Oxygen- and Glucose-Dependent Expression of *Trhxt1*, a Putative Glucose Transporter Gene of *Trichoderma reesei*[†,‡]

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Received December 14, 2005; Revised Manuscript Received April 1, 2006

ABSTRACT: The filamentous fungus *Trichoderma reesei* is adapted to nutrient-poor environments, in which it uses extracellular cellulases to obtain glucose from the available cellulose biomass. We have isolated and characterized *Trhxt1*, a putative glucose transporter gene, as judged by the glucose accumulation phenotype of a Δ*Trhxt1* mutant. This gene is repressed at high glucose concentrations and expressed at micromolar levels and in the absence of glucose. The gene is also induced during the growth of *T. reesei* on cellulose when the glucose concentration generated from the hydrolysis of cellulose present in the culture medium is in the micromolar range. We also show that oxygen availability controls the expression of the *Trxht1* gene. In this regard, the gene is down-regulated by hypoxia and also by the inhibition of the flow of electrons through the respiratory chain using antimycin A. Intriguingly, anoxia but not hypoxia strongly induces the expression of the gene in the presence of an otherwise repressive concentration of glucose. These results indicate that although the absence of repressing concentrations of glucose and an active respiratory chain are required for *Trhxt1* expression under normoxic conditions these physiological processes have no effect on the expression of this gene under an anoxic state. Thus, our results highlight the presence of a novel coordinated interaction between oxygen and the regulatory circuit for glucose repression under anoxic conditions.

Glucose is a primary energy source for most organisms, and it also acts to modulate many vital cellular processes, such as growth, metabolism, and development. Because different microorganisms evolved in different environments and show vast nutritional diversity, they must have developed adaptations to ensure an adequate supply of glucose in the face of the varying levels of extracellular glucose encountered in their natural habitats. Habitats are often grouped according to their nutrient content into nutrient-rich and nutrient-poor environments (*1*). One well-studied eukaryotic microorganism that adapted to an environment rich in sugars is the unicellular yeast *Saccharomyces cerevisiae*. For example, grapes are one of the natural habitats of *S. cerevisiae*, and grape-juice contains up to 1.5 M glucose and fructose (*2*).

In contrast to the nutrient-rich environments in which *S. cerevisiae* evolved, eukaryotic soil microorganisms such as the multicellular filamentous fungus *Trichoderma reesei* (*3*) are adapted to nutrient-poor environments in which the concentration of glucose is below 200 μM (*4*). To ensure an adequate supply of glucose in its natural habitat, *T. reesei* exploits extracellular hydrolases such as cellulases (*5*) to obtain glucose from cellulose. At high glucose concentrations and in the presence of oxygen, *S. cerevisiae* and *T. reesei* utilize two distinct ATP-producing pathways: the unicellular *S.* cerevisiae primarily uses an anaerobic pathway (fermentation), whereas the multicellular fungus *T. reesei* uses an aerobic pathway (respiration) (*6*−*8*). Fermentation generates a low yield of ATP at high rates, whereas respiration gives a higher yield of ATP but proceeds at a lower rate.

Using a combination of model simulations and biochemical observations, Pfeiffer et al. (*9*) have shown that energetic limitation and the rise in the concentration of oxygen in the atmosphere, which allowed organisms to produce ATP at a higher yield by respiration, may have been implicated in the evolutionary transition from unicellular fermenters to undifferentiated, multicellular, respiratory organisms. Thus, the selective pressures to which eukaryotic organisms have been subjected during evolution will have favored the emergence of regulatory signals that respond to changes in the availability of oxygen and glucose to control the high-yield production of ATP in mitochondria.

Indeed, it has been shown that variations in glucose levels (*6*, *10*), oxygen availability (*11*), and mitochondrial activity (*12*, *13*) control the transcription of many nuclear and mitochondrial genes in eukaryotic organisms. However, although many components of the corresponding regulatory processes have been identified in yeast and in mammalian cells (*14*, *15*), the question of how the relevant signals are generated and transduced has not been fully answered.

[†] This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo grant FAPESP 2003/00042-3.

[‡] The nucleotide sequence of the *Trhxt1* gene has been deposited in the GenBank database (accession no. AY444343).

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10.1021/bi052553y CCC: $33.50
Published on Web 06/08/2006

T. reesei can be considered to be a model for eukaryotic microorganisms that have adapted to nutrient-poor environments in which oxygen played a crucial role in the regulation of metabolic pathways for energy production. Therefore, we have begun to analyze the transcriptional response of this microorganism to variations in glucose and oxygen levels. For this purpose, we have recently established an EST database for *T. reesei* (http://trichoderma.iq.usp.br) and used cDNA microarray techniques to address the basis for the fundamental differences observed between *S. cerevisiae* and *T. reesei* in the utilization of glucose for ATP production (*8*). This work showed that although both microorganisms possess conserved gene sets for ATP production by aerobic and anaerobic pathways the regulation of gene transcription by a glucose-rich medium in *T. reesei* differs from that seen in *S. cerevisiae* (*7*) in the case of critical genes whose products control the flow of metabolites between aerobic and anaerobic metabolism. These studies strongly implied that the regulatory machinery controlled by glucose in *T. reesei* has been the target of evolutionary pressures that directed the flow of metabolites into a pathway with a low rate but high yield of ATP production.

Because transport across the plasma membrane is the first step in the utilization of hexoses and to understand how microorganisms adapted to environments of energetic limitation, we decided to study glucose transport in *T. reesei*. In this article, we describe the isolation and functional characterization of a putative glucose transporter gene, *Trhxt1*, of *T. reesei*. We demonstrated that cellulose induces the transcription of both *Trhxt1*[1] and *cbh1* (which encodes cellobiohydrolase I, the major cellulase in *T. reesei*) under conditions in which *T. reesei* is utilizing cellulose to obtain glucose, indicating a functional role for this gene in the utilization of glucose in vivo. We also show that unlike the glucose transporters of *S. cerevisiae*, which require millimolar levels of glucose for induction (*16*) and are insensitive to variation in oxygen levels (*17*, *18*), *Trhxt1* is expressed in the presence of micromolar levels of glucose, and its expression is modulated by oxygen, just like that of the mammalian glucose transporter GLUT1 (*19*). Interestingly, we found that anoxia[2] but not hypoxia relieved the repression of the *Trhxt1* gene by glucose, resulting in the upregulation of its expression. Moreover, we investigated whether the oxygen sensing mechanisms described in yeast and mammalian cells (*15*, *20*, *21*) might control the expression of *Trhxt1*. In this regard, our results reveal a complex regulatory system controlled by oxygen and glucose availability.

