The Filamentous Fungus Aspergillus niger Contains Two “Differentially Regulated” Trehalose-6-phosphate Synthase-encoding Genes, tpsA and tpsB*

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Two genes encoding trehalose-6-phosphate synthase were cloned from Aspergillus niger. tpsA was cloned using the Saccharomyces cerevisiae GGS1/TPS1 gene as a probe. It encodes a 517-amino acid polypeptide with 64–70% similarity to trehalose-6-phosphate synthase of S. cerevisiae, Kluyveromyces lactis, and Schizosaccharomyces pombe. Its transcription occurs constitutively and is enhanced on carbon derepressing carbon sources, coinciding with the presence of a CREA-binding nucleotide motif in the 5'-unconing region of tpsA. Disruption of tpsA only weakly reduces growth on glucose, and neither influences the glucose induction of a low affinity glucose permease nor interferes with the catabolite repression of a pectinase; it causes reduced the heat tolerance of conidia. tpsB was cloned by a polymerase chain reaction-based strategy. Its 480 amino acid sequence showed 76.5% identity to tpsA. Its transcription was hardly detectable at ambient temperatures but was enhanced strongly upon heat shock, which agrees with the presence of several copies of a C/T stress-responsive element in its 5'-upstream sequences. Hence the function of yeast GGS1/TPS1 has been split into two differentially regulated genes in A. niger, of which none appears to be involved in glucose sensing.

Trehalose (α-glucosido-1,1-glucose) is a nonreducing disaccharide found in such diverse organisms as bacteria, fungi, algae, plants, invertebrates, and insects (1). In fungi, trehalose is accumulated by mycelia during the stationary phase and by conidia, and it is metabolized rapidly once growth is resumed or germination initiated. For a long time, therefore, trehalose has been considered a reserve carbohydrate (2). However, an additional role of trehalose as a protectant against various conditions has emerged more recently (3–5). Osmotic shock has emerged more recently (3–5).

A. niger

\textit{Saccharomyces cerevisiae GGS1/TPS1}

was cloned (9), which in \textit{S. cerevisiae} GGS1/TPS1 mutants would lead to an unrestricted flux through hexokinase and consequently inhibition of growth.

\textit{Aspergillus niger} is a filamentous fungus that has been used for decades for the industrial production of enzymes and organic acids (10, 11). Both product accumulations are strongly, yet divergently, influenced by the presence of glucose (12–14). Several enzymes have been shown to take part in the control of glycolysis in \textit{A. niger} (15), but under citric acid-producing conditions a major control point occurs at the level of hexokinase (16). Interestingly, the hexokinase of \textit{A. niger} is inhibited only weakly by trehalose-6-phosphate (17). We have therefore investigated the role of trehalose-6-phosphate synthase in the growth and glucose metabolism by \textit{A. niger}.

EXPERIMENTAL PROCEDURES

**Strains and Media**—A. niger ATCC 11414 was used throughout this study. Conditions for strain maintenance and preparation of conidia were described previously (18). Growth tests were performed on minimal medium (19), containing 2.0% (w/v) agar without biotin. Liquid cultures of the fungus were grown at 30 °C in 1-liter flasks containing 400 ml of complete medium, consisting of minimal medium plus 0.5% (w/v) yeast extract and 0.5% (w/v) peptone, and a carbon source (3% (w/v)). Flasks were inoculated with $2 \times 10^6$ conidia and incubated on a rotary shaker at 250 rpm for 14–18 h.

To induce heat stress response, \textit{A. niger} cultures were pregrown in complete medium for 18 h, and aliquots of 20 ml of culture broth in 100-ml flasks were then transferred to a shaking water bath and incubated at 40 °C for 60 min and 120 rpm.

**Escherichia coli** strain DH5a was used for propagation of plasmids and grown under standard conditions (20).

**Plasmids and Vectors**—A plasmid containing a 1.9-kb\(^1\) BamHI/XhoI fragment of the \textit{S. cerevisiae} GGS1/TPS1 gene locus cloned into YEPlac 181 (8) was obtained from J. Thevelein, Leuven, Belgium. pAN7–1(21) was obtained from P. Punt, Rijswijk, The Netherlands.

