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Disruption of *TPS2*, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae*, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity

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Preparations of the trehalose-6-phosphate synthase/phosphatase complex from *Saccharomyces cerevisiae* contain three polypeptides with molecular masses 56, 100 and 130 kDa, respectively. Recently, we have cloned the gene for the 56-kDa subunit of this complex (*TPS1*) and found it to be identical with *CIF1*, a gene essential for growth on glucose and for the activity of trehalose-6-phosphate synthase. Peptide sequencing of the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex (TPS2) revealed one sequence to be 100% identical with the deduced amino acid sequence of the upstream region of *PPH3* on the right arm of chromosome IV. This sequence was used to clone an upstream region of *PPH3* containing an open reading frame of 2685 nucleotides, predicted to encode a polypeptide of 102.8 kDa. The N-terminal sequence, as well as three internal amino acid sequences, obtained from peptide sequencing of the 100-kDa subunit, were identical with specific regions of the deduced amino acid sequence. Thus, the sequence cloned represents *TPS2*, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex. Interestingly, a stretch of about 500 amino acids from the first part of TPS2 was 33% identical with the entire TPS1 sequence. Disruption of *TPS2* had no effect on trehalose-6-phosphate synthase activity but caused complete loss of trehalose-6-phosphate phosphatase activity, measured *in vitro*, and accumulation of excessive amounts of trehalose-6-phosphate instead of trehalose upon heat shock or entrance into stationary phase *in vivo*. These results suggest that *TPS2* codes for the structural gene of the trehalose-6-phosphate phosphatase. Heat shock induced an increase in trehalose-6-phosphate phosphatase activity and this was preceded by an accumulation in *TPS2* mRNA, suggesting that the trehalose-6-phosphate phosphatase is subjected to transcriptional control under heat-shock conditions.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranose) is a disaccharide found in as diverse organisms as algae, bacteria, insects, invertebrates and yeasts (Elbein, 1974). Since trehalose accumulates in most of these organisms upon nutrient limitation as well as upon physical and chemical stresses, it probably functions not only as a carbohydrate reserve but principally as a protectant against damage imposed by stresses (for review see Van Laere, 1989; Wiemken, 1990). We have become interested in the close correlation between the accumulation of trehalose and the acquisition of tolerance to short term heat stress in yeast (Hottiger et al., 1987a and b, 1989 and 1992; Attfield, 1987; De Virgilio et al., 1990 and 1991). This prompted us to study the regulation of trehalose biosynthesis.

Trehalose is known to be formed in a two step process (Cabib and Leloir, 1958). In the first step, trehalose-6-phosphate synthase (UDP-glucose:D-glucose-6-phosphate-1-glucosyl transferase) transfers a glucosyl residue from UDP-glucose to glucose-6-*P* to yield trehalose-6-phosphate (Tre6*P*). In the second step, this compound is split into trehalose and P_i by a specific trehalose-6-phosphate phosphatase (trehalose-6-phosphate phosphohydrolase). Several studies have dealt with the purification and kinetic properties of Tre6*P* synthase and Tre6*P* phosphatase in yeast (Elander, 1968; Vandercammen et al., 1989; Londesborough and Vuorio, 1991; Vuorio et al., 1992; Bell et al., 1992). Fructose-6-*P* was found to be a strong activator of Tre6*P* synthase (Vuorio et al., 1992), whilst inorganic P_i inhibited Tre6*P* synthase and activated Tre6*P* phosphatase (Vandercammen et al., 1989; Vuorio et al., 1992). Furthermore, evidence was presented that both Tre6*P* synthase and Tre6*P* phosphatase were subjected to catabolite inactivation and repression during growth on glucose (François et al., 1991). In addition, substrate

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Abbreviations. Tre6*P*, trehalose-6-phosphate; PCR, polymerase chain reaction; ORF, open reading frame; TPS2, 100-kDa subunit of trehalose-6-phosphate synthase/phosphatase complex.

Enzymes. UDP-glucose: D-glucose-6-phosphate-1-glucosyltransferase (EC 2.4.1.1.5); trehalose-6-phosphate phosphohydrolase (EC 3.1.3.12).

Note. The novel nucleotide sequence data published here have been submitted to the EMBL Data Bank and are available under accession number X70694.

availability (Winkler et al., 1991) and post-translational modification (Panek et al., 1987; De Virgilio et al., 1990) may regulate activities of these enzymes. In contrast to trehalase, Tre6*P* synthase activity is probably not regulated by protein phosphorylation (François et al., 1991).

Recently, a proteolytically modified Tre6*P* synthase/phosphatase complex of 800 kDa has been purified from *Saccharomyces cerevisiae*, consisting of three polypeptides of 57, 86 and 93 kDa (Londesborough and Vuorio, 1991). Using a different purification protocol, we have purified an enzyme complex with less proteolytic modifications, consisting of three major polypeptides of 56, 100 and 105 kDa and we have cloned *TPS1*, the gene for the 56-kDa subunit (Bell et al., 1992). *TPS1* is identical with *CIF1*, a gene reported to be essential for growth on glucose (Gonzáles et al., 1992). Hence, TPS1 seems to play an important role in the regulation of glucose and trehalose metabolism.

In the present study, we employ the previously reported purification protocol (Bell et al., 1992) with special care to prevent proteolytic modification, and we report the isolation of a Tre6*P* synthase/phosphatase complex with three major polypeptides of 56, 100 and 130 kDa. Tre6*P* synthase and Tre6*P* phosphatase co-purified throughout the purification. Using this preparation, we identified and cloned *TPS2*, the gene encoding the 100-kDa subunit of the Tre6*P* synthase/phosphatase complex. We show that disruption of *TPS2* causes loss of the activity of Tre6*P* phosphatase but not of Tre6*P* synthase *in vitro* and accumulation of Tre6*P* *in vivo*, suggesting that *TPS2* is the structural gene for the Tre6*P* phosphatase.