EXPERIMENTAL PROCEDURES

Cultivation of T. reesei. The *T. reesei* strain used in this work, QM 9414, was obtained from the American Type Culture Collection (ATCC 26921). Inoculum preparation, culture media, and growth conditions for *T. reesei* have been described previously (*22*). For the experiments on glucose accumulation, the conidia of *T. reesei* were inoculated into the culture medium supplemented with 0.05% glycerol, and grown for 8 h. Germinating conidia were then collected by centrifugation and transferred to a fresh medium supplemented with 2 mM sophorose (2-*O*-β-glucopyranosyl-D-glucose). Cells were induced with sophorose for 3 h, collected by filtration, and washed three times with ice-cold 100 mM potassium phosphate buffer (pH 5.0). After washing, the cells were resuspended in the same potassium phosphate buffer and used immediately in the glucose accumulation assays. *T. reesei* chemostat cultures were performed in a 2-l Bioflo III bioreactor (New Brunswick) and fed at a dilution rate (d) of 0.1 h^{-1} with the culture medium (*22*) supplemented with 0.1% yeast extract and 100 mM glucose. Cultivations were carried out at 28 °C, 900 rpm, and pH 6.0. The dissolved oxygen (DO) was monitored with an InPro 6100 oxygen sensor (Ingold). After steady-state conditions were achieved, the DO was reduced from its initial value (5 mg/L) to zero in 1 h steps. The vessel was purged with pure nitrogen for 2 h, and then, the oxygen level was restored to the initial value. Aliquots were withdrawn from the culture medium at different DO levels for the determination of cellular dry weight, extracellular glucose concentration, and RNA analyses. The results presented in this work are from at least two independent experiments.

Transformation of T. reesei. The conidia of *T. reesei* were transformed by microprojectile bombardment using M5 tungsten particles (an average diameter of 0.4 μm). Briefly, microprojectiles (10 mg) were first sonicated in 0.1 M HNO_3 for 20 min and washed once with distilled water and then with absolute ethanol. The particles were re-suspended in 600 μL of distilled water and stored at −20 °C. Precipitation of DNA onto the particles was carried out as follows. To a 50 μL aliquot of particles, 10 μL of DNA (0.5−1 μg/μL) and 50 μL of 2.5 M $CaCl_2$ and 20 μL of 0.1 M spermidine were added under agitation. The projectiles were then sonicated briefly and washed with 250 μL of absolute ethanol. Finally, the beads were resuspended in 60 μL of absolute ethanol, sonicated, and vigorously mixed by vortexing. A 5 μL drop of the mixture was placed in the center of each flying disk and left to dry. The conidial suspension was spread on potato−dextrose agar (PDA) plates ((1−2) $\times 10^7$ conidia per plate) and air-dried under sterile conditions for 4 h before bombardment. Particle bombardment was carried out under vacuum at 1200−1600 psi at a target distance of 3 or 6 cm. The plates were then incubated at 28 °C for 7 h. A layer of PD broth containing 1% agarose and hygromicin B (final concentration 100 μg/mL) was then deposited on the plates. Transformants were observed after 3 days. Colonies of transfomants were restreaked three times on selective media and then transferred to PDA plates for sporulation. After sporulation, the conidia were again spread on selective media. Colonies that were able to germinate in the presence of the antibiotic were used for further analyses.

Glucose Accumulation Assays. Glucose accumulation assays were based on the procedures described by Stambuk et al. and Coons et al. (*23*, *24*) with the following modifications. Glucose accumulation was assayed in the range between 2.5 and 200 μM (0.02−0.2 μCi/nmol). In each

[1] Abbreviations: *cbh1*, cellobiohydrolase I gene; DO, dissolved oxygen; EST, expressed sequence tag; *glut1*, glucose transporter 1 gene; *hph*, hygromycin B phosphotransferase gene; LORE, low oxygen response element; MFS, major facilitator superfamily; *Trhxt1*, *Trichoderma reesei* hexose transporter 1 gene; *trpC*, glutamine amidotransferase gene/N-(5′-phosphoribosyl)anthranilate isomerase gene/indole-3-glycerol phosphate synthase gene; PCR, polymerase chain reaction.

[2] In this work, we considered normoxia (normal oxygen concentration) to be 5 mg/L, hypoxia (low oxygen concentration) to be any oxygen concentration below 5 mg/L, and anoxia to be the absence of oxygen.

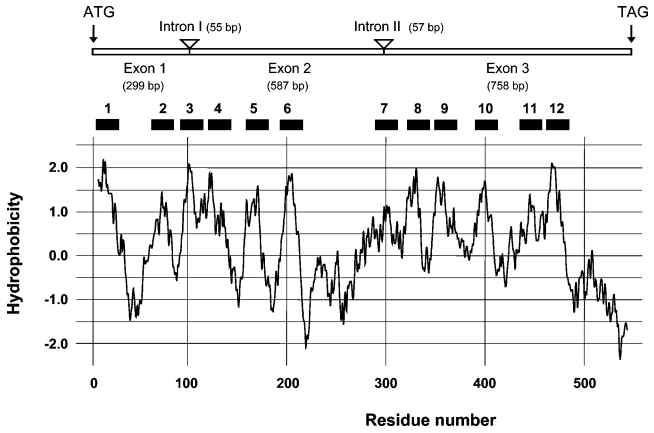

FIGURE 1: Hydrophobicity analysis of *Trhxt1*. A scheme depicting the organization of the *Trhxt1* gene is presented at the top of the figure. Introns and start and stop codons are indicated. Boxes numbered 1−12 represent the hydrophobic transmembrane domains predicted by the algorithms of TMHMM (*30*) and SOSUI (*31*). The hydropathy profile of the protein was evaluated using a window of 15 residues as described by Kyte and Doolittle (*29*).