The vectors pGEM-5Z(+) \((\text{Promega, Madison, WI})\) and pBluescript SK\(^+\) (Strategene) were used for cloning.

**Cloning of the tpsA Gene**—A BamHI/XhoI fragment of GGS1/TPS1

\(^\text{1}\) The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; bp, base pair(s); GST, glutathione S-transferase; ORF, open reading frame.
was labeled with $^{32}$PdCTP by random priming and used to screen 40,000 plaques of the genomic library. Hybridization was carried out at 60°C. The final washing was done in 2 × SSC, 0.1% SDS at the same temperature. Inserts of hybridizing clones were analyzed by restriction mapping and subcloned.

Cloning of the tpsB Gene—Using sequence similarities of the GGS1/TPS1/tps1 genes from S. cerevisiae (8), K. lactis (22), S. pombe (9), and A. niger tpsA (see “Results”) two degenerated primers were designed for amplification: ggsdegI (5'-GGGTAGATTATAYTNTGT-3') and ggsdegII (5'-GGNGACNCYTATTCAT-3'). PCR products were amplified in a total volume of 50 μl containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 4 mM MgCl₂, 1% Triton X-100, 0.5 mg of genomic DNA, 25 pmol of each primer, 200 μM dNTPs, and 0.5 units of Taq polymerase (Biomedica). The program for amplification consisted of a 1-min incubation at 95°C followed by 35 cycles of 1 min at 95°C, 1.5 min at 55°C and 1 min at 72°C and was terminated by a final extension for 7 min at 72°C. Protruding 5′-termini of PCR products were removed with Vent polymerase (New England Biolabs) according to the manufacturer’s instructions and were analyzed by restriction cleavage with Tqi. A 450-bp fragment, verified as a fragment of tpsB by sequencing, was consequently used as a probe to screen 40,000 plaques of the genomic library. Hybridization was done at 64°C. The final washing was performed in 0.2 × SSC, 0.1% SDS at the same temperature. Appropriate fragments of hybridizing clones were subcloned, subjected to restriction mapping, and sequenced.

DNA Sequencing and Analysis—Sequencing was performed with the Sequenase version 2.0 system (U. S. Biochemical Corp.) and by means of an automatic sequencer (Applied Biosystems), using both universal primers as well as a series of primers specific for both strands of the tpsA locus. Sequence similarities were investigated using the BLAST server (23). Alignments were performed using the program MACAW (24) and improved by visual inspection.

Gene Disruption—A 4.5-kb NdeIVnol fragment of the tpsA locus containing the whole tpsA structural gene and 2.3 kb and 0.38 kb of its 5′- and 3′-noncoding sequences, respectively, was cloned into pBlue-script SK+ (previously cut with SalI and NotI) to yield pGARP10. To disrupt the tpsA locus of A. niger, a 2.6-kb fragment of the T. gigantea tpsA gene flanked by the Aspergillus nidulans gpdA promoter and the trpC terminator was released from pAN7–1 (21) by restriction cleavage with SacI and HindIII. Protruding 5′- and 3′-termini were filled or removed, respectively, with Klenow enzyme, and the fragment was ligated into the unique EcoRV site of pGARP10, located within the tpsA coding sequences (395 bp) to yield pGARP11 (see Fig. 4A). The NotI/Vhol insert of pGARP11 was introduced into A. niger protoplasts by transformation as described by Yelton et al. (25). They were regenerated in liquid medium for 3 h at 30°C containing 2% (w/v) glycerol, 0.5% (w/v) yeast extract, and 0.5% (w/v) peptone for 3 h at 30°C followed by plating on solid minimal medium containing 300 μM/ml hygromycin B (Calbiochem) and glycerol as a carbon source.

