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Transcription of *NTH1* is controlled in response to stress recovery signals in *Saccharomyces cerevisiae*

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Abstract

Trehalose is an important storage carbohydrate for *Saccharomyces cerevisiae*. It protects yeast cells from physiological stresses. Trehalose also controls glucose transport to yeast cells by acting on hexokinase. When trehalose is over accumulated or during the recovery stage of the yeast cells from stress, neutral trehalase enzyme which is encoded by *NTH1* gene degrades trehalose to restore normal growth conditions. Hence trehalase enzyme is essential for balanced levels of trehalose and for normal metabolic activities of the yeast cells. TOR (Target of rapamycin) signaling pathway activates many cellular processes and signaling pathways under normal growth conditions in yeast. Besides rapamycine, caffeine treatment and certain stress conditions inhibits TOR pathway. In this research the effects of various signal transduction pathways on the promoter activities of *NTH1* gene and on the reserve carbohydrate metabolism were investigated during nutrient limitation and nutrient replenishment periods in *S. cerevisiae*. The results showed that the transcription of *NTH1* gene is transiently activated during nutrient replenishment period. Our results also suggest that cAMP dependent PKA and TOR signaling pathways may involve in the transcriptional regulation of *NTH1* gene.

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Introduction

Trehalose is a multi-functional nonreducing disaccharide in *Saccharomyces cerevisiae*. It is stored in response to unfavorable conditions, such as nutrient limitations and stress conditions like oxidative stress and temperature stress (Haunsa *et al.,* 1998; Eleutherio *et al.,* 1993). One of the biological functions of trehalose is to prevent protein denaturation in the cytoplasm under stress inducing conditions (Singer ve Lindquist, 1998; Jain and Ipsita, 2009). However, when the stress conditions alleviated, cytoplasmic trehalose should be rapidly degraded to glucose by neutral trehalase enzyme to resume normal metabolic conditions (Londesborough and Varimo, 1984; Elbein *et al,* 2003). Trehalose biosynthesis is also connected to glucose uptake by means of trehalose 6-phosphate, that forms during trehalose biosynthesis. It restricts glucose flux by acting on Hexokinase-II (Blazquez *et al.,* 1993). Trehalose biosynthesis and degradation is carried out by two different enzymes, *TPS* and *NTH1*, respectively.

Trehalose synthesis is catalyzed by the trehalose synthase complex encoded by *TPS1*, *TPS2*, *TPS3* and *TSL1* (Bell *et al.,* 1992; De Virgilio *et al.,* 1993; Bell *et al.,* 1998). The hydrolysis of trehalose into two glucose molecules is catalyzed mainly by the neutral trehalase Nth1, encoded by *NTH1* (Kopp *et al.,* 1993; Nwaka *et al.,* 1995; Van Dijck *et al.,* 1995). Enzymatic functions of Nth1p is regulated by post-translational modifications. Four amino acid residues in Nth1p is phosphorylated by cAMP-PK, and upon phosphorylation, 14-3-3 proteins Bmh1p and Bmh2p binds to Nth1p (Veisova *et al.,* 2012; Panni *et al.,* 2007). Interactions of Nth1 with either one of the Bmh proteins is required for the full level enzymatic activity of Nth1p (Panni *et al,* 2007).

It is known that transcription of *NTH1* gene is also regulated by stress induced transcriptional activators Msn2p and Msn4p (Zahringer *et al.,* 2000). Msn2/4 complex that binds to STRE sequences on the promoter region, activates *NTH1* transcription in response to physiological stresses such as heat stress and osmotic stress (Zahringer, 2000). However, there is not much information on the transcriptional regulation pattern of *NTH1* gene during the stress recovery. It has been reported that trehalase activity is required during recovery from heat stress (Wera *et al.,* 1999).

TOR complex regulates the transcription of many genes involved in growth and metabolism in response to nitrogen and carbon source availability (Beck and Hall, 1999). TOR pathway is inhibited by rapamycin, caffeine and poor nitrogen source, proline (Wullschleger *et al.,* 2006; Reinke *et al.,* 2006; Stracka *et al.,* 2014). Inactivation of TOR pathway results in activation of Tap42 which interacts with catalytic subunits of the type 2A protein phosphatases (PP-IIA) (Dicomo and Arndt, 1996). Tap42 dependent PP-IIA activation causes the dephosphorylation of Msn2/4 and translocation to nucleus (Santhanam *et al.,* 2004).

The Ras-cAMP-dependent protein kinase (Ras-cAMP-PKA) pathway in S. cerevisiae is essential for growth and cell cycle progression. The deficiency in PKA activity results in physiological changes