconeogenesis, the tricarboxylic acid cycle, the glyoxylate cycle, and respiration is enhanced, whereas expression of genes involved in glycolysis and protein synthesis is decreased.

The extent of these changes raises the question of which regulatory mechanisms lead to a coordinated expression of the genome in response to glucose depletion. The problem can be addressed by investigating the involvement of a particular transcription factor. Another possible method can be the alteration of transduction systems that transfer the signal of glucose starvation to the transcription factors. In both cases identification of up- and down-regulated genes can be achieved by using global approaches.

In this way the role of the transcriptional activator Cat8p during the diauxic shift has been defined by the identification of its target genes (5). It appears that although this factor is essential for growth on ethanol as the carbon source, only a limited part of the genes induced during the diauxic transition belongs to the Cat8p regulon.

Cat8p is involved in the Snf1p-dependent pathway that is required in the response to glucose starvation. The protein kinase Snf1p is the catalytic subunit of a complex containing a regulator subunit Snf4p and a third element that can be Gal83p, Sip1p, or Sip2p. This heterotrimeric complex is the homologue of the mammalian AMP-activated protein kinase (for review, see Ref. 6). Like its mammalian homologue, Snf1p is activated in response to glucose limitation, a process that requires its own phosphorylation by an upstream kinase and its interaction with the regulatory subunit Snf4p (7). The activated form of Snf1p phosphorylates target proteins including transcriptional activators, such as Cat8p and Sip4p, and the repressor Mig1p (8).

The aim of the present study was to characterize new functions of the Snf1p-dependent pathway in the transcriptional changes occurring at the diauxic shift. We compared the transcriptional profiles of wild type and *snf4Δ* strains at the time of glucose exhaustion by using a genome-wide scan. We showed that genes involved in iron uptake are subject to a positive control by the Snf1p pathway in response to glucose starvation. This induction requires the transcriptional activator Aft1p. The results presented here suggest a link between the regulation of iron uptake and utilization and the adaptation to the respiratory growth during the diauxic transition.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—The wild type strains *S. cerevisiae* FY5 (*MATa*, *mal*) and FY2 (*MATa*, *mal*, *ura3–52*) derived from S288C were kindly provided by F. Winston (Harvard Medical School). The *SNF4*, *CAT8*, *SNF1*, and *AFT1* deletions were constructed in the wild type strains FY5 or FY2 by replacing the gene with the *kanMX4* cassette (9). The different yeast strains obtained are named YP3 (MATa, mal, *cat8Δ::KanMX4*), YP4 (MATa, mal, *snf4Δ::KanMX4*), YP11 (MATa, mal, *snf1Δ::KanMX4*), YP14 (MATa, mal, *ura3–52, snf4Δ::KanMX4*), YP17 (MATa, mal, *aft1Δ::KanMX4*), and YP31 (MATa, mal, *ura3–52, snf1Δ::KanMX4*). The single chromosomal integration of *KanMX4* at these loci was controlled by PCR or Southern blot analyses. The strains expressing Aft1p-HA* were created by the transformation of pRS416-AFT1-HA, generously supplied by Y. Yamaguchi-Iwai (10), to FY2, YP14, and YP31.

Cultures were performed at 22 °C in a 500-ml Erlenmeyer flask containing 50 ml of supplemented minimal medium YNBS (0.17% yeast nitrogen base without ammonium sulfate and amino acids, 0.5% ammonium sulfate, 2% glucose, 25 μg/ml inositol, 85 mM succinate/NaOH, 0.5% glucose). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviation used is: HA, hemagglutinin.
pH 5.8) or complete medium YM1 (0.5% yeast extract, 0.17% yeast nitrogen base without ammonium sulfate and amino acids, 2% glucose, 1% Bacto-peptone, 0.5% ammonium sulfate, 85 mM succinate/NaOH, pH 5.8). To test the response to iron limitation, cultures were performed in the same conditions as mentioned above but in rich media YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) supplemented or not with the iron chelator ferrozine to a final concentration of 1 mM. Cultures were shaken at 300 rpm, and growth was monitored by measuring their absorbance at 600 nm (an A_{50} of 1 corresponds to 2.4 × 10^{10} cells/ml).