Standard Molecular Biological Techniques—Plasmid constructions, E. coli transformation, plasmid isolations from E. coli, restriction enzyme usage, isolation of chromosomal DNA, Southern and Northern blotting, preparation of $^{32}$P-labeled DNA probes, hybridization of blots, and construction of the gene library in EMBL3 were performed according to standard protocols (20). Isolation of DNA fragments from agarose gels was performed with a Quiaex II kit (Quiagen) according to the manufacturer’s protocols. RNA was isolated as described by Chomczynski and Sacchi (26) Polyclonal antibodies were isolated from total RNA using the FastTrack 2.0 system (Invitrogen, San Diego, CA) according to the manufacturer’s instructions.

Gel Mobility Shift Assays and Footprinting Techniques—A ScaI/HpaII fragment of the 5′-noncoding region of tpsA was prepared and radioactively labeled with $^{32}$PdCTP and appropriate dNTPs by filling in with the Klenow fragment of DNA polymerase. A CreA::GST fusion protein was obtained by overexpression of a NcoI fragment (from nucleotides 103–722) of the A. nidulans creA gene (27) containing the two C2H2 zinc fingers and an alanine-rich region as a GST fusion in E. coli, contained in vector pGEX-CreA, followed by the expression and purification protocol of Kullberg et al. (28). Polyacrylamide gel shifts and methylation protection footprinting were performed as described previously.

Enzyme Assays—Cell-free extracts were prepared by grinding mycelium in liquid nitrogen, suspending the powder in 20 mM HEPES, pH 7.1, containing 20% (w/v) glycerol, 2 mm EDTA, 1 mm dithiothreitol, and 0.1 mm phenylmethylsulfonyl fluoride, and centrifugation (15 min, 5,000 × g, 4°C). Trehalose-6-phosphate synthase was measured as described by Cahill and Leloir (6) with the modifications introduced by Vanderammen et al. (30). Assay of glucose transport was carried out as described previously (32). Protein concentration was determined by the dye binding method (31).

Determination of Trehalose—Mycelia were thereafter harvested by filtration, ground under liquid nitrogen, and trehalose extracted by their suspension in distilled water at 95°C for 30 min. After centrifugation for 10 min, 12,000 × g, 18°C, the extracts were passed through a 0.45-μm filter. Trehalose was determined using the method in Ref. 33 except that commercial porcine kidney trehalase (Sigma) was used.

Electrophoretic Techniques—For demonstration of individual pectinases in the supernatant, samples from the culture broth were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting to nitrocellulose and immunological detection as described previously (34). A polyclonal antiserum was used to detect pectinase PL II (35).

RESULTS

Cloning of the A. niger tpsA Gene—To clone the A. niger equivalent of GGS1/TPS1 we screened a genomic λEMBL3 library of A. niger by heterologous hybridization with the S. cerevisiae GGS1/TPS1 gene and obtained seven positive clones. Restriction mapping and Southern hybridization showed that all clones were part of the same gene locus (data not shown). Hybridizing DNA fragments were subcloned into pGEM-5Zf(+) and sequenced. The clones revealed the presence of an ORF of 1,825 bp (Fig. 1), which was interrupted by four introns (identified by the presence of consensus sequences of splicing signals for filamentous fungi; (36)). High stringency Southern hybridization of A. niger genomic DNA cleaved with XhoI, which does not cut within the gene, with a tpsA-specific probe yielded a single band, suggesting that tpsA occurs as a single copy in the A. niger genome. The predicted 517-amino acid sequence shows high identity to the GGS1/TPS1 gene products of S. cerevisiae (64.0%), K. lactis (64.3%), and S. pombe (69.5%).