MATERIALS AND METHODS

Yeast strains and culture conditions

As in our previous work (Bell et al., 1992), strain C13-ABYS86 (MATα, *his3, leu2, ura3, can*^{r}, pra1-1, prb1-1, prc1-1, cps1-3), kindly supplied by D. H. Wolf, University of Freiburg, FRG, was used for purification of the Tre6*P* synthase/phosphatase complex. Strain RH 144-3A (MATα, *ura3, his4, leu2, bar1-1*), a kind gift of H. Riezman, Biocenter Basel, Switzerland was used for gene disruption.

S. cerevisiae media were prepared according to Sherman et al. (1986). For purification of the Tre6*P* synthase/phosphatase complex, cells were grown to stationary phase in liquid YPD media (1% yeast extract, 2% bactopeptone, 2% glucose). Cultures well adapted to exponential growth (at least five generations of exponential growth) at a density of $0.5-1.0\times10^7$ cells/ml were used for the heat-shock experiments (temperature shift of 27−40°C).

Bacterial strains and media

Escherichia coli JM 101 (*sup*E, *thi*, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]) was used as host for plasmid constructions. Bacterial media were used as described by Sambrook et al. (1989).

Purification of the trehalose-6-phosphate synthase/phosphatase complex and protein sequences

The purification of the Tre6*P* synthase/phosphatase complex was performed exactly as described in Bell et al. (1992), using stationary-phase cells of the *S. cerevisiae* strain C13-ABYS86 lacking proteases A and B and carboxypeptidases Y and S. In order to minimize proteolytic degradation of the Tre6*P* synthase/phosphatase complex, special care was taken to speed up the overall purification procedure from approximately one week to three days.

The intact purified Tre6*P* synthase/phosphatase complex was subjected to polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970) using 10% acrylamide. After staining (0.1% Coomassie brilliant blue R-250), the band containing the 100-kDa subunit of the Tre6*P* synthase/phosphatase complex was cut out from the gel, equilibrated in 0.1 M NH_4HCO_3, 0.1% SDS and subjected to electroelution (Electro eluter 422, BioRad). The dried eluate was resuspended in 90% ice-cold ethanol and incubated overnight at −20°C. After centrifugation (10 min at 10000 *g*), the dried protein pellet (15 µg) was dissolved in 50 µl 0.4 M NH_4HCO_3, 8 M urea and treated with 5 µl 45 mM dithio-DL-threitol at room temperature. Cysteine residues were alkylated in the dark with 5 µl 0.1 M iodoacetamide for 15 min at room temperature. Subsequently, the protein solution was diluted with 140 µl water and digested overnight at 37°C with 0.75 µg trypsin (Boehringer, sequence grade). The peptides in the incubation mixture were separated by reverse-phase HPLC on a Vydac 218TP 52 (2.1 mm×250 mm) column with a linear acetonitrile gradient (0−70%) in 0.05% trifluoroacetic acid over three hours at a flow rate of 150 µl/min, using a Hewlett Packard 1090 apparatus. Ultraviolet-absorbing peaks were collected manually and selected peaks were then subjected to sequence analysis.

Microsequencing was performed using automatic Edman degradation with on-line HPLC analysis of phenylthiohydantoin (PTH)-labelled amino acids on an Applied Biosystems 477A microsequenator and a 120A PTH amino acid analyser.

Cloning of the *S. cerevisiae* TPS2 gene

The peptide sequences were used for a search of homologous sequences in the data bank at EMBL (Heidelberg, FRG) using the TFASTA program. One sequence (*LLSFTDXFD*-LEVM) was 100% identical with the deduced amino acid sequence of the upstream region of *PPH3* on the right arm of chromosome IV (Ronne et al., 1991; EMBL accession no. X 58858). The length of the potential open reading frame (ORF) in the data bank was 870 bp. A 565-bp fragment of this region was isolated by the polymerase chain reaction (PCR) method with the two synthetic oligonucleotides TAACTTGGCATTATAGACGTACCGTT and TAAAAAGGGTGTTTCAAACCTTTGCG as primers and genomic DNA of RH 144-3A as template, using the PCR Kit (Perkin Elmer Cetus) according to the manufacturers instructions. The fragment obtained was labeled with [α-^{32}P]dATP (Amersham), using the Random Primed DNA Labeling Kit from Boehringer Mannheim (FRG), and subsequently used to screen a genomic DNA library (in Psey8; kindly provided by M. Hall, Biocenter, Basel, Switzerland) as described in Sambrook et al. (1989). Positive clones were confirmed by nucleotide sequencing. We found two overlapping clones (pSEY-TP6 and pSEY-TP12; Fig. 1), both about 8 kb in length, which together covered the whole *PPH3* gene and an additional 7 kb of the upstream region of the latter gene. A segment of 3915 bp, covering the whole sequence of the ORF upstream of *PPH3*, was sequenced in both directions.