assay, 80 μL of cell suspension (germinated conidia of *T. reesei* suspended in 100 mM potassium phosphate buffer (pH 5.0) and equilibrated at 28 °C for 4 min) was mixed with 20 μL of radiolabeled D-(U-^{14}C) glucose (Amersham Biosciences) and incubated for 20 s. The medium was rapidly removed by filtration through MF-Millipore membrane filters, and the cells were immediately washed with 2.5 mL of ice-cold water. The filters were then placed in 5 mL of ACS scintillation cocktail (Amersham Biosciences), and radioactivity was measured in a 1214 Rackbeta liquid scintillation counter. It is important to note that glucose accumulation was linear with time for at least 30 s and that no more than 5% of the substrate was consumed during the assays.

Glucose Assay. Extracellular glucose concentrations in the culture filtrate were determined using a SERA-PAK kit (Bayer).

Plasmids. The cassette used to disrupt the *Trhxt1* gene was constructed as follows. A 2 kb *Sal*I-*Xba*I fragment containing the hygromycin B phosphotransferase gene (*hph*) flanked by the promoter and terminator of the *trpC* gene of *Aspergillus nidulans* was isolated from pCSN43 (*25*) and ligated to a 1.7 kb *Bam*HI-*Sal*I fragment extending from − 600 to + 1115 of *Trhxt1*. (The positions are given relative to that of the start codon.) The ligation product was then cloned between the *Bam*HI and *Xba*I sites in the pBluescript KS (−) vector (Stratagene). The resulting plasmid was named pH1. A 3 kb fragment from the 3′-flanking region of *Trhxt1* was amplified by PCR and inserted into the blunted *Sac*I site of pH1 to generate pH2. The 7 kb *Bam*HI fragment used as a targeting vector (Figure 2) for the homologous deletion of *Trhxt1* was isolated from pH2 by cleavage with *Bam*HI.

Cloning and Sequencing Trhxt1. The *Trhxt1* gene was identified in an EST database of *T. reesei* developed in our laboratory (http://*trichoderma*.iq.usp.br). A total of seven cDNA clones were obtained and analyzed. Four of them were found to contain the entire *Trhxt1* coding sequence. Using a 1.9 kb cDNA fragment as a probe, genomic clones were obtained by screening a genomic library of *T. reesei* constructed in the lambda DASH II vector (Stratagene). Two *Bam*HI fragments identified by Southern analyses of the genomic clones were subcloned in pBluescript KS (−) (Stratagene) and used for the construction of the deletion cassette for *Trhxt1*. Sequencing reactions were performed using the BigDye terminator cycle sequencing kit and an ABI 377 DNA sequencer (Perkin-Elmer Life Sciences).

RNA Isolation and Analyses. The frozen mycelium of *T. reesei* was ground to a fine powder under liquid nitrogen, and the total RNA was isolated with the TRIzol Reagent (Invitrogen). Aliquots (10 μg) of RNA were fractionated by electrophoresis on a 1.2% agarose gel, after denaturation with glyoxal and dimethyl sulfoxide. The RNA was transferred to Hybond-N+ membranes (Amersham Biosciences) and hybridized with probes radiolabeled with [α-^{32}P]dATP (Amersham Biosciences) by random priming.

RESULTS

Isolation and Structural Analysis of the Trhxt1 Gene. We have established an EST database for the filamentous fungus *T. reesei* (*8*) and searched it for sequences that show similarity to genes involved in sugar uptake. We identified a gene, *Trhxt1*, which exhibits significant similarity to glucose transporters from other organisms. Genomic and full-length cDNA clones of *Trhxt1* were isolated. Analyses of their sequences showed that *Trhxt1* has a single open reading frame of 1644 bp that encodes a polypeptide of 548 amino acids. The greatest similarity was observed in the high-affinity glucose transporters from *T. harzianum* (GTT1, 96.5%) (*26*) and *Kluyveromyces lactis* (Hgt1, 55.7%) (*27*). In addition, *Trhxt1* is also highly similar to hypothetical protein sequences identified in the genome sequence of *Neurospora crassa* (*28*).

Hydrophobicity plots (*29*) of the deduced amino acid sequence of *TrHXT1* based on a window size of 15 residues and the sequence analysis according to the algorithms of TMHMM (*30*) and SOSUI (*31*) are presented in Figure 1. The results show the presence of 12 putative transmembrane domains, a characteristic feature of the major facilitator superfamily (MFS) (*32*), which comprises a diverse variety of transport protein families with members from all of the major groups of living organisms including the well-studied hexose transporters from *S. cerevisiae* (*16*). Other characteristic motifs found in members of the MFS were identified in *Trhxt1*, including a long cytoplasmic loop connecting transmembrane domains 6 and 7 and the two sugar transporter signatures (*33*).

Taken together, the results of this analysis strongly indicate that the product of this gene has structural features characteristic of a membrane protein involved in glucose transport.

The genomic sequence shows that the *Trhxt1* coding sequence is interrupted by two small introns (of 55 and 57 bp; Figure 1), a feature found in many genes in filamentous fungi (*34*). The sequence of the gene and its flanking regions presented in this work were submitted to the NCBI database (accession no. AY444343) before the genome sequence of *T. reesei* was publicly available (http://gsphere.lanl.gov/trire1/trire1.home.html). Our data are in perfect agreement with those obtained from the genome sequence. Although several genes with similarities to glucose transporters were predicted in the genome of *T. reesei*, computational analysis and high stringency southern analyses using the full-length *Trhxt1* coding sequence as a probe indicate that *Trhxt1* is present in a single copy in the genome of *T. reesei* (data not shown).