The 5′-noncoding region of tpsA contains two putative CreA binding sites (5′-SYGGRG-3′; (28, 37)) at positions −169 and −177, organized as a tandem repeat. Binding sites for the yeast carbon catabolite repressor protein MIG1, whose DNA binding domain shows high similarity to that of A. nidulans and A. niger CreA (27, 38), are also found in the promoter of GGS1/TPS1 from S. cerevisiae (8) and K. lactis (22). Using a CreA::GST fusion protein, binding to a tpsA restriction fragment resembling bp −58 to −265 (ScaI/HpaII) was shown by gel retardation assays and by methylation protection footprinting in vitro (Fig. 2). To investigate whether transcription of tpsA was indeed regulated by carbon catabolite repression, Northern analysis of mRNA, harvested from A. niger grown on various carbon sources, was carried out (Fig. 3A). tpsA expression was observed on all carbon sources tested, but the ratio between the tpsA and the actA (control) transcripts was higher during growth on citrate and arabinose than on glucose and lactate, which would be consistent with partial regulation by carbon catabolite.

Transcription of the S. cerevisiae and the S. pombe GGS1/TPS1 is known to be inducible by heat stress (8, 9). We did not find any of the consensus sequences for binding of heat shock regulating proteins in the tpsA promoter. However, a single copy of the C2T motif (consensus core sequence CCGCTC), which mediates transcription in response to various conditions of stress in S. cerevisiae (39), was present at −386. Northern analysis of A. niger cultures subjected to 40°C heat shock showed that tpsA transcription declined immediately upon transfer to 40°C (Fig. 3B). We conclude that A. niger transcription is not triggered by heat shock. Sequences resembling the consensus for binding of yeast GCR1 (CCTTC (40)) were found at −249 and −457, but those for binding of other activators of glycolytic genes were not found.

Disruption of the tpsA Gene—To prove that tpsA encodes a trehalose-6-phosphate synthase of A. niger and to study its
function in this fungus, we replaced it with a tpsA derivative into which the E. coli hph gene from pAN7–1 had been inserted by transformation (Fig. 4A). Transformants, in which the tpsA gene had been replaced by the disrupted gene, were identified by the presence of a 9.6-kb DNA fragment, which replaced the wild type 7.0-kb band (Fig. 4B). One of these, strain *A. niger* tpsA*D*1–3, was selected for further analysis. When grown at 30°C on glucose or glycerol its trehalose-6-phosphate synthase activity was below the detection limit (Fig. 5), which proves that the gene encodes trehalose-6-phosphate synthase. Interestingly, the mycelial trehalose content at 30°C was reduced strongly compared with the parental strain, but it was not depleted completely. In view of the clear reduction of trehalose-6-phosphate synthase activity, this suggests the presence of alternative enzymes/pathways for trehalose biosynthesis in *A. niger*. This assumption was supported strongly by the findings that very high trehalose-6-phosphate synthase activity was observed in the disruptant strain upon growth of *A. niger* at 40°C, which was virtually similar to that of the parent strain where it represented a 6- and 15-fold increase (on glucose and glycerol as carbon source, respectively). This was also reflected in drastically increased mycelial trehalose concentrations. Interestingly, although this trehalose-6-phosphate synthase activity was twice as high on glycerol as the carbon source, the trehalose content on this carbon source was 30–40% lower, which may be due to a lower glucose-6-phosphate supply and/or a different regulation of trehalose-6-phosphatase by glycerol and glucose.

Disruption of *GGS1/TPS1* in *S. cerevisiae* and *K. lactis* results in an impairment of growth on glucose, fructose, and other rapidly fermentable sugars (8, 22). To see whether there was a similar phenotype in *A. niger* tpsA*D*1–3, the wild type as well as the disruptant were pregrown for 24 h at 30°C on solid minimal medium under derepressing conditions (0.1% glycerol) and then transferred to fresh medium containing different carbon sources.

