Induction of the gal Pathway and Cellulase Genes Involves No Transcriptional Inducer Function of the Galactokinase in Hypocrea jecorina*

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Lukas Hartl 1, Christian P. Kubicek, and Bernhard Seiboth

From the Molecular Biotechnology Group, Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Technische Universität Wien, Getreidemarkt 9–166.5, A-1060 Vienna, Austria

The Saccharomyces cerevisiae galactokinase ScGal1, a key enzyme for D-galactose metabolism, catalyzes the conversion of D-galactose to D-galactose 1-phosphate, whereas its catalytically inactive parologue, ScGal3, activates the transcription of the GAL pathway genes. In Kluyveromyces lactis the transcriptional inducer function and the galactokinase activity are encoded by a single bifunctional KlGal1. Here, we investigated the cellular function of the single galactokinase GAL1 in the multicellular ascomycete Hypocrea jecorina (= Trichoderma reesei) in the induction of the gal genes and of the galactokinase-dependent induction of the cellulase genes by lactose (1,4-O-β-D-galactopyranosyl-D-glucose). A comparison of the transcriptional response of a strain deleted in the gal1 gene (no putative transcriptional inducer and no galactokinase activity), a strain expressing a catalytically inactive GAL1 version (no galactokinase activity but a putative inducer function), and a strain expressing the Escherichia coli galK (no putative transcriptional inducer but galactokinase activity) showed that, in contrast to the two yeasts, both the GAL1 protein and the galactokinase activity are fully dispensable for induction of the Leloir pathway gene gal7 by D-galactose and that only the galactokinase activity is required for cellulase induction by lactose. The data document a fundamental difference in the mechanisms by which yeasts and multicellular fungi respond to the presence of D-galactose, showing that the GAL1/Gal3–Gal4–Gal80-dependent regulatory circuit does not operate in multicellular fungi.

The enzymes of the Leloir pathway are responsible for the conversion of D-galactose to D-glucose 1-phosphate and have been identified in all biological kingdoms. In the model organism Saccharomyces cerevisiae, the genes encoding the three main Leloir pathway enzymes, GAL1 (galactokinase), GAL7 (UDP-galactose uridylyltransferase), and GAL10 (UDP-galactose epimerase and aldose 1-epimerase), are clustered and coordinately regulated at the level of transcription. The S. cerevisiae GAL genes are repressed by D-glucose, expressed only at a very low basal level on other respiratory carbon sources, and highly induced (up to 1000-fold) when the cells are switched to a medium containing D-galactose. This transcriptional activation is mediated by the interplay of three proteins; in the presence of D-galactose and ATP, the transcriptional inducer ScGal3 associates with the ScGal80 repressor thereby alleviating its repressing effects. This allows the transcriptional activator ScGal4 to recruit the RNA polymerase II to each of the GAL genes (reviewed in Refs. 1–3).

The S. cerevisiae galactokinase ScGal1 displays a 73% amino acid identity to the ScGal3, but galactokinase activity is absent in ScGal3. However ScGal3 can be converted into a catalytically active galactokinase through the insertion of the two amino acids, Ser and Ala, into its sequence (4). Overexpression of ScGal1, or a phenomenon termed long-term adaptation, can recover a gal3 phenotype in S. cerevisiae (5). The presence of ScGal3 seems to be a unique trait of S. cerevisiae, and its role as a transcriptional inducer is fulfilled in other yeasts such as in Kluyveromyces lactis by a single galactokinase KlGal1. The regulatory function of KlGal1 does not require the galactokinase activity (6), which is consistent with the recent demonstration that the enzymatic and the weak transcriptional inducer properties of ScGal1 are located in separate parts of the protein (7).