Gene disruption and Southern-blot analysis

Gene disruption was accomplished by the one step procedure of Rothstein (1983). The *Eco*RI--*Bgl*II fragment of

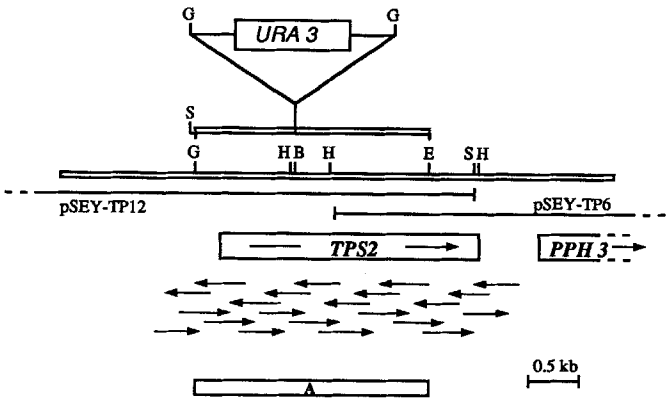

Fig. 1. Restriction map, sequencing strategy and gene disruption of *TPS2* of *S. cerevisiae*. Top, position of the gene disruption of *TPS2* by insertion of a *BglII*–*BglII* fragment containing *URA3*. *tps2::URA3* was constructed by transforming strain RH 144-3A with the linearized 3.4-kb *Eco*RI–*Sal*I fragment containing *URA3*. Positions of the overlapping clones pSEY-TP6 and pSEY-TP12 and the ORF of *TPS2* and *PPH3* are shown below the restriction map. Horizontal arrows indicate the direction and length of each individual sequencing reaction. Oligonucleotides were used to promote DNA synthesis for the sequencing, thus allowing the coverage of both strands. Bottom, fragment A was used as probe for DNA-blot analysis. B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; S, *Sal*I.

TPS2 was cloned at the *Eco*RI–*Bam*HI site in the multi cloning site of Ycplac 111 (Rose et al., 1987). A 1.18 kb *Bgl*II–*Bgl*II fragment, containing the *URA3* gene (obtained from pVT101-U, Vernet et al., 1987) was cloned into the *Bam*HI site (situated in the first third of the coding sequence of *TPS2*) of the *Eco*RI–*Bgl*II fragment. The disrupted sequence was then excised from the corresponding plasmid with *Eco*RI and *Sal* I (polylinker of Ycplac 111) and transformed into strain RH 144–3A. The resulting gene-disruption mutant was called CDV 64 (MATα *ura3, leu2, his4, bar1, tps2::URA3*). Gene disruption was confirmed by DNA blot analysis (Southern, 1975), probing genomic DNA digested either with *Bam*HI or *Hind*III with the *Eco*RI–*Bgl*II fragment of *TPS2* labelled with [α-^{32}P]dATP.

DNA sequencing and sequence analyses

Sequences were obtained from plasmids prepared by CsCl gradients, using the dideoxy chain termination method (Sanger et al., 1977) with sequenase (United States Biochemical Corporation) and [α-^{35}S]dATP (Amersham). Oligonucleotides were synthesized with an Applied Biosystem DNA synthesizer. Sequence analyses were carried out with the GCG package (Devereux et al., 1984).

RNA isolation and analysis

RNA was extracted as described by Domdey et al. (1984). Briefly, log-phase cells (60 ml; 1×10^7 cells/ml) were harvested by filtration, washed three times with 10 ml diethylpyrocarbonate-treated water, suspended in 0.5 ml water, and frozen in liquid nitrogen. Cells were disrupted in 0.3 ml lysis buffer (10 mM EDTA, 150 mM LiCl, 20 mM NaCl, 0.1% SDS, 20 mM Tris/HCl, pH 7.5) and 0.5 ml phenol at 60°C for 10 min and rapidly frozen in liquid nitrogen. Following centrifugation for 10 min at 15000 *g*, the phenol phase was removed, leaving the interphase intact. A second aliquot of phenol (0.5 ml) was added and the extraction was repeated. The mixture was rapidly chilled in liquid nitrogen. After centrifugation (10 min at 15000 *g*), the aqueous phase was transferred to a new tube and extracted once with a 1:1 mixture of phenol/chloroform at room temperature. Then, after centrifugation for 10 min at 15000 *g*, the aqueous phase was brought to 0.8 M LiCl, and 2.5 volumes of ethanol were added to precipitate RNA. The pellet was washed once with ice-cold 70% ethanol and dissolved in 50 µl diethylpyrocarbonate-treated water. The RNA concentrations were equalized after the A_{260} was measured, and their quality was confirmed in a 'pre-run' with ethidium bromide stained probes on a 1% formaldehyde/agarose gel. Total RNA (10 µg) was then subjected to electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, blotted onto nitrocellulose filters (BA 83; Schleicher and Schuell AG) and hybridized with radioactive probes at 42°C (Maniatis et al., 1989). The radioactive DNA probes were generated with the Random Primed DNA Labeling Kit (Boehringer Mannheim), using PCR-generated fragments covering either the whole *ACT1*-coding or *TPS2*-coding regions as templates.

Quantification of bands on autoradiograms was done using a Bio-Profil scanning densitometer (Vilbert Lourmat).

Enzyme assays and determination of metabolite levels

The activity of Tre6*P* synthase was measured in permeabilized cells (Miozzari et al., 1978). 40 ml log-phase (1 ml stationary-phase) cultures were filtered (Whatman GF/C), resuspended in 1 ml 0.2 M Tricine (Na^+), pH 7.0, 0.05% Triton X-100, and immediately frozen in liquid nitrogen. After thawing (2 min at 30°C), the cells were centrifuged (1 min at 3000 *g*), washed twice with ice-cold 0.2 M Tricine (Na^+), pH 7.0, and immediately used for the assay of Tre6*P* synthase activity at 50°C (temperature optimum) in a total volume of 240 µl, using the procedure of Hottiger et al. (1987).