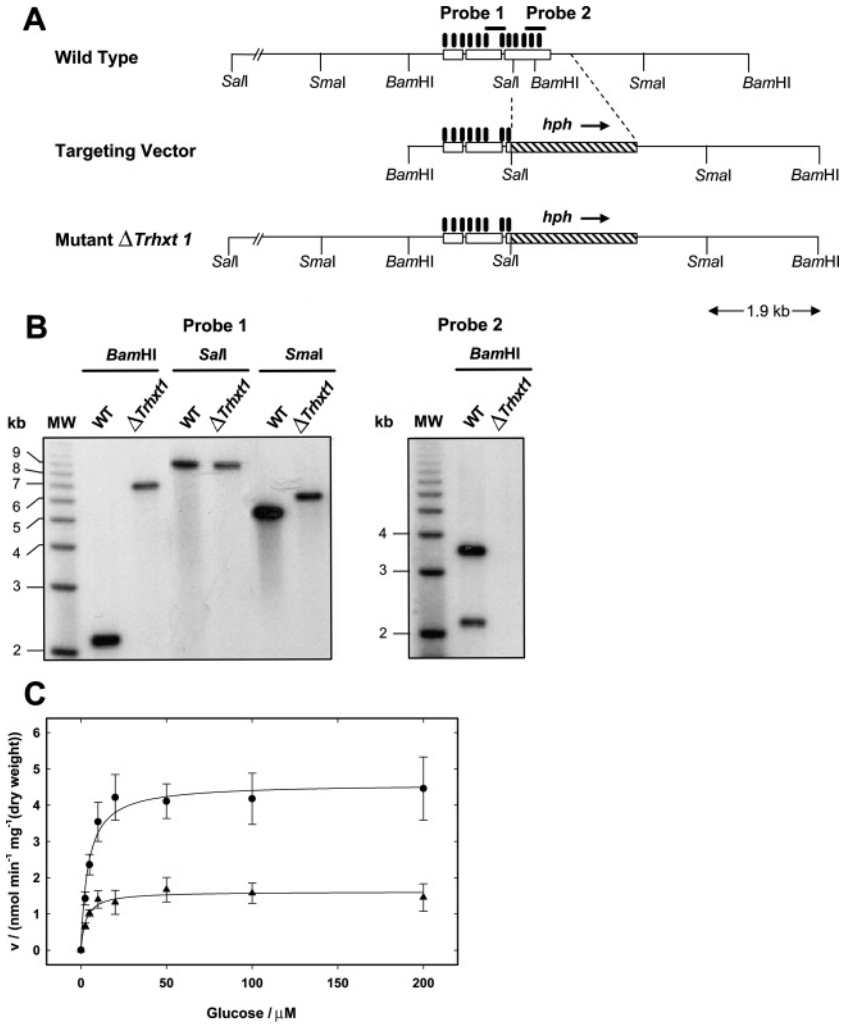

FIGURE 2: Functional analysis of the *Trhxt1* gene. (A) Structure of the *Trhxt1* gene (top diagram) showing the organization of the exons (open boxes), the restriction map, and the 12 putative transmembrane domains (filled boxes). The targeting vector (second diagram) contains 1.7 and 3.4 kb segments of the 5′ and 3′ ends, respectively, of *Trhxt1* flanking the hygromycin B phosphotransferase resistance gene (*hph*) (hatched box). The structure of the locus after the integration of the targeting vector is shown in the bottom diagram. (B) Identification of the Δ*Trhxt1* mutant by Southern analysis. Hybridization of wild-type and mutant genomic DNAs digested with the indicated restriction enzymes was performed with probe 1 (left blot) or 2 (right blot). The position of each probe in the WT sequence is indicated in (A) (top diagram). (C) Glucose accumulation by the wild type (●) and the Δ*Trhxt1* mutant (▲). Germinated conidia from the wild-type and Δ*Trhxt1* strains were induced with 2 mM sophorose for 3 h and washed before glucose uptake assays were performed as described in Experimental Procedures. Bars represent standard deviations.

Glucose Accumulation in a T. reesei Mutant Strain Lacking Functional Trhxt1. To determine if the product of this gene is involved in glucose accumulation, we constructed a *T. reesei* mutant in which the coding sequence of the *Trhxt1* is interrupted. To that end, we used homologous recombination in *T. reesei* cells to replace a major portion of *Trhxt1* exon 3, encoding the last four putative transmembrane domains, with the *E. coli hph* gene for hygromycin B phosphotransferase, which confers hygromycin B resistance (Figure 2A). The hygromycin B resistance gene was controlled by the promoter and terminator of the *trp*C gene of *A. nidulans* (*25*). In addition to its role in disrupting the *Trhxt1* gene, the resistance cassette can also serve as a selection marker for targeted deletion. Stably transformed cells were purified by three rounds of single colony isolation from plates containing hygromycin B. The *T. reesei* transformants were isolated and found to remain resistant to hygromycin B after successive passages through nonselective media. A Southern analysis of one of the transformants is presented in Figure 2B. Two probes (Figure 2A) were designed to analyze whether the targeting vector (Figure 2A, middle) had been correctly integrated into the wild-type *Trhxt1* (Figure 2A, top), deleting the coding sequence for the last four putative transmembrane domains and replacing them with the hygromycin B gene (Figure 2A, bottom). The results presented in Figure 2B show that the fragments that hybridized with probe 1 had the sizes expected had the hygromycin B gene inserted within the *Trhxt1* locus. Hybridization with probe 2 gave no signal, indicating that the region encoding the last four transmembrane domains of *Trhxt1* had been replaced by the hygromycin B gene (Figure 2B). It is also important to mention that the targeting vector did not integrate anywhere else in the genome (data not shown). We named this *T. reesei* mutant T36-Δ*Trhxt1*.