In contrast to S. cerevisiae and K. lactis, the gal genes of filamentous fungi, such as Aspergillus nidulans, Hypocrea jecorina, and Neurospora crassa, are not clustered (www.broad.mit.edu/annotation/fungi; genome.jgi-psf.org/), and an already high basal level of expression is found during growth on all carbon sources including D-glucose, indicating some major differences in the regulation of D-galactose metabolism between yeasts and filamentous fungi (8–11). In H. jecorina only gal1 and gal7, but not gal10, are further induced by D-galactose and in addition also by L-arabinose. The H. jecorina gal1 has been shown to be essential for the induction of the cellulase genes by the D-galactose-containing disaccharide lactose (1,4-O-β-D-galactopyranosyl-D-glucose), an important carbon source for many fungal fermentations including the production of cellulases and recombinant proteins in H. jecorina (12). A search of the H. jecorina genome data base shows that this fungus does not possess a GAL3 orthologue. Therefore, it would be possible that regulation of the gal genes in H. jecorina follows the model of the K. lactis GAL/LAC regulon.

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1 To whom correspondence should be addressed. Tel.: 43-1-58801-17210; Fax: 43-1-58801-17299; Web site: www.vt.tuwien.ac.at; E-mail: lhartl@mail.zserv.tuwien.ac.at.

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Induction of the gal Pathway in H. jecorina

The objective of this paper was to test whether the induction of the Leloir pathway genes by D-galactose and of the cellulase function of H. jecorina GAL1. We will show that induction by lactose requires solely the galactokinase activity and that GAL1 is completely dispensable for the basal as well as the induced transcriptional level of the Leloir pathway genes. These data highlight a significant difference in D-galactose regulation between single and multicellular ascomycetes.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions

H. jecorina strains QM9414 (ATCC 26921), Δgal1 (8), and Δxyl1 (13) were maintained on malt extract agar (Merck, VWR International, Vienna, Austria). Strains were grown in 1-liter Erlenmeyer flasks on a rotary shaker (250 rpm) at 30 °C in the medium described elsewhere (14) with the respective carbon source at a final concentration of 10 g l⁻¹.

Southern analysis was performed to verify the integration of the promoter region. All constructs were verified by sequencing.

Construction of Fungal Strains

Plasmids and Plasmid Constructions

pGAL1 contains a 4.8-kb EcoRV–HindIII clone of the H. jecorina gal1 (8) in pUC19 (15) and pGALSH a 2.1-kb SacII–HindIII fragment of the terminator region, which was amplified by PCR. The resulting PCR product was cut with SacII/SfiI to give a 140-bp fragment, which was ligated in the SacII/SfiI-restricted pGAL1 and designated pGAL1-SA.

pGALK—The E. coli galK coding region (1.1 kb) was placed under the promoter and terminator region of the H. jecorina gal1. E. coli galK was amplified from genomic E. coli K12 DNA with oligonucleotides EcGAL1rv inserting Acc65I/HindIII sites by PCR. The HindIII-restricted galK fragment was ligated into the HindIII site of pGAL1. The introduced Acc65I site was used to insert a 0.6-kb fragment of the terminator region, which was amplified by PCR using oligonucleotides GAL1termfw and GAL1termrv.

pLH1hph—A plasmid expressing the E. coli hygromycin B phosphotransferase gene (hph) under the promoter and terminator region of H. jecorina gpd1 (glyceraldehyde 3-phosphate dehydrogenase; genome.jgi-psf.org/Trire2/Trire2.home.html, scaffold 1:1562760–1564086) was constructed. 800 bp of the gpd1 promoter region was amplified using the primer pair Tgpdf and Tgpdpr. The XbaI/NsiI fragment of the resulting vector was used for the insertion of the promoter region. All constructs were verified by sequencing (Table 1).

Construction of Fungal Strains

Protoplast preparation, DNA-mediated transformation, and purification of the conidia were described previously (17). Hygromycin B (50 μg ml⁻¹) was used for selection of the transformants.

Transformations of the Δgal1 strain were done as co-transformations either with the circular pLH1hph and a 4.2-kb PvuII fragment of pGALK or with the excised Pgd1-hph-Tgpd1 cassette of pLH1hph together with the circular pGAL1 or pGAL1-SA. The Pgd1-hph-Tgpd1 cassette was excised with BamHI, Sall, and PdmI.