The activity of Tre6*P* phosphatase was measured in permeabilized cells prepared as described above, but resuspended in 50 mM (Na^+/K^+) phosphate buffer, pH 6.0, in an assay mixture containing 1.6 mM Tre6*P* (Sigma), 10 mM $MgCl_2$, 25 mM (Na^+/K^+) phosphate buffer, pH 6.0, and 50 µl extract in a total volume of 0.1 ml. After a 20-min incubation at 30°C, the reaction was stopped in a boiling water bath for 3 min. The assay mixtures were then centrifuged (5 min at 15000 *g*) and the amount of trehalose formed was directly measured by HPLC analysis on a CarboPac PA-100 anion-exchange column, using a DIONEX DX-300 Gradient Chromatography System coupled with pulsed amperometric detection. Before injection, the column was equilibrated with 0.1 M NaOH for at least 10 min. The column was eluted with 0.1 M NaOH for 3 min, followed by a sodium acetate gradient of 0–0.2 M in 0.1 M NaOH over 3–8 min, at a flow rate of 1 ml/min. Under theses conditions, the retention times of trehalose and Tre6*P* were 2.8 min and 7.9 min, respectively. In most cases, a small peak of glucose (retention time 4.2 min) was also present, probably because the trehalose formed was hydrolyzed by the trehalases which are also present in permeabilized cells. However, this amount did not exceed 5% of the trehalose formed in any of the assays. Glucose 6-phosphate (retention time 10.4 min) was not present in any of the assays performed. For determination of trehalose and Tre6*P*, 10 ml exponentially growing cells or 1 ml stationary-phase cells were filtered (Whatman GF/C), washed four times with 5 ml distilled H_2O, resuspended in 1 ml H_2O and transferred to a boiling water bath for 5 min. After centrifugation (5 min at 15000 *g*), trehalose and Tre6*P*

Table 1. Amino acid sequences of the N-terminus and of tryptic fragments of the 100 kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex. The first amino acid sequence was derived from the N-terminus, whilst the other three sequences were obtained from tryptic fragments of TPS2. X denotes cycles in which amino acids were not unequivocally identified. Each peptide sequenced could be unequivocally assigned to the positions indicated of the deduced amino acid sequence of the open reading frame of *TPS2* (Fig. 2).

Number	Amino acid sequence	Corresponding amino acid position in *TPS2*
1	XXXAQDNXXK	2– 11
2	DAIVVNPWDXVAVA	485–498
3	XVSCQDXVNLTEXVD	645–659
4	LLSFTDXFDLEVM	712–724

were determined in the supernatant by HPLC analysis as described above. Protein was quantified by the method of Lowry et al. (1951) using bovine albumin as standard.

RESULTS

Purification of the yeast trehalose-6-phosphate synthase/phosphatase complex

In previous work we reported that SDS/PAGE analysis of the Tre6*P* synthase/phosphatase enzyme complex revealed three main bands with apparent molecular masses of 56, 100 and 105 kDa (Bell et al., 1992). However, despite of the use of the C13-ABYS86 strain, the larger polypeptide displayed signs of proteolytical degradation during the purification (data not shown). To eliminate this problem, special care was taken in the present study to reduce the time for purification. The purification, carried out as described in Bell et al. (1992), was performed within 3 days, whereas previously it took more than a week. SDS/PAGE analysis of the purified Tre6*P* synthase/phosphatase complex revealed three major polypeptides with apparent molecular masses of 56, 100 and 130 kDa. The specific activities of both Tre6*P* synthase and Tre6*P* phosphatase were increased up to 300-fold during the purification of the Tre6*P* synthase/phosphatase complex (data not shown).

Protein-sequence analysis and cloning of the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex

The 100-kDa polypeptide was eluted from the gel and an N-terminal sequence was obtained as well as three sequences of peptides generated by tryptic digestion (Table 1). A data bank search showed one sequence (number 4 in Table 1) to be identical with the deduced amino acid sequence of the upstream region of *PPH3* on the right arm of chromosome IV (Ronne et al., 1991). This sequence was used to clone the whole upstream region of *PPH3* (see Materials and Methods). Subsequently, we sequenced a segment of 3915 bp of this upstream region, containing an ORF of 2685 nucleotides (Fig. 2). Upstream of the predicted start site, there was no in-frame ATG not followed by stop codons. The stop codon of this ORF was situated 575 bp in front of the *PPH3* start codon. The N-terminal sequence of the 100-kDa polypeptide, with five amino acids unequivocally determined in 10 sequencing cycles, corresponded to the predicted N-terminal sequence of codons 2–11 of the ORF, based on initiation at the first ATG (Table 1; Fig. 2). We therefore conclude that the start codon is at position 972, as numbered in Fig. 2, and that the mature protein has lost its N-terminal methionine. In addition, all three tryptic peptides sequenced could be unequivocally assigned with 100% identity at all positions identified, to specific regions of the deduced amino acid sequence (Table 1; Fig. 2). From these results, we infer that we have cloned and sequenced the gene coding for the 100-kDa subunit of the Tre6*P* synthase/phosphatase complex.

The deduced amino acid sequence corresponds to a protein of molecular mass 102.8 kDa (Fig. 2), which is in accordance with the apparent molecular mass of the subunit determined on denaturing gels. The p*I* value of the predicted gene product is 7.90. According to our previous nomenclature, we named the gene *TPS2*.

To our surprise, we found that the first 552 amino acids of the *TPS2* gene product were 33% identical, upon optimal alignment with the entire TPS1 (= CIF1) amino acid sequence (Fig. 3). Furthermore, a search for homologous amino acid sequences in the data bank revealed a third deduced gene product, described as a CIF homolog [Manning, A. M., Rosenbloom, C. L. & Beaudet, A. L. (1992) EMBL accession number M88172], which shared 36% and 29% identity with TPS1 and TPS2, respectively, over a stretch of about 500 amino acids (Fig. 3). In contrast to the situation in TPS1 and TPS2, this stretch was an internal part of the deduced sequence.