To analyze the function of *Trhxt1*, we compared the rates of glucose accumulation by wild-type *T. reesei* and Δ*Trhxt1* cells. Because the *Trhxt1* gene can be induced with the disaccharide sophorose (2-*O*-*β*-glucopyranosyl-D-glucose) (Figure 3B), geminated conidia of *T. reesei* were exposed to sophorose for 3 h and washed. Then, glucose accumulation

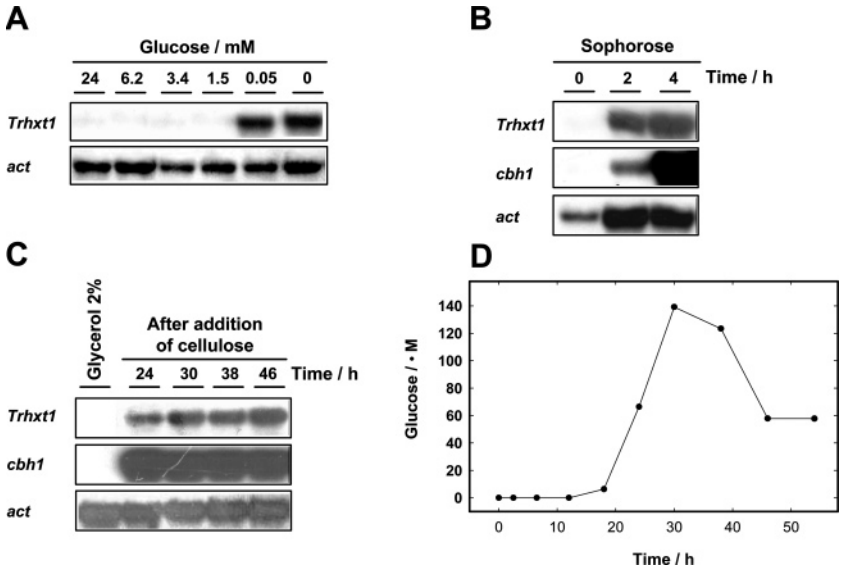

FIGURE 3: Effect of glucose and inducers of the cellulase system on the expression of *Trhxt1*. Mycelia of *T. reesei* were grown in shake-flask cultures as described previously (*22*). Aliquots were withdrawn as indicated for Northern blot analyses and glucose determination in the culture medium. Total RNA was isolated, and 10μg aliquots were fractioned electrophoretically and analyzed with a labeled *Trhxt1* probe as described in Experimental Procedures. The actin gene of *T. reesei* (*48*) was used as an internal control. (A) Effect of glucose concentration on the expression of *Trhxt1*. *(*B) *T. reesei* cells were grown on 2% glycerol and then treated with 2 mM sophorose, and the expression of the *Trhxt1* and *cbh1* transcripts was analyzed at the times indicated. (C) *T. reesei* cells were grown as described in the legend to Figure 3B except that cellulose (0.8%) was added instead of sophorose. (D) Glucose concentration in the culture medium during the growth of *T. reesei* on cellulose.

was measured in a sophorose-free medium. The cells were withdrawn and analyzed for the expression of the *Trhxt1* transcript. The transcript was expressed during the period in which the cells were assayed for glucose accumulation (data not shown). Under these conditions and at micromolar levels of glucose, the rate of glucose accumulation by the Δ*Trhxt* mutant is 50 to 70% lower than that of the wild-type strain (Figure 2C). Compared with the wild-type strain, the Δ*Trhxt1* mutant shows no significant difference in growth rate with different glucose concentration (50 μM−50 mM). This behavior is similar to that of *S. cerevisiae* in which the deletion of one glucose transporter affects glucose uptake but not growth rate (*16*). These results indicate that *Trhxt1* is most probably involved in glucose accumulation in *T. reesei*.

Effect of Glucose Concentration and Inducers of the Cellulase System on the Expression of the Trht1 Transcript. To examine the effect of glucose on the expression of this gene, the abundance of the *Trhxt1* transcript was analyzed in cells grown in the presence of different concentrations of glucose. The results presented in Figure 3A show that the *Trhxt1* mRNA was repressed in *T. reesei* cells exposed to high glucose concentrations. The transcript, however, was highly expressed in the presence of less than 100 μM glucose and upon the complete depletion of glucose.

Soil, the natural habitat of *T. reesei*, is considered to be a nutrient-poor environment (*4*). In this environment, *T. reesei* can obtain glucose by exploiting an efficient cellulolytic system that hydrolyses cellulose biomass to glucose. Cellulose, an insoluble polymer of glucose, also serves as the natural inducer of this cellulase system. Compelling evidence has been presented, which demonstrates the importance of constitutive low-level expression of cellulase activity in triggering the induction of the cellulase system by at least 1000-fold (*22*, *35*, *36*). The constitutive basal level of cellulase is believed to hydrolyze cellulose to soluble products from which an active inducer such as the disaccharide sophorose is produced by the transglycosylation activity of a β-glucosidase present in *T. reesei* (*37*).

To determine whether *Trhxt1* is induced by the soluble disaccharide inducer sophorose under conditions in which the cellulose is being hydrolyzed to glucose, we analyzed the expression of the *Trhxt1* transcript in the presence of sophorose and during the cellulose-mediated induction of the cellobiohydrolase I gene (*cbh1*), the major member of the cellulase system in *T. reesei*. Figure 3B and C shows that the *cbh1* and *Trhxt1* transcripts can both be detected in the presence of sophorose and also in cells growing in the presence of cellulose. It is noteworthy that the concentration of glucose in the culture medium during the growth of *T. reesei* on cellulose never exceeded 140 μM (Figure 3D), ensuring the continued expression of the *Trhxt1* transcript during the hydrolysis of cellulose to glucose.

These results show that *Trhxt1* is induced by two well-established inducers of the cellulase system, the insoluble polymer cellulose and the soluble disaccharide sophorose, indicating the potential physiological involvement of this gene in glucose accumulation during the growth of *T. reesei* on cellulose.