Southern analysis was performed to verify the integration of the constructs. Only strains showing a single ectopic integration of the construct were used for further analysis. Genomic
Induction of the gal Pathway in H. jecorina

DNA of the Δgal1 retransformed strains with pGAL1 or pGAL1-SA was digested with HindIII. Hybridization of these strains with a 2.6-kb HindIII gal1 fragment resulted in H. jecorina QM9414 in a 2.6-kb band, in the Δgal1 strain in a 4.7-kb band, and in retransformed strains in a 4.7-kb band, as well as an additional band that represents either the gal1 or gal1-SA fragment. Integration of E. coli galK was verified by digestion with MluI, which cuts once within the galK coding region. For hybridization a 1.1-kb PCR fragment of galK amplified with EcGAL1fw and EcGAL1rv was used. Strains that showed two hybridizing bands, indicating the integration of one copy of E. coli galK, were used for further analysis.

Transcript Analysis

Digoxigenin-labeled probes for the Northern analyses of cbh1, cbh2, and 18s rRNA with the respective sizes of 1.2 kb, 1 kb, and 300 bp were amplified from chromosomal DNA using the primers CBH1SF, CBH1SR, CBH2SF, CBH2SR, 18sRF, and 18sRR. In the case of gal7, the probe was amplified from a 1.1-kb cDNA fragment (9) using the primers gal7for and gal7rev.

Nucleic Acid Isolation and Hybridization

DNA and RNA preparation was described previously (8). Standard methods were used for nucleic acid electrophoresis and blotting (18). Target DNAs were detected with probes labeled with [α-32P]dCTP by random priming. Northern analysis was performed with the digoxigenin nonradioactive system from Roche Applied Science.

Galactokinase Assay

Strains were pregrown as described above, and after transfer, cultivation was continued for 12 h (D-galactose) or 20 h (lactose), respectively. Mycelia were harvested by filtration, washed extensively with cold sterile tap water, blotted dry with paper towels, and ground to a fine powder under liquid N2 with mortar and pestle. About 1 gram (wet weight) of mycelium was suspended in 3 ml of extraction buffer (0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol) and homogenized by sonication 10 times for 30 s at 2 °C with intermittent 1-min cooling periods. The resulting homogenate was centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant (average protein concentration 10–15 mg ml−1) was used as a cell extract for measuring intracellular galactokinase activity as described previously (19). Specific activities are reported as nanokatal/mg protein, which was determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

RESULTS

A Catalytically Inactive Galactokinase Is Unable to Induce Cellulase Formation by Lactose—The necessity of the gal1 gene for cellulase induction by lactose has been demonstrated (8). To test whether cellulase induction by GAL1 depends on a putative transcriptional inducer function, we constructed a strain that expressed a catalytically inactive version of GAL1 (GAL1-SA) in a Δgal1 background. This recombinant GAL1-SA lacks the two amino acids, Ser170 and Ala171, that are found in the conserved GLSSSAA motif of fungal galactokinases and are essential for their activity (4). Northern analysis confirmed that these strains had gained the ability to form a gal1 (−SA) transcript on D-galactose (Fig. 1A). In contrast to H. jecorina QM9414 or control strains that had been retransformed with the wild-type gal1, strains expressing the GAL1-SA fully lacked galactokinase activity and showed the same growth behavior as a Δgal1 strain on D-galactose (Fig. 1B), thus confirming that the deletion of the two amino acids leads to an inactive galactokinase. Consequently, these strains were tested for their ability to induce transcription of the two major cellulase genes, cbh1 and cbh2, by lactose. Fig. 1C shows that only strains expressing the wild-type gal1 fully restored cbh1 and cbh2 transcript formation, whereas strains expressing the GAL1-SA did not. From this we conclude that the presence of the SA amino acid doublet in the H. jecorina GAL1 is essential for galactokinase activity and induction of cellulases by lactose.