Glucose Measurement—Glucose measurements were performed with a Sigma Diagnostic kit (catalog number 510-A).

**RNA Isolation**—Total RNA was extracted as previously described (11).

**Mini-array Filter Hybridization**—Mini-array analyses were performed using genomic filters (Research Genetics) as described (5). Two independent experiments of comparison were performed with a single set of membranes. Between hybridizations, the efficiency of stripping (≥95%) was checked by scanning the filters. To allow comparison between separate filter hybridizations, the intensity of each spot was normalized to the total hybridization signal intensity. A gene was considered as potentially Snf4p-dependent when the spot displayed at least a 2-fold difference between the mutant and the wild type strains in the two experiments.

**Northern Blot Hybridization**—15 μg of each RNA preparation was electrophoresed under denaturing conditions (12) and blotted onto nylon membranes (13). Hybridization probes used were PCR-amplified with appropriate primers (sequences available upon request). Hybridization signals were detected using a Molecular Dynamics Phosphor-Imager, and quantification was achieved with the ImageQuant software (Molecular Dynamics).

**Preparation of Protein Extracts and Immunoblot Analysis**—Proteins extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14). Samples of 2.4 × 10^6 cells were harvested by centrifugation, rinsed with water, and then extraction was performed as previously described (14).

**RESULTS**

**Global Pattern of Gene Expression in snf4Δ Strain**—A functional Snf1p-Snf4p complex is required for growth on alternative carbon sources. To identify genes under the control of the Snf1/Snf4 pathway during the diauxic shift, we chose to investigate gene expression of a snf4-deleted strain rather than a snf1-deleted strain. Indeed, under the culture conditions used in our laboratory, i.e. minimum medium containing 2% glucose as carbon source, it was observed that the growth rate and the rate of glucose consumption of the snf4Δ strain were similar to those of the wild type strain during the approach to glucose exhaustion (Fig. 1). In contrast, under the same culture conditions, the snf1Δ mutant strain displayed a strong alteration in growth parameters. The growth rate and the glucose consumption of snf1Δ progressively slowed down before glucose exhaustion, indicating that the physiology of this strain is already altered before the diauxic shift. In a rich medium this phenomenon was reduced but not abolished (not shown).

Message RNAs were obtained from cells harvested 25 min after glucose depletion. Extracts obtained from wild type, and mutant cells were used to synthesize 32P-labeled cDNA hybridized to high density filters.

Fifty open reading frames or genes were found under-expressed at least 2-fold in the mutant strain. As expected, Cat8p target genes were found among these genes. Among the other genes under-expressed in the mutant strain, we found five genes belonging to the iron regulon: **FTR1**, **FET3**, **FIT2**, **SIT1**, and **TIS11**. These five genes are known to be induced under conditions of iron limitation in an Aft1p-dependent manner (16–19). Their defect of expression at the diauxic shift in absence of Snf4p suggested that they could be targets of the Snf1p-dependent pathway and that Aft1p could mediate this response.

**Genes Involved in the Iron Uptake Pathway Are Induced at the Diauxic Shift**—Our transcriptome analysis was performed from extracts of cells harvested at a unique time of growth, 25 min after the glucose exhaustion. Thus, we cannot rule out the possibility that differences between wild type and mutant were present well before the diauxic transition. To answer this question we compared the temporal profiles of miRNA levels in wild type and snf4Δ strains from 30 min before glucose exhaustion...
Snf1p Control during the Diauxic Shift

This Induction Is Due to Glucose Exhaustion—The snf4Δ strain is unable to use ethanol after glucose exhaustion even if we add more iron in the culture medium (data not shown). The defect of expression of genes belonging to the Aft1 regulon in this mutant could be a consequence of its inability to adapt to ethanol utilization. To address this question, expression of FTR1 was monitored in a cat8Δ strain. The CAT8 gene encodes a transcriptional activator essential for the growth on ethanol. In this strain expression of FTR1 is induced at the diauxic shift at the same level than in the wild type strain (data not shown). Thus, it is not the growth defect on ethanol that inhibits the induction of the Aft1 regulon in snf4Δ strain.