Effect of Oxygen Availability on the Expression of the Trhxt1 Transcript. Studies aimed at understanding the adaptive response of mammalian cells to low oxygen concentration have identified numerous genes whose expression is enhanced in response to oxygen limitation. One such gene is the glucose transporter gene *glut1*. Chronic exposure to hypoxia results in an increase in the level of *glut1* mRNA (*19*). In contrast, genome-wide transcriptional analysis of the unicellular yeast *S. cerevisiae* upon exposure to anaerobic conditions (*17*, *18*) has revealed that not one of the genes related to glucose transport is induced by this treatment.

To examine if the *Trhxt1* gene of *T. reesei* is controlled by oxygen availability, we evaluated the expression of the

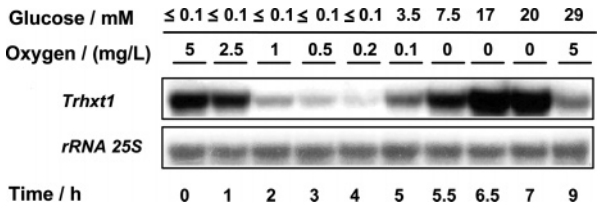

FIGURE 4: Effect of oxygen concentration on the expression of *Trhxt1*. Glucose-limited continuous cultures of *T. reesei* were performed as described in Experimental Procedures. After reaching the steady-state (first lane), the oxygen concentration was reduced gradually until it reached zero and was subsequently restored to its original value (last lane). Aliquots were withdrawn as indicated for glucose determination and Northern blot analyses. rRNA 25S was used as the internal control gene.

Trhxt1 transcript by Northern analysis under different concentrations of oxygen availability and in the complete absence of oxygen. Because the *Trhxt1* transcript is expressed at micromolar levels of glucose, we started this evaluation with a steady-state culture in which the cells were exposed to a constant level of glucose concentration at micromolar levels. Under conditions in which the growth rate was constant, with dissolved oxygen at 5 mg/L and glucose below 100 μM, the *Trhxt1* transcript was found to be expressed in a steady-state manner (Figure 4). The transcript was down-regulated when the oxygen concentration dropped to below 2.5 mg/L. At oxygen concentrations below 0.2 mg/L, glucose began to accumulate in the culture medium to levels that would be expected to repress the expression of the *Trhxt1* transcript. Surprisingly, at this stage, that is, at 0.1 mg/L oxygen (extreme hypoxia) with extracellular glucose at 3.5 mM, the transcript was expressed at a level comparable to that seen in the original state (time zero, 5 mg/L oxygen and below 100 μM glucose) (Figure 4). In fact, the transcript was induced at least 2-fold (relative to the original state) when the oxygen concentration reached zero, and the glucose concentration was between 7.5 and 20 mM. As expected, restoration of the oxygen concentration to the normal level (5 mg/L) resulted in the strong repression of the transcript because of the relatively high concentration of glucose still present in the culture medium (compare the first and the last lanes in Figure 4). These results show four different metabolic conditions triggered by different levels of oxygen availability, which altered glucose utilization by the cells and influenced the expression of the *Trhxt1* transcript: (1) under normoxic conditions in the presence of nonrepressive glucose concentrations, the transcript is expressed; (2) under hypoxic conditions at nonrepressive glucose concentrations, the cell reduces its metabolic activities and the transcript is down-regulated; (3) under anoxic conditions in the presence of repressive concentrations of glucose, when the cell is metabolically unable to consume glucose, the transcript is induced; (4) under normoxic conditions with repressive concentrations of glucose, when the cells utilize glucose, the transcript is repressed. The gene's behavior under conditions 1 and 4 make it a classical example of a glucose-repressible gene (*6*). Its response to condition 2 indicates that *Trhxt1* belongs to a class of genes that are influenced by oxygen availability and are under extensive investigation in many laboratories (*11*). The striking finding of this work, however, is the expression profile under condition 3, in which anoxia completely relieves glucose repression and, actually, induces the expression of the *Trhxt1* transcript.

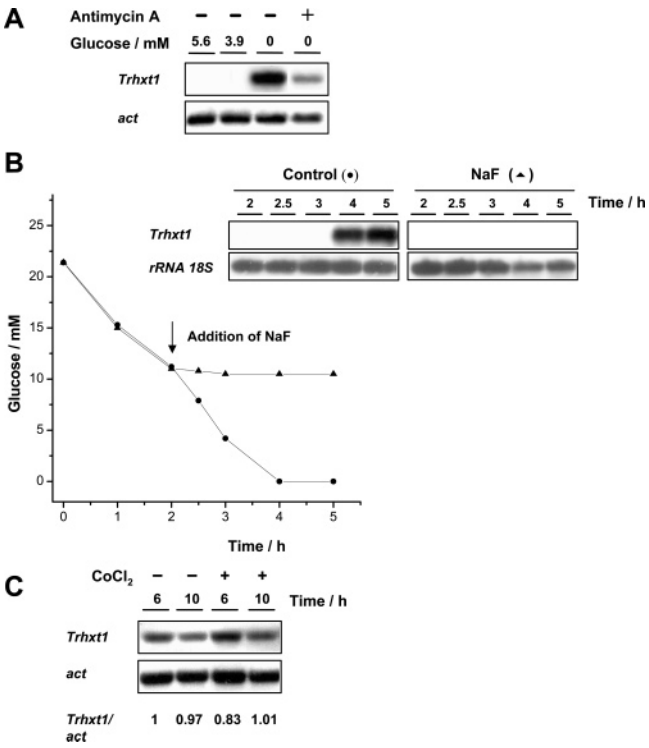

FIGURE 5: Effects of metabolic inhibitors and cobalt ions on the expression of *Trhxt1*. (A) and (B) *T. reesei* mycelia were grown aerobically in shake-flask cultures in the presence of glucose as the sole carbon source. At a concentration of ~10 mM glucose, the mitochondrial inhibitors antimycin A (1 μg/mL) and sodium fluoride (NaF) (10 mM) were added separately, and aliquots were then withdrawn as the glucose was consumed. Because of the repression of the actin transcript in the presence of NaF, rRNA 18 S was used as an internal control. (C) Cobalt chloride (400 μM) was added to shake-flask cultures of *T. reesei* grown in the presence of 2% glycerol. The normalized ratio of *Trhxt1* expression is presented in relation to the transcript level of the actin gene.