The E. coli Galactokinase galK Can Rescue Cellulase Induction by Lactose—The loss of galactokinase activity in GAL1-SA mimics the H. jecorina Δgal1 phenotype with respect to the induction of cellulases by lactose. To test for whether it is solely the galactokinase activity that is essential for cellulase induction, we examined whether the expression of a bacterial galactokinase, which possesses no transcriptional inducer function, would be able to rescue the Δgal1 phenotype. Therefore we
used the E. coli galK gene, which is able to complement the loss of the ScGAL1 gene but not the loss of ScGAL3 gene in S. cerevisiae (5). An H. jecorina Δgal1 strain was transformed with the E. coli galK gene under control of H. jecorina gal1 expression signals. The galK-transformed Δgal1 strains grew better than the Δgal1 strains on D-galactose but were not able to reach the level of the parental strain QM9414 or gal1 retransformed strains (Fig. 2A). Measurements of galactokinase activity in these galK-transformed strains showed that they produced somewhat lower enzyme activities (about 80–90%) compared with QM9414 and H. jecorina retransformed strains (Fig. 2B). However, when grown on lactose, galK-expressing strains regained their ability to fully induce cbh1 and cbh2 transcription (Fig. 2C), thus proving that the galactokinase activity was sufficient for the induction of cellulases by lactose.

**Gal1 Is Dispensable for Induction of gal7 by D-Galactose**—
The results discussed above have shown that galactokinase activity per se (but not a putative transcriptional inducer function of the GAL1 protein as found in the ScGal3 or KGal1) is necessary for cellulase induction by lactose. Consequently, we investigated whether the induction of the Leloir genes would follow the same trend. Unlike in S. cerevisiae or K. lactis, only the H. jecorina gal1 and gal7 (but not gal10) genes are induced to still higher levels by the addition of D-galactose. Therefore, we compared the transcriptional level of gal7 in our set of recombinant strains during growth on D-glucose and D-galactose. As seen from Fig. 3 induction by D-galactose led to an ~3-fold increase in gal7 transcript levels compared with D-glucose, and this result was also obtained with the parental strain, a Δgal1 strain, and Δgal1 strains that expressed the E. coli GalK or the H. jecorina Gal1-SA. These results imply that the GAL1 protein as well as the galactokinase activity is dispensable for the basal as well as the inducible level of gal7 transcription.

**Galactitol Formation Is Dispensable for gal7 Induction by D-Galactose**—D-Galactose can be catabolized by filamentous fungi such as H. jecorina or A. nidulans via a second pathway absent in the yeasts S. cerevisiae and K. lactis (Fig. 4A) (8, 20). The first step in this pathway is the reduction to galactitol by analdose reductase, which in the case of H. jecorina was identified as the D-xylulose reductase XYL1. Galactitol formation by the D-xylulose reductase in this pathway has been shown to be responsible for the induction of extracellular β-galactosidase formation by D-galactose (13). Because neither GAL1 protein nor galactokinase activity is required for the induction of gal7 by D-galactose, we tested whether catabolism of D-galactose via this second pathway would also create the inducer of the Leloir pathway. To this end, we tested the induction of gal7 by D-ga-
lactose in a Δxyl1 strain. The results shown in Fig. 4B document that gal7 induction is not affected in this strain, implying that the alternative pathway and galactitol formation are not involved in induction. We concluded from these results that D-galactose induction of gal7 in H. jecorina occurs independently of a blockage in the first step of either of the two pathways for D-galactose catabolism and is independent of galactitol formation.

**DISCUSSION**

The *S. cerevisiae* GAL regulon has become a paradigm for transcriptional control in lower eukaryotes and a model system for gene regulation (1–3). Although its importance is uncontested, the present results illustrate that this regulatory network may be restricted to yeasts and not representative for other ascomycetous fungi in general. The present data, that the Leloir pathway is regulated in an independent way in *H. jecorina*, are consistent with previous findings such as nonclustered genomic organization, lack of carbon catabolite repression by D-glucose, induction of gal7 and gal1 by L-arabinose, the existence of a second D-galactose catabolic pathway, and lack of a mutarotase (aldose 1-epimerase) domain in GAL10 (reviewed in Ref. 21). This and the fact that we were not able to detect orthologues of GAL4 in the genome sequences of *H. jecorina* and other filamentous fungi (*N. crassa*, *Magnaporthe grisea*, *Fusarium graminearum*, *A. nidulans*) support the present data that the Leloir pathway is controlled in a different way. Although the present study has been performed with only one ascomycetous species from the Pyrenomycetes, we also have preliminary data from *A. nidulans* and *Aspergillus niger* that are fully consistent with those obtained for *H. jecorina*. Consequently, we conclude that the GAL regulon is a yeast-specific trait but cannot be used to explain D-galactose regulation in filamentous fungi.