The Aft1 regulon is induced under conditions of iron deprivation. The induction of Aft1p target genes observed at the diauxic shift could be due to an iron starvation concomitant with the glucose starvation. Thus, the expression of FTR1 and FET3 was assayed in an identical medium but supplemented with 350 μM of ferrous sulfate. At this concentration, the defect of growth of a aft1Δ strain on ethanol is suppressed (data not shown). Even when adding iron in the medium, the induction of FTR1 and FET3 was still detected when glucose became exhausted. Moreover, the level of induction was the same with or without iron addition (Fig. 4). In summary the induction of Aft1p target genes at the diauxic shift is independent to elevated extracellular iron concentrations and appears to be a response to the glucose limitation occurring at this stage of the culture.

The Induction Requires a Snf1p Functional Protein—The established role of Snf4p is as a positive regulator of the kinase activity of Snf1p. To know if the Snf4p-dependent induction of the Aft1 regulon is also Snf1p-dependent, the mRNA patterns of FTR1 and FET3 in wild type and snf1Δ strains at the diauxic shift were compared (Fig. 5). A rich medium was used because, as we mentioned before, the growth of wild type and snf4Δ strains are almost similar in this medium.

In the wild type cells, the induction of FTR1 and FET3 was observed, and the use of rich medium did not influence the kinetic of this induction. In contrast, in the snf1Δ strain the mRNA levels of these two genes were not enhanced at the time of glucose deprivation. We concluded from this experiment that the protein kinase Snf1p is essential in the Snf4p-dependent pathway, which leads to the induction of the Aft1p target genes at the diauxic transition. Thus, the defect observed initially in a SNF4-deleted strain is caused by a defect in kinase activity of Snf1p.

The Induction Is Aft1-dependent—The five genes FTR1, FET3, FIT2, SIT1, and TIS11 belong to the Aft1 regulon. Aft1p activates transcription under low iron conditions, as its binding...
to a conserved promoter sequence and its subcellular localization are regulated in an iron-dependent manner (10, 16). To test the involvement of Aft1p during the diauxic shift, we evaluated the expression of FTR1 and FET3 in a aft1Δ strain when glucose is exhausted (Fig. 6). FET3 mRNA was not detected in the mutant strain, and the level of induction of FTR1 was strongly reduced compared with the level in the wild type strain. Thus the transcriptional activator Aft1p is required for the induction of these two genes at the diauxic shift, and it is involved in the Snf1/Snf4-dependent pathway.

**Phosphorylation of Aft1p Occurring at the Diauxic Shift Is Snf4p- and Snf1p-independent—Casas et al.** showed that Aft1p was phosphorylated during the diauxic transition. We tried to reproduce this result in our strain and culture conditions. A wild type strain FY2 was transformed with a centromeric plasmid expressing an HA-tagged Aft1p protein. Cells lysates were prepared from samples harvested at different times before and during the diauxic shift. The mobility of Aft1p on SDS-PAGE was examined by Western blotting using an anti-HA antibody. Cells harvested before the glucose exhaustion exhibit mainly one form of Aft1p (Fig. 7). When glucose becomes exhausted, this form disappears, and cells exhibit at least one additional slower-migrating form of Aft1p. When extracts from cells harvested 2 h after glucose exhaustion were treated with alkaline phosphatase in absence of specific inhibitor, this additional form of Aft1p was not detected anymore (Fig. 7C). This result confirmed that the post-translational modification of Aft1p is a phosphorylation event.

Knowing that Snf1p is responsible for the phosphorylation of several transcriptional factors such as Cat8p or Mig1, we can presume that Aft1p could be a direct target of this kinase. We tried to reproduce this result performed with extracts from snf4Δ cells gave an identical result; the same post-translational modification was examined by Western blotting using an anti-HA antibody.

**FIG. 4.** Northern blot assay of the expression of FET3 and FTR1 in wild type strain (FY5) during the diauxic shift. Wild type cells were grown in YNBS medium supplemented (+Fe) or not (-Fe) with 350 μM (Fe(II)SO4). RNAs were extracted at different times during the diauxic shift: 15 min before glucose exhaustion (1), glucose exhaustion (2), 15 min after (3), 30 min after (4), 45 min after (5). The graphics represent relative mRNA levels, normalized to ACT1 mRNA, at each time point.