Because our results indicate that hypoxia lowered the metabolic activities of the cells and anoxia results in the disruption of the flow of electrons and metabolites in mitochondria and the glycolytic pathway, respectively, we reasoned that the use of well-known inhibitors of the respiratory chain and the glycolytic pathway, which would mimic the metabolic situation under hypoxia and anoxia, might shed light on how oxygen availability regulates *Trhxt1* expression.

Effects of Inhibitors of the Respiratory Chain and the Glycolytic Pathway on the Expression of the Trhxt1 Transcript. We tested the effect of a mitochondrial inhibitor, antimycin A, on the expression of *Trhxt1* expression. In the presence of antimycin A (1 μg/mL), there was a clear reduction in the expression of the *Trhxt1* transcript (Figure 5A).

In the presence of sodium fluoride (NaF) (10 mM), which inhibits the enzyme enolase in the glycolytic pathway, cells ceased to consume glucose, which was present in the culture medium at repressing concentrations, indicating the interruption of the flow of metabolites through the glycolytic pathway (Figure 5B). This interruption of the utilization of glucose mimics the effects of the anoxic conditions in which *Trhxt1* was induced in the presence of otherwise repressing concentrations of glucose (Figure 4). However, as shown in Figure 5B, the *Trhxt1* transcript was not induced in the presence of NaF. The result indicates that the inhibition of

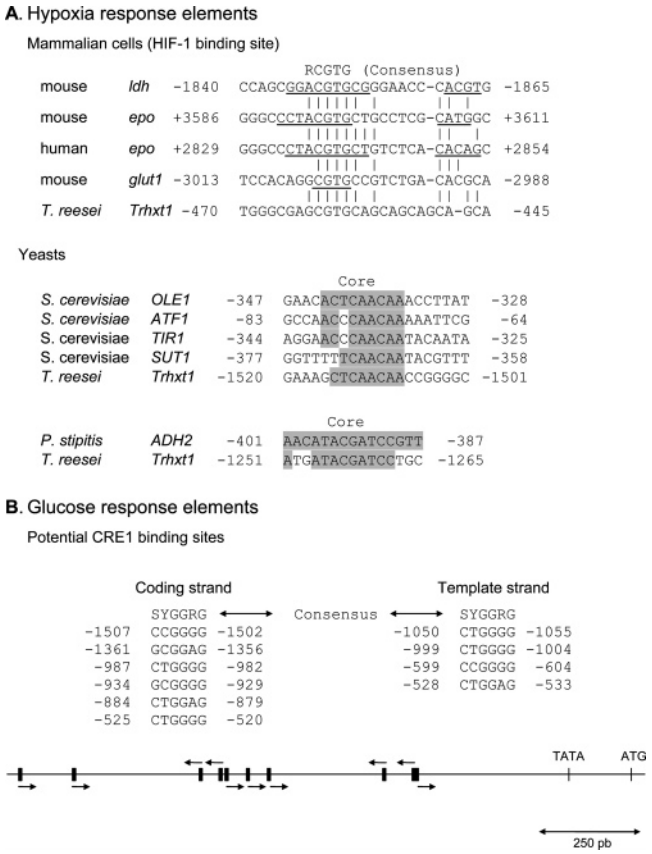

FIGURE 6: Potential cis-acting elements in the *Trhxt1* promoter. (A) Sequences identified in the *Trhxt1* promoter of *T. reesei* that are similar to hypoxia response elements from mammalian cells, *S. cerevisiae*, and *P. stipitis*. Nucleotides demonstrated to be critical for the function of the HIF-1 binding sites are underlined. The core sequences essential for the hypoxic induction of *OLE1* from *S. cerevisiae* and *ADH2* from *P. stitpitis* are shaded. Representative genes containing potential LOREs in *S. cerevisiae* are also indicated. (B). Glucose response elements. Schematic representation of potential CRE1 binding sites located in the promoter region of *Trhxt1* on the basis of the consensus sequence for *A. nidulans* CREA binding. The distribution (boxes) and orientation (arrows) of these elements are indicated.

the glycolytic pathway alone is not enough to overcome the repression of *Trhxt1* transcription by glucose.

Finally, transition metals, such as cobalt and nickel, and specific iron chelators are known to mimic low oxygen availability in mammalian cells, causing the stabilization and activation of HIF-1 and thus resulting in the induction of hypoxia-responsive genes in the presence of oxygen (*14*). In *S. cerevisiae*, the hypoxia-responsive gene *OLE1* coding for Δ9 fatty acid desaturase is also induced by transition metals in a similar way (*12*). To determine if a similar mechanism is involved in the induction of *Trhxt1* in *T. reesei*, we analyzed the effect of cobalt ions on the expression of its transcript. *Trhxt1* expression is not induced by cobalt ions over a wide range of concentrations (100 μM−5 mM) in cells grown in the presence of either glycerol or glucose (Figure 5C). The effect of the iron chelator 2,2′-dipyridyl on *Trhxt1* expression was also evaluated. No induction of the gene by this treatment was observed (data not shown).

Potential cis-Acting Elements Identified in the Promoter of Trhxt1. To identify potential elements that might be involved in the complex pattern of *Trhxt1* regulation in response to hypoxia and anoxia, the 5′-flanking region of the gene was sequenced and analyzed. Sequences similar to the consensus HIF-1 binding site (*19*, *38*) were identified in the *Trhxt1* promoter (Figure 6). In *S. cerevisiae* a hypoxia response element that is functionally related to the HIF-1 binding site has been reported. This so-called low oxygen response element (LORE) is essential for the induction of the *OLE1* gene by hypoxia (*39*). A sequence closely related to the LORE core was identified in the *Trhxt1* promoter and is shown in Figure 6. We also localized a sequence on the template strand of the *Trhxt1* promoter that is similar to a recently characterized element, which has been shown to be essential for the hypoxic induction of the *ADH2* gene in *Pichia stipitis* (Figure 6) (*40*).