TPS2 codes for the trehalose-6-phosphate phosphatase

In order to determine the phenotype of a null mutant of *TPS2*, a gene disruption was carried out (see Materials and Methods and Fig. 1). Southern-blot analysis confirmed that integration of the 1.18-kb *Bgl*II–*Bgl*II fragment containing *URA3* occurred at the *TPS2* locus (data not shown). Since viable *tps2* haploids could be isolated, the *TPS2*gene is not essential under normal growth conditions.

When a haploid yeast strain bearing the *tps2* gene disruption (CDV 64) was either heat shocked or grown to stationary phase, Tre6*P* synthase was almost as active as in the wild-type strain (Table 2). However, Tre6*P* phosphatase activity was almost completely absent in log-phase, stationary-phase and heat-shocked cultures (Table 2). The low basal activities in CDV 64 were probably due to the activity of unspecific cleavage of Tre6*P* by other phosphatases present in permeabilized cells, since they were similar to the phosphatase activities with glucose-6-*P* as substrate (data not shown). In contrast to cells of the strain CDV 64, wild-type cells showed normal activity of Tre6*P* phosphatase in the log phase and displayed a three-fold increase in Tre6*P* phosphatase after 1 h heat shock or upon entry into stationary phase (Table 2).

In accordance with these observations, cells of the strain CDV 64 were only able to accumulate low amounts of trehalose (less than 8% when compared with the wild-type cells) under heat-shock conditions (Fig. 4A). However, they accumulated up to 0.45 g Tre6*P*/g protein after 1 hour at 40°C (Fig. 4B). Wild-type cells accumulated trehalose normally upon heat shock, whilst Tre6*P* levels were below the detection limit. Additionally, we also observed a large increase in the total Tre6*P* content in CDV 64 cells entering the stationary phase. Whilst wild-type cells accumulated about 1.0 g trehalose/g protein and no Tre6*P*, CDV 64 cells accumulated

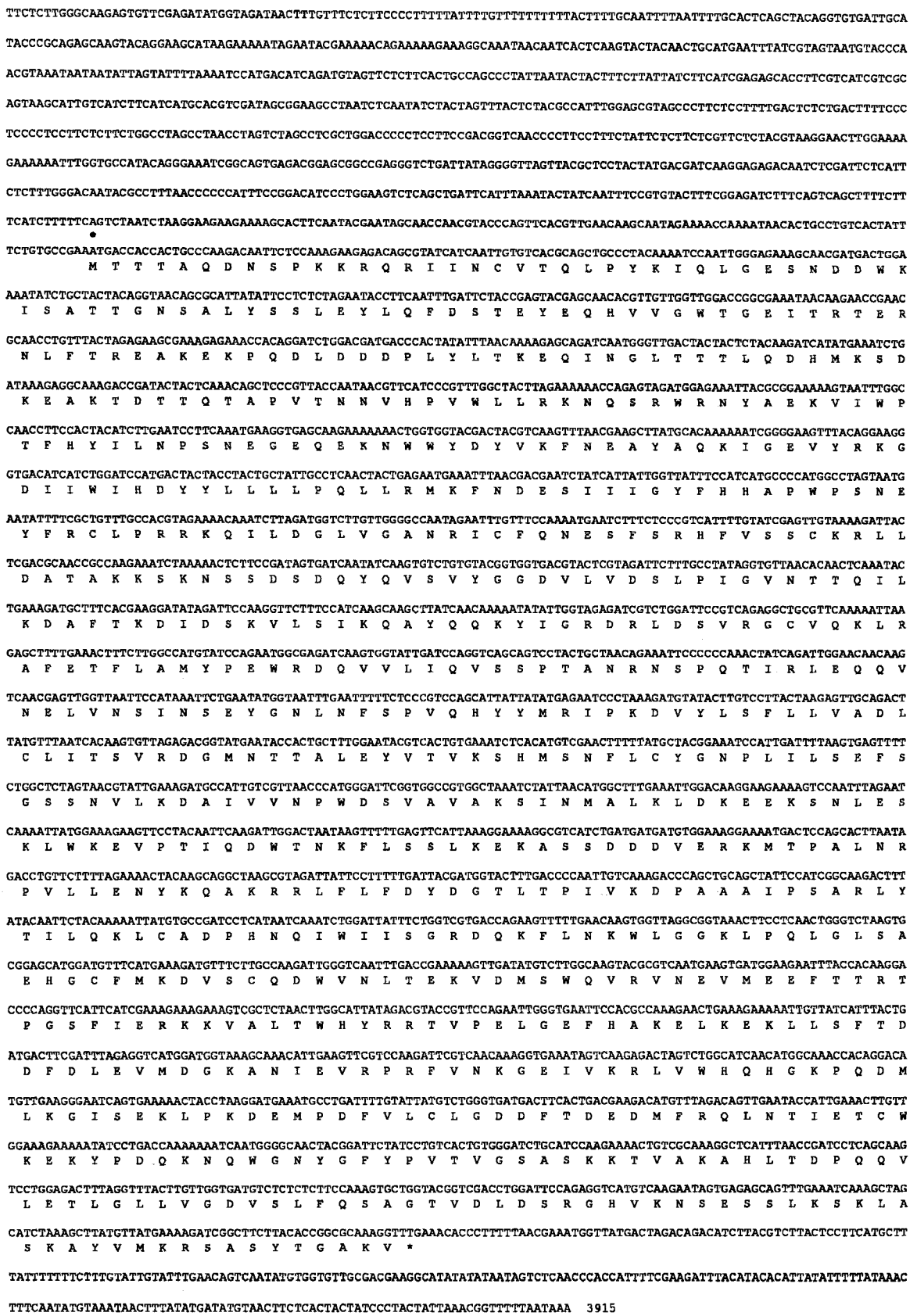

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the gene *TPS2* of *S. cerevisiae*. The predicted amino acid sequence is shown in the single-letter code. The start codon is marked with an asterisk.