**FIG. 2. In vitro binding of CreA::GST to the CreA binding motif in tpsA.** Panel *A*, gel mobility shift assay using a Scal/HpaII fragment of the 5′-noncoding sequences of *tpsA* comprising the two adjacent CreA binding motifs as a probe. *fp*, free probe (2 ng, no protein added); *nc*, 2-ng probe plus 100 ng of the CreA::GST fusion protein; *10x*, same as lane *nc* but with an additional 20 ng of unlabeled probe; *50x*, same as lane *nc* but with an additional 100 ng of unlabeled probe. Panel *B*, confirmation of binding sites by methylation protection footprinting. Lanes *pp* and *fp* indicate results in the presence and in the absence of the CreA::GST fusion protein, respectively. Protected guanidines are indicated by asterisks, and the hypersensitive site is indicated by a plus sign. Panel *C*, schematic summary of the binding information as obtained from panels *A* and *B*. Symbols are as in panel *B*.
concentrations of glucose; the increase in colony diameter was measured over a period of 5 days. Virtually the same growth rate on glucose was observed with the wild type strain and strain tspAΔ1–3 at 30°C, but the disruptant grew slightly lower at 37°C and at 40°C. Similar results were also obtained with glycerol as a carbon source. No significant differences in the morphology of the growing hyphae were observed between the parent strain and the disruptant at either temperature.

In contrast to S. cerevisiae and K. lactis, the only effect of disruption of tsp1 in S. pombe was a failure to germinate in a medium with glucose as a carbon source (9). A. niger tspAΔ1–3 showed no retardation or even deficiency in germination on glucose, however (data not shown).

The data described above suggest a role of trehalose-6-phosphate synthase A for mycelial growth at higher temperature. To study the possibility that the reduced trehalose content in A. niger tspAΔ1–3 also affects its viability at temperatures where growth no longer takes place, we incubated conidiospores of the wild type and the disruptant strain at 50 and 55°C, respectively, and thereafter analyzed their ability to germinate at 30°C. At 50°C germination of the disruptant exhibited a 27% reduction compared with the parent strain (94% versus 70% of the control), and this effect was even more dramatic at 55°C (7.4 versus 2.6% of the control; i.e. 32%). Considering the fact that the trehalose content in A. niger tspAΔ1–3 is reduced by 56%, these data support the assumption that trehalose contributes to the heat stability of A. niger conidiospores.

To learn whether the functional impairment of tspA leads to glucose derepression (as has been shown for yeast invertase and α-glucosidase (8)), A. niger tspAΔ1–3 and its parent strain were grown on glucose as a carbon source, and the formation of pectinase A (which is subject to glucose repression (35)) was determined by Western blotting and immunostaining. Under these conditions, pectinase A remained below the limit of detection in both strains, whereas it was demonstrated clearly during growth on pectin (data not shown). We conclude that a disruption of tspA in A. niger does not lead to carbon catabolite derepression.

A. niger has been shown to respond to the presence of elevated concentrations of glucose or sucrose (>5%, w/v), by increasing the glycolytic flux and citric acid accumulation (14, 18). This effect is, among others, reflected in the induction of a low affinity glucose permease by elevated glucose concentrations (32). However, in the presence of 10% glucose the low affinity transporter was formed in the wild type as well as the disruptant strain, and both its Km (4.1 m) as well as Vmax (0.14 μmol/min/mg dry weight) were virtually the same. We therefore conclude that tspA is not involved in the signaling of high glucose concentrations to A. niger.

Cloning and Transcriptional Analysis of the A. niger tspB Gene—The fact that A. niger tspA was only expressed constitutively, yet the disruptant strain still contained high trehalose-6-phosphate synthase activity at 40°C, suggested the presence of a second, heat-inducible trehalose-6-phosphate synthase-encoding gene in A. niger. Using the predicted protein sequences of the three GGS1/TPS1 genes from S. cerevisiae, K. lactis, and S. pombe as well as of tspA, a pair of degenerated oligonucleotide primers was designed which corresponded to the nucleotide sequences encoding at nucleotides 145–156 and 190–198 in the tspA polypeptide. PCR amplified a single nucleotide fragment of 0.5 kb. Restriction fragment length polymorphism analysis with HinII and TaqI showed that this fragment corresponded to two different amplicons and that the restriction fragments of one of them were in accordance with those predicted from the tspA sequence. The other amplicon was unique. Using a 450-bp fragment of this amplicon as a probe, the A. niger library was screened under stringent con-
and three positive clones were obtained. Subcloning and sequencing showed that the \( tpsB \) gene is contained in an ORF of 1,732 bp (Fig. 6). Four putative introns occur at the same relative position as in \( tpsA \). The predicted 480-amino acid sequence shows 76.5% identity to \( tpsA \), and 64.0, 64.0, and 65.1% identity to the \( GGS1/TPS1 \) gene products from \( S. \) cerevisiae, \( K. \) lactis, and \( S. \) pombe, respectively (Fig. 7).