**FIG. 5.** Northern blot assay of the expression of FET3 and FTR1 in wild type (FY5) and snf1Δ (YP11) strains during the diauxic shift. Cells were grown in complete medium. RNAs were extracted at different times during the diauxic shift: glucose exhaustion (1), 15 min after (2), 30 min after (3), 45 min after (4), 60 min after (5). The graphics represent relative mRNA levels, normalized to ACT1 mRNA, at each time point. wt, wild type.

**FIG. 6.** Northern blot assay of the expression of FET3 and FTR1 in wild type (FY5) and aft1Δ (YP17) strains during the diauxic shift. Cells were grown in YNBS medium. RNAs were extracted at different times during the diauxic shift: glucose exhaustion (1), 15 min after (2), 30 min after (3), 45 min after (4), 60 min after (5). The graphics represent relative mRNA levels, normalized to ACT1 mRNA, at each time point. wt, wild type.
pattern of Aft1p was observed in mutant and in wild type strains (Fig. 7, A and C). Thus, Snf4p is not necessary for the phosphorylation of the protein Aft1p, concomitant with glucose depletion.

This observation suggests that Aft1p is not a substrate of the Snf1p kinase. To verify this hypothesis, we tested the phosphorylation of Aft1p-HA in a snf4Δ strain (Fig. 7B). Of the growth defect of this strain in a minimal medium, we performed this experiment from cells cultured in a rich medium (see above). Despite a difference in the kinetics of appearance of the phosphorylated form of Aft1p-HA according to the glucose concentration between wild type and snf4Δ strains, which could be due to a slowing of glucose consumption in the mutant strain (see above), it appears that Aft1p-HA is still phosphorylated when glucose becomes exhausted, even in absence of the kinase Snf1p.

We can conclude from these results that, first, the phosphorylation of Aft1p happens at the entrance into the diauxic transition, in the same time as the induction of its target genes. Second, neither Snf4p nor Snf1p is required for the phosphorylation of Aft1p. Thus, Aft1p is not a target, even indirectly, of the kinase activity displayed by the complex Snf1p-Snf4p. The nature of the link between Snf1p kinase activity and activation by Aft1p remains to be clarified. And third, because it is observed in the snf1Δ and snf4Δ strains, the phosphorylation event of Aft1p is not sufficient to induce the Aft1 regulon.

The Snf1 Pathway Is Not Involved in the Response to Iron Deprivation—The Aft1p transcriptional factor regulates the iron regulon in response to iron availability. We showed that genes belonging to this iron regulon were induced during the diauxic shift in a Snf1p/Snf4p-dependent manner. This response observed at the time of glucose exhaustion did not change according to the iron status (see above). We tested the reciprocity of this fact, i.e. the involvement of the Snf1p pathway in the regulation of gene expression in response to iron starvation when glucose is available in the medium. To achieve this goal, yeast strains were grown inYPD-rich medium containing glucose. During the exponential growth phase, iron was withdrawn by the addition of the iron chelator ferrozine in the culture medium at a final concentration of 1 mM. When medium was also supplemented with iron, Fe(NH2)(SO4)2 was added at a final concentration of 0.3 mM. Cultures were then performed for 2 h in the usual conditions. At 0 and 2 h after the addition of ferrozine (+ferr) or both ferrozine and ferrous sulfate (+ferr +iron), a sample of culture was taken, and the cells were harvested and frozen. From each sample, RNAs were extracted and analyzed by Northern blot for the amount of FET3 mRNA. The levels of FET3 mRNA were normalized to ACT1 mRNA. The histogram represents the results obtained by this manner.

We showed here that several Aft1p target genes, FET3, FTR1, TIS11, SIT1, and FIT2, are induced during the diauxic shift. This induction happens when glucose in the media is exhausted, i.e. at the entrance into the diauxic transition. This induction is positively controlled by a Snf1p/Snf4p-dependent pathway, and Aft1p is absolutely required. It appears that Snf1p/Snf4p, activated at the diauxic shift, would positively regulate Aft1p function.