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1  leTDAtKkynvpkfggysnnAkLraSlmrNsyelfkhLPwTIvd--SdkgngsLk-navniavAektvKepvswvgt--mGiPtdElPhevchk 363
2  MTTDnaK-------------AQLtsSsggNiivvsnrLPvTItknsStgqyEYam-sSgglvtAleGlKktyTf-kw--FGwPglEiPdDekDq  77
3  MTTtAqdnspkkrqriincvtQLpykiqlgesnddwkisaTtgnsalysslEYLqfdSteyeqhvvGwtgeiTrternlFtreakEkPqDldDd  94
C  MTTDA.K.............AQL..S...N.......LP.TI....S....EYL...S.....A..G.K...T......FG.P..E.P.D..D.

1  isKkLeqdFssfPvvtdDit----------------------------fkgaYkNYAkqILWPTlHYqIpdnPNskaFEdhsWdyYqKvNQkFs 429
2  vrKdLlEkFNavPifLsDei----------------------------adlhYngfsnsILWPlFHYh----PgEinFdeNaWfgYneaNQtFt 139
3  plyltkEqiNgltttLqDhmksdkeaktdttqtapvtnnvhpvwllrknqsrwrNYAekviWPTFHY-IlnpsNEgeqEkNwWydYvKfNeaya 187
C  ..K.L.E.FN..P..L.D.............................Y.NYA..ILWPTFHY.I...PNE..FE.N.W..Y.K.NQ.F.

1  drIvsVYkpGDtIWIHDYHLMLVPQMvReK-----LpkaKIGfFLHvsFPSSEvFRCLanRerILeGiiGANfVGFQTkeYkRHFLqtCnRLLa 518
2  neIaktmnhnDlIWvHDYHLMLVPeMLRvKihekqLqnvKvGwFLHtPFPSSEiyRiLPvRqeILkGvlscdlVGFhTydYaRHFLSSvqRvLn 233
3  qkIgeVYrkGDiIWIHDYyLlLlPQlLRmKfnd---esiiIGyFhHaPwPSnEyFRCLPrRkqILdGlvGANricFQnesfsRHFvSSCkRLLd 278
C  ..I..VY..GD.IWIHDYHLMLVPQMLR.K.....L...KIG.FLH.PFPSSE.FRCLP.R..IL.G..GAN.VGFQT..Y.RHFLSSC.RLL.

1  Adv-----SnDeVkYhcni----VsVmyaPIGIDyyhlTsqLrngSVlewrQlIKErwrnKKlIVcRDqfDrIRGlqkKmlAyErFLiEnPEyi 603
2  vnt-----lpngVeYQgrf----VnVgafPIGIDvdkfTdgLkkeSVqkriQqlKEtfkgcKiIVGvDRLDyIkGvpQKLhAmEvFLnEhPEWR 318
3  AtakksknSsDsdqYQvsvyggdVlVdslPIGvnttqilkdaftkdidskvlsIKqayqqK--yiGRDRLDsvRGcvQKLrAfEtFLamyPEWR 370
C  A.......S.D.V.YQ.......V.V...PIGID....T..L...SV....Q.IKE....KK.IVGRDRLD.IRG..QKL.A.E.FL.E.PEWR

1  eKVVLIQicigk----SsdpeYeRQim-vvVdRINSlssNisiSqPVvFlHqdldFaqYLaLnceADVfLVdalReGMNLTchEfIvssfEKn- 692
2  gKVVLvQVavPs-rgdveeyqYLRsvVNELVgRINgqfGtveFv-PihFmHksIPFeeliSLyaVsDVCLVsStRDGMNLvsyEYIacqeEKk- 409
3  dqVVLIQVssPtanrnSpqtirLeQqVNELVnsINSeyGNlnFS-PVqhyymrIPkdvYLSfllVADlCLitSvRDGMNTtalEYvtvkshmsn 463
C  .KVVLIQV..P.....S....YLRQ.VNELV.RINS..GN..FS.PV.F.H..IPF..YLSL..VADVCLV.S.RDGMNLT..EYI....EK..

1  -----aPLlLSEFTGSSsVLKeGAIliNPWDinhVAqSIkrsLemspEEKrrrWkKLfKsViehdSdnWitKcfeyindaweSnqetStvfN  779
2  -----gsLILSEFTGaaqsL-nGAIiVNPWntddlsdaINeALtLpdvkKevnWeKLyKyiskyTSafWqenFvheLystsSSstssSatkN  495
3  flcygnPLILSEFsGSSnVLKd-AIvVNPWDsvaVAkSINmALkLdkEEKsnlesKLwKeVp--TiqdWtnKFlssLkekaSSdddverkmt  552
C  ......PLILSEFTGSS.VLK.GAI.VNPWD...VA.SIN.AL.L..EEK...W.KL.K.V...TS..W..KF...L....SS....S...N
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Fig. 3. Comparison of the amino acid sequences of the CIF homolog (1), TPS1 (2) and TPS2 (3). Gaps (−) were introduced to optimize the alignment. Bottom line, consensus sequence (C) showing residues identical in at least two sequences (capital letters). Amino acids conserved in all three amino acids are underlined. The sequences aligned correspond to the following positions in the original polypeptides: 1−495 in TPS1; 1−552 in TPS2; 275−779 in the CIF homolog.

Table 2. Activities of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in wild-type yeast (RH 144-3A) and the *tps2* disruption mutant (CDV 64) under various conditions.