In addition to elements involved in the hypoxic response, we also identified the sequence required for the binding of glucose repressors in fungi (*41*) including *T. reesei* (*42*).

DISCUSSION

Several properties of glucose transporters in eukaryotic microoorganisms reflect their adaptation to the environments in which the cells have evolved and to which they are adapted. For example, unlike heterotrophic organisms, photosynthetic organisms such as most microalgae, which evolved in aquatic environments, are unable to transport glucose for growth and energy needs. These microalgae such as *Phaeodactylum tricornutum* trap solar energy and form ATP and NADPH, which they use as energy sources to reduce CO_2 and H_2O to carbohydrates. Interestingly, introducing a human gene that encodes a glucose transporter, *glut1*, into the plasma membrane of *P. tricornutum* enables it to utilize glucose for energy production and growth in the absence of light (*43*). The unicellular microorganism *S. cerevisiae*, however, is mainly found in habitats that are rich in sugar, such as flowers and fruits, and it is adapted to transport glucose when exposed to a broad range of glucose concentrations (*16*).

The hexose transport system of *S. cerevisiae* is among the best characterized. It comprises 20 different hexose transport-related proteins (*16*). These HXT proteins belong to the major facilitator superfamily of transporters (*32*), and constitute two glucose uptake systems in *S. cerevisiae*: a low-affinity system (K_m = 25−45 mM) and a high-affinity system (K_m = 1−2 mM) (*16*, *24*). Although considerable information is available regarding the genes for and the properties of glucose transporters in *S. cerevisiae*, much less is known about the glucose transporters of microorganisms that evolved in nutrient-poor environments.

To investigate the properties of glucose transporters that evolved in nutrient-poor environments, we have isolated and characterized *Trhxt1*, the first gene involved in glucose accumulation from *T. reesei*, an economically important microorganism that finds application in the textile, food, and paper industries (*44*−*46*). Its product TrHXT1 has 12 (putative) transmembrane domains, a feature characteristic of proteins of the major facilitator superfamily. The involvement of TrHXT1 in glucose accumulation was demonstrated using a *T. reesei* mutant in which the sequence encoding the last four putative transmembrane domains of TrHXT1 were deleted; this mutant was found to have a 50−70% lower capacity for glucose accumulation at external glucose concentrations between 2.5 and 200 μM.

The transcript encoding the glucose transporter that is the subject of this report is expressed at micromolar levels of glucose and also induced during the induction of the major member of the cellulase system, *cbh1*, by cellulose and sophorose. The simultaneous expression of both genes, therefore, is most likely to be the result of the hydrolysis of cellulose to glucose, which gradually accumulates in the culture medium to the micromolar levels that induce the *Trhxt1* transcript, and the subsequent formation of the inducer of the cellulase system. Thus, the results argue for the involvement of *Trhxt1* in glucose utilization when *T. reesei* is growing on cellulose.

The complex pattern of gene expression controlled by oxygen and glucose presented in this work most probably represents the regulatory process that developed during the evolution of respiratory multicellular organisms from unicellular fermenting organisms. The alterations in the expression of *Trhxt1* mediated by oxygen and glucose could be explained by analyzing the metabolic state of the cell in the two phases of glucose utilization. In the first phase, under hypoxic conditions (between 1 and 0.2 mg/L of dissolved oxygen), although the cells were utilizing all of the available glucose as in normoxic conditions, metabolic activity was reduced by at least 25% as judged by CO_2 production and protein synthesis activity measured by genome-wide expression using microarrays (*47*). Under these hypoxic conditions, *Trhxt1* is down-regulated, most probably because of the lower metabolic and/or mitochondrial activity of the cells. Below the threshold oxygen concentration of 0.2 mg/L and in anoxia, the cells are impaired in glucose utilization because of the repression of oxidative phosphorylation and the inability of *T. reesei* to utilize glucose by anaerobic metabolism. The high-level expression of the transcript in this second phase is most probably triggered by an internal metabolic signal generated by the perturbation of glucose utilization. It is also conceivable that the signal responsible for glucose repression is inactivated as a result of the total absence of oxygen. Thus, at oxygen levels that facilitate the total utilization of glucose the expression of *Trhxt1* was dependent on the flow of electrons down the respiratory chain. This conclusion is supported by the downregulation of the *Trhxt1* transcript after the inhibition of complex III by antimycin A.

The strong expression of the transcript in the absence of oxygen seems not to be dependent on the level of metabolic flux through the glycolytic pathway because inhibitors of glycolysis, such as sodium iodoacetate and sodium fluoride, did not affect *Trhxt1* expression. The induction of the *Trhxt1* transcript below 0.2 mg/L oxygen resembles the expression of mammalian GLUT1, which is up-regulated in response to chronic hypoxia (*19*). However, the mechanisms controlling the expression of *Trhxt1* and mammalian hypoxic genes are most probably different because *Trhxt1* expression is not up-regulated by transition metal ions or iron chelation, and unlike *Trhxt1*, the *glut1* gene is up-regulated by the inhibitors of oxidative phosphorylation (*19*).

To the best of our knowledge, *Trhxt1* is the first gene for which the fact that glucose-mediated repression can be relieved by anoxia is demonstrated. Finally, it is tempting to speculate that *Trhxt1* played a crucial role in the adaptation of *T. reesei* to nutrient-poor environments and that the regulatory circuit controlling oxygen-dependent *Trhxt1* gene expression evolved to adapt the utilization of glucose in this microorganism during the evolutionary transition from a unicellular fermenter to an undifferentiated respiratory organism (*9*).

ACKNOWLEDGMENT

We thank Boris Stambuk for valuable discussions.

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BI052553Y