In contrast to \( tpsA \), the 5\(^{\prime}\)-nontranscribed sequences of \( tspB \) did not contain nucleotide sequences homologous to CreA binding sites. In analogy with \( tpsA \), sequences resembling the consensus for binding of yeast GCR1 (CCTTC (40)) were also present at 2,17, 2,152, 2,300, 2,375, and 2,412. Interestingly, the C4T motif identified in \( tpsA \) occurred in five copies in \( tspB \), i.e. at 2,14, 2,103, 2,224, 2,230, and 2,343, respectively. Probing the Northern blots of Fig. 3 with the \( tspB \) gene revealed only a low level of transcript at 30°C, whose level was even lower on arabinose and citrate, but the transcript increased strongly upon transfer to 40°C (Fig. 8). These findings are consistent with the assumption that \( tspB \) encodes the heat-induced trehalose-6-phosphate synthase activity of \( A. \) niger.

**DISCUSSION**

We have isolated from \( A. \) niger two different genes encoding trehalose-6-phosphate synthase (\( tpsA \) and \( tspB \)), as indicated by the fact that the genes have roughly 65% identity at the amino acid level with the \( GGS1/TPS1 \) genes of \( S. \) cerevisiae (8) and \( K. \) lactis (22) and the \( TPS1 \) gene of \( S. \) pombe (9). Furthermore, disruption of \( tspA \) virtually reduces the trehalose-6-phosphate synthase activity under conditions where this gene is selectively expressed (e.g. growth on glucose as a carbon source) to zero.

Since \( tpsA \) is expressed more strongly during vegetative growth on a variety of carbon sources at ambient temperatures than \( tspB \) we considered it a more likely candidate for exerting a regulatory effect on glucose metabolism similar to that of

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**Fig. 5.** Effect of \( tpsA \) disruption on trehalose-6-phosphate synthase activity and trehalose content in \( A. \) niger. Data show trehalose-6-phosphate synthase activity and trehalose content of mycelia of \( A. \) niger ATCC 11414 (wild type, WT) and of \( A. \) niger \( tpsA \), respectively, grown at 30°C for 18 h on glucose (G) and glycerol (Y) and after a further 1-h incubation at 40°C. Bars indicate standard deviations (n = 3).

**Fig. 6.** Nucleotide sequences of the \( A. \) niger \( tspB \) gene and the deduced amino acid sequence. The derived amino acid sequences are given at the second position of each codon by the one-letter code. Putative CreA binding sites are printed in bold and are underlined. Nucleotide sequences homologous to C(T) stress response elements are double underlined. Binding sites for GCR1 (CCTTC) are printed in bold and are underlined by dots. Intronons are printed in lowercase letters. The stop codon is indicated by an asterisk.
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GGS1/TPS1 in yeast. The phenotype of tpsA gene disruption in A. niger, however, was quite different from that produced by the disruption of the GGS1/TPS1 genes in S. cerevisiae and K. lactis and resembled that observed in S. pombe in some aspects. In agreement with the latter but in contrast to S. cerevisiae and K. lactis, a disruption of tpsA did not influence the capacity of A. niger to grow on glucose. Blázquez et al. (9) attributed this difference between S. cerevisiae (and K. lactis) and S. pombe to the different behavior of the hexokinases of these yeasts toward trehalose 6-phosphate, a strong inhibitor of S. cerevisiae and K. lactis hexokinases, but not that of S. pombe (9). Such an explanation also may be valid for A. niger, whose hexokinase has a rather low $K_i$ for trehalose 6-phosphate (17) and is virtually not inhibited by glucose 6-phosphate (41). The low $K_i$ for trehalose 6-phosphate is reflected by the findings that disruption of tpsA leads to an increase in glycolytic flux at very high sugar concentrations (>5%, w/v) only, apparently because an intracellular concentration of trehalose 6-phosphate, sufficient for inhibiting hexokinase, can only be accumulated under these conditions (17). All of these findings argue against a regulatory role of tpsA in A. niger glycolysis during vegetative growth on conventional media containing low carbon source concentrations.