Culture condition	Tre6*P* synthase activity for		Tre6*P* phosphatase activity for	
	strain			
	RH 144-3A	CDV64	RH 144-3A	CDV64
	nkat/g protein			
Log phase (27 °C)	390.0	240.0	57.1	3.9
Heat shock (40 °C)	3100.0	2610.0	186.2	4.0
Stationary phase	2540.0	2000.0	185.7	6.3

up to 1.4 g Tre6*P*/g protein but almost no trehalose upon entrance in the stationary phase (data not shown).

These results suggest that *TPS2* is the structural gene of the *S. cerevisiae* Tre6*P* phosphatase.

Kinetics of TPS2 mRNA accumulation after a heat shock

The elevated levels of the Tre6*P* phosphatase activity in heat-shocked *S. cerevisiae* cells could be due to either transcriptional regulation or to post-transcriptional processes. As an initial step towards elucidating the control of Tre6*P* phosphatase activity under heat-shock conditions, we studied the kinetics of *TPS2* mRNA accumulation after a heat shock. For this purpose, logarithmically growing cells of the wild-type strain RH 144−3A were removed from cultures at various times after a shift over 27−40°C, and RNA was isolated and

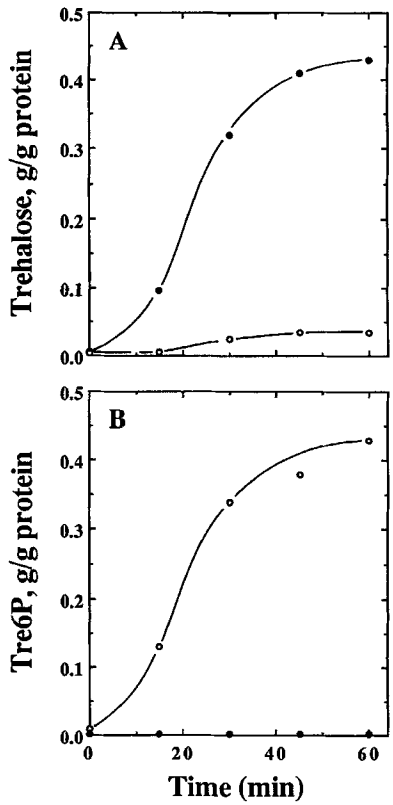

Fig. 4. Trehalose (A) and trehalose-6-phosphate (B) levels of wild-type cells and a *tps2* disruption mutant during heat shock. Log-phase cultures were shifted over 27−40°C at 0 min. Full circles, wild-type (RH 144−3A); open circles, *tps2* disruption mutant (CDV 64).

analyzed as described in Materials and Methods. Exponentially growing cells displayed a very low level of *TPS2*

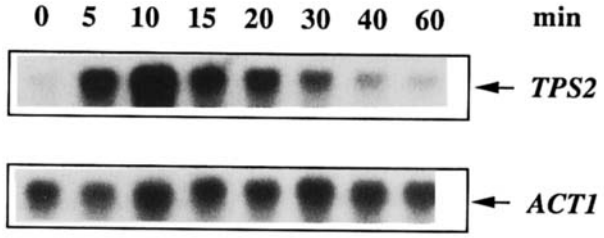

Fig. 5. Autoradiograms of blots of total cellular RNA probed with either *TPS2* DNA or *ACT1* DNA as a function of time after a temperature shift of 27–40°C. RH 144-3A wild-type cells were grown in YPD at 27°C and transferred to a water bath at 40°C at time zero. RNA isolated from these cells, harvested at various times after heat shock, was electrophoresed and blotted as described in Materials and Methods. Blots were probed with ^{32}P-labeled *TPS2* DNA (upper lane) and *ACT1* DNA (lower lane) genes. The time after the upshift of the culture to 40°C is indicated at the top of the figure.

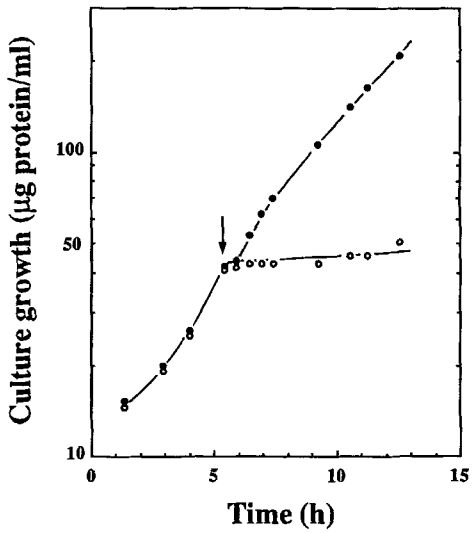

Fig. 6. Growth of wild-type cells and a *tps2* disruption mutant after a shift of 27–40°C. Log-phase cells of RH 144−3A (wild-type cells; closed circles) and CDV 64 (*tps2* disruption mutant; open circles) were shifted over 27−40°C at the time indicated by the arrow.

mRNA. However, the level of *TPS2* mRNA increased drastically after the temperature upshift, reaching the maximum approximately after 10 min (Fig. 5). Quantification of the band on the autoradiogram by scanning densitometry revealed that the level of *TPS2* mRNA increased more than 20-fold within 10 min after the shift to 40°C (data not shown). Thereafter, the mRNA levels decreased again and reached almost the level attained before heat shock 60 min after the temperature upshift. As an internal control, the RNA blot used for probing with the *TPS2* gene was additionally probed with the radiolabelled *ACT1* gene (Fig. 5, lower lane). The RNA blot analysis with the *tps2* disruption mutant revealed that the 3.0-kb *TPS2* mRNA was absent and that a smaller 1.2-kb fragment hybridyzed to the *TPS2* probe (data not shown). This result confirms the disruption of the *TPS2* gene in the *tps2* disruption mutant.