These results raise the question of the nature of the functional link between the Snf1p kinase and the Aft1p transcriptional factor. In accordance with the previous report (20), we showed that Aft1p is phosphorylated during the course of the diauxic shift. We showed that this phosphorylation happens at the time of glucose exhaustion and, therefore, is concomitant with the induction of Aft1p-dependent genes. This result indicated that this post-translational modification may be a mechanism for Aft1p activation. One possibility would be that the Snf1p kinase could be responsible of this phosphorylation. But we showed that neither Snf1p nor Snf4p is necessary for the phosphorylation of Aft1p. Because the induction of Aft1p targets requires a functional Snf1p-Snf4 complex, this modification is not sufficient to induce genes activation.

Recently, it was shown that Aft1p is localized in the cytoplasm and shows nuclear retention in conditions of iron deprivation. This nuclear localization is sufficient for the transcription of FTR1, a gene belonging to the Aft1p regulon (10). It is not known if in response to glucose exhaustion Aft1p is controlled in the same way; if that is the case, Snf1p could be involved at this level. Another hypothesis would be that Snf1p affects the expression of Aft1p-dependent genes by an indirect way, for example, by regulating an additional regulator/pressor of these genes. Indeed, the Snf1p-dependent pathway is already involved in the regulation of transcriptional factors at different levels; the localization of Mig1p is controlled in a Snf1p-dependent manner (23-25), and Snf1p plays a dual role...
in the control of the transcriptional activator Cat8p at the level of transcription and at the level of activity (26).

This functional link between Snf1p/Snf4p complex and Aft1p can be integrated with a previous report (27) that Aft1p target genes are under a negative control exerted by Tpk2p, one of the three protein kinase A enzymes of the yeast (cAMP-activated proteins kinase A). Glucose depletion is associated with a drop in the intracellular cAMP level leading to the inactivation of protein kinase A. This decrease of cAMP is required for the switch from growth on fermentable carbon source to growth on non-fermentable carbon source (the diauxic shift) (28). Together these results also suggest a link between Tpk2p and the Snf1/Snf4-dependent pathway.

Another question is why there is the induction of iron high affinity uptake genes at the entrance into the diauxic shift? The induction of these genes suggests that cells have to reply to an exceptional need of iron. In that case the signal does not seem to be a deprivation of extracellular iron. We showed that increasing the concentration of iron in the media such that an aft1Δ strain does not stop growing after glucose exhaustion has no influence on the induction. One possible explanation is that the signal comes from the intracellular iron. It has been reported that the major storage compartment of iron in the cell is the vacuole and could also be the transit compartment for iron (29). When cells are shifted from glucose to ethanol media, the vacuolar iron is mobilized for mitochondriogenesis. FTH1 and FET5 encode the vacuolar iron oxidase and the vacuolar iron transporter, respectively, and may be responsible for mobilizing intravacuolar stores of iron (30). These two proteins are associated in a complex. The loss of this complex compromises the ability of the cell to switch from fermentative to respiratory metabolism. Also, it has been shown that the use of the membrane permeant Fe(II) chelator, 2,2′-bipyridyl, caused a strong and rapid induction within 5 min of FET3 and FTR1 transcripts under anaerobic conditions, indicating that a relatively small, labile intracellular pool of Fe(II) regulates the expression of these activities (31). Compiling these results, we can postulate that when cells enter the diauxic shift there will be an increasing need for iron, essentially toward the mitochondria, and the depletion of the cytosolic iron pool or the vacuolar iron mobilization will be a signal for the induction of genes coding for iron high affinity uptake proteins. Alternatively, the iron threshold for induction of the Aft1 regulon could be increased at the diauxic shift.

Our results show that not all members of the Aft1 regulon are induced at the diauxic shift since we were unable to detect mRNA products of the FRE1 and the FIT1 genes at this stage by Northern blot. One hypothesis to explain this result was proposed above. The Snf1/Snf4 pathway would not control directly the Aft1 activity but could regulate an additional regulator/repressor, which would control a different set of genes.

Last, we showed that the Snf1 pathway is not involved in the response to iron deprivation, resulting in the induction of the Aft1 regulon. Thus, two different signals, iron and glucose starvation, can give a similar result, which is the induction of the iron uptake system by the transcriptional activator Aft1p through distinct signaling pathways.

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