In a previous study we failed to detect the induced level of the gal7 transcript in a Δgal1 strain of *H. jecorina*; however, we used only a single time point and different conditions (8). In contrast, here we showed that D-galactose clearly induces gal7 in the Δgal1 strain. Although this implies that D-galactose induces gal7 without being metabolized through one of the two pathways, the underlying mechanism is unclear. It could be D-galactose itself, either bound to its permease (a Gal2 orthologue is present in the *H. jecorina* genome; scaffold 10:1127812–1130067) or its intracellular steady state concentration. It should be noted that the constitutive expression of the Leloir pathway genes could create a reverse pathway of galactose 1-phosphate formation by the action of GAL10 (forming UDP-galactose from UDP-glucose) and its subsequent conversion to D-galactose 1-phosphate by gal7. Therefore, we cannot exclude at the moment that the inducer could still be D-galactose 1-phosphate.

In contrast to gal7 induction, galactokinase activity was required for the induction of the cellulase genes by lactose. This implies that the regulation of the Leloir pathway and that of cellulases are not linked, which is corroborated also by the findings that D-galactose does induce cellulase gene expression only at a low level at low growth rates (22). In theory, this would be consistent with a role for D-galactose 1-phosphate as an inducer of cellulase formation. However, such an assumption raises the question of why galactokinase activity promotes high cellulase gene expression during growth on lactose. As emphasized previously, a major difference between growth on D-galactose and on lactose is that the latter is accompanied by a stronger participation of the alternative, reductive D-galactose-degrading pathway (21, 22). Blockage of the latter in a Δxyl1 also strongly impairs cellulase induction on lactose, and the effect is not additive with that in a Δgal1, as seen in a Δgal1/Δxyl1 strain. In other words, D-galactose 1-phosphate, as well as galactitol or catabolites thereof, is needed for high induction of cellulase formation. Whether they both cooperate in the induction themselves, or first need to be converted to another metabolite, which then acts as an inducer, is currently investigated.

In *S. cerevisiae* the GAL genes are repressed by D-glucose, derepressed (noninduced) on other respiratory carbon sources, and highly induced (1000-fold) in the presence of D-galactose. In comparison with *S. cerevisiae*, the basal level of GAL gene expression is higher in other yeasts, e.g. *K. lactis* (23). In *H. jecorina* no repression by D-glucose was found, transcription on D-glucose occurred to a similar extent as on other noninducing carbon sources, and only a relatively weak induction (three to 4-fold) was found during growth on D-galactose. These differences in the transcriptional regulation raise the question of why the Leloir pathway is regulated in a different way by filamentous fungi. An explanation for this difference may be derived from the difference in the importance of D-galactose for fungal cell biology; D-galactose is an important component of the cell wall of many filamentous fungi and also of their glycosylated proteins, whereas it does not occur in the cell walls of *S. cerevisiae* and is only a minor component of its glycoproteins (24). The importance of D-galactose for the cell wall and glycoproteins is higher in other yeasts, including *K. lactis* and *Schizosaccharomyces pombe*, than in *S. cerevisiae* (25–27). It seems, therefore, especially important for filamentous fungi to metabolize D-galactose, which results from the normal turnover of the cell wall and glycoproteins, before it accumulates intracellularly and would interfere with the regulation of other pathways. This may also explain the constitutive expression of gal10, the gene product of which is needed for the production of the activated precursor UDP-galactose for the synthesis of the carbohydrates in cell wall components and glycoproteins. Although there may still be other reasons, we speculate that these differences gave rise to the evolution of the different patterns of D-galactose regulation in fungi.

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Induction of the gal Pathway in H. jecorina