TPS2 gene disruption results in temperature-sensitive growth

Whilst wild-type cells (after a short lag-phase of 30 min) were able to resume growth upon a shift of 27−40°C, CDV 64 cells stopped growth permanently at 40°C (Fig. 6). We suggest that the accumulation of high levels of the metabolic intermediate Tre6*P* may be inhibitory to growth. CDV 64 was also unable to grow at 37°C, even after a prolonged incubation time (two days). However, after two days at 37°C, colonies able to grow at this temperature appeared at a relatively high frequency of 6/10^3 cells.

DISCUSSION

To minimize proteolysis during purification, a problem encountered in previous work (Londesborough and Vuorio, 1991), we used the strain C13-ABYS86 (Achstetter et al., 1984) which lacks four major vacuolar proteases (Bell et al., 1992), and we took special care to reduce the overall purification time. We found the Tre6*P* synthase/phosphatase complex to be composed of three polypeptides, namely TPS1, TPS2 and TPS3, with molecular masses of 56, 100 and 130 kDa, respectively. In our previous work (Bell et al., 1992), we found three polypeptides with similar molecular masses except for the largest one, which was only 105 kDa, suggesting that this subunit was particularly sensitive to proteolysis. The polypeptides we describe now are probably the same as the ones reported by Vourio et al. (1992), although these authors suggested that only the two major polypeptides of 56 kDa and 130 kDa, but not the weakly stained 99-kDa band, belonged to the purified Tre6*P* synthase/phosphatase complex.

In our earlier work, we have already identified the gene *TPS1* coding for the 56-kDa subunit of the Tre6*P* synthase/phosphatase complex (Bell et al., 1992). We showed that *TPS1* is identical with *CIF1* which was originally isolated by complementation of the *cif1* mutant deficient in growth on fermentable carbon sources (Navon et al.,1979; Gonzáles et al., 1992). The *cif1* mutation was found to be allelic to *fdp1* and *byp1* (Hohmann et al., 1992) and the gene has been renamed *GGS1* (Thevelein, 1992). All these mutants have low levels of Tre6*P* synthase activity, indicating that *TPS1* is an essential component of the Tre6*P* synthase/phosphatase complex. One additional characteristic property of all these mutants is that they accumulate hexose phosphates upon addition of glucose, although they do not have any defect in glycolytic enzymes (van de Poll and Schamhart, 1977). It is most unlikely that the Tre6*P* synthase activity itself is involved in this phenotype, since Tre6*P* synthase activity is very low in growing wild-type cells. Therefore, we suggest that *TPS1* has strictly regulatory functions. These functions may be related to the control of the Tre6*P* synthase/phosphatase complex (Bell et al., 1992) as well as to the control of glucose influx and glucose-induced signalling pathways (Thevelein, 1992).

In this study, we report the identification of the gene *TPS2* which codes for the 100-kDa subunit of the Tre6*P* synthase/phosphatase complex. Disruption of *TPS2* results in the almost complete absence of Tre6*P* phosphatase activity in log-phase, stationary-phase and heat-shocked cells. In contrast, under the same conditions, Tre6*P* synthase activities in the *tps2* disruption mutant differ only slightly from those in the wild type. *In vivo*, the *tps2* disruption mutant accumulates high amounts of Tre6*P* under conditions that normally lead to the accumulation of trehalose in wild-type cells. Taken together, these results suggest that *TPS2* codes for the structural gene of Tre6*P* phosphatase.

The large increase in *TPS2* mRNA upon heat shock indicates that the heat-induced increase in Tre6*P* phosphatase activity might be due mainly to transcriptional regulation. The rapid and transient accumulation of the mRNA resembles the pattern described for members of the heat-shock gene family (Werner-Washburne et al., 1989). These results provide further evidence for our previously reported suggestion that trehalose is an important factor of the heat-shock response in *S. cerevisiae* (Hottiger et al., 1989 and 1992; De Virgilio et al., 1990 and 1991).

Disruption of *TPS2* caused temperature-sensitive growth and the permanent stop of cell proliferation upon a temperature shift over 27–40 °C. This might be due to the excessive accumulation of Tre6*P* at elevated temperatures. Alternatively, the fixation of a great part of the cells P_i into Tre6*P* might result in a shortage of the intracellular P_i and ATP, even though cells are not starved for P_i.

A further interesting observation is that incubation of a *tps2* disruption mutant for more than two days at 37 °C resulted in the appearance of growing colonies. This observation has also been made in the *tre1.1* mutant, formerly reported to be possibly defective in the structural gene of Tre6*P* phosphatase (Piper and Lockheart, 1988). It might be that spontaneous mutations arise which can overcome the metabolic consequences of Tre6*P* accumulation. In this context, it is worth noting that inhibition of growth on glucose in *cif1* mutants can be suppressed by second-site changes in expression of *FPS1* and *FPS2* (Thevelein, 1992).

Finally, as the *tps2* disruption mutant had normal Tre6*P* synthase activities, we hypothesize that the polypeptide responsible for Tre6*P* synthase activity may be the 130-kDa subunit of the Tre6*P* synthase/phosphatase complex. Interestingly, the CIF homolog [Manning, A. M., Rosenblom, C. L. & Beaudet, A. L. (1992) EMBL accession number M88172] which shares a homologous stretch of about 500 amino acids with both TPS1 and TPS2, would have a predicted molecular mass of 118.8 kDa. We are currently investigating whether the CIF homolog is involved in the trehalose metabolism and especially, whether TPS3 might be encoded by the latter gene. This would mean that the Tre6*P* synthase/phosphatase complex is composed of three different proteins which share a long stretch of sequence homology.

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