Trehalose has been attributed a dual role as a reserve carbohydrate (2) and as a protectant of proteins against denaturation by dehydration (5). Consistent with the first role, disruption of tpsB in S. pombe prevented germination of conidia (9). In contrast, disruption of tpsA in A. niger had no effect on the germination of conidia. These differences may be because disruption of tpsA does not produce a null phenotype of trehalose-6-phosphate synthase activity because of the presence of tpsB. Interestingly, tpsA disruption and the accompanying reduction of the conidial trehalose concentration lead to a reduction in the heat stability of the conidia. This suggests that tpsA contributes to trehalose formation during conidiation and that its lack cannot be fully compensated by tpsB.

In contrast to tpsA, the tpsB transcript was hardly detectable during vegetative growth of A. niger but accumulated strongly during heat shock. A similar behavior has been described for S. cerevisiae GGS1/TPS1 (8) and S. pombe tps1 (9) gene expression. This heat shock-triggered gene expression is consistent with the presence of an element with the sequence AAGGGGAT in the 5'-noncoding sequences of GGS1/TPS1 (8), which confers regulation of certain S. cerevisiae genes by heat shock, nitrogen starvation, and the RAS-cAMP pathway (39, 42). Several copies of this motif are also present in the 5'-upstream sequences of tpsB, and it is tempting to speculate that this motif confers regulation by heat shock also in A. niger. However, as the functionality of this motif in Aspergillus and other filamentous fungi has not yet been demonstrated, this assumption must still be treated cautiously. The fact that a single AAGGGGAT motif also occurs in the 5'-upstream regions of tpsA, whose expression is not triggered by heat shock, indicates that its mere presence in A. niger does not imply functionality and suggests at least that they may be subject to position effects. Kobayashi and McEntee (42) reported that a single copy of the C4T motif is active in S. cerevisiae, yet the presence of two such motifs evokes a more than additive response.

We have shown here for the first time that the biological function of a gene that is present in a single copy in several yeasts (S. cerevisiae, K. lactis, S. pombe) is fulfilled by two genes in the filamentous fungus A. niger, which encode highly similar polypeptides but are regulated differently. A comparison of the amino acid sequences of the tpsA and tpsB gene products does not provide a clue as to the reason for this. Interestingly, while this paper was prepared for publication, Borgia et al. (43) reported on the presence of two trehalose-6-phosphate phosphatases in A. nidulans but did not investigate whether the two gene products may have different functions. It
will be interesting to see if this is a general feature of multicellular fungi or a feature unique to aspergilli.

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FIG. 8. Northern blot analysis of tpsB transcription during growth on different carbon sources (panel A) and upon exposure to heat stress (panel B). Panel A, A. niger wild type was grown for 18 h on complete medium containing the carbon sources as indicated. 100 μg of total RNA was applied to each lane. A 1.56-kb HindIII/SacI fragment (corresponding to position −295 and +1283 of the tpsB sequence) was used as a probe. The blot was rehybridized with a restriction fragment containing the whole ORF of the A. nidulans actA gene as control. Hybridization was carried out at 42°C. The final washing was generally done in 0.3 × SSC, 0.1% SDS at 60°C for 40 min, except for the actA control where the temperature was lowered to 55°C. Panel B, A. niger wild type was grown at 30°C on complete medium containing either glucose or glycerol as carbon source and the temperature then rose to 40°C for 1 h. RNA was isolated at the times indicated, and poly(A)+ RNA corresponding to 500 μg of total RNA was applied to each lane. Hybridization conditions were the same as in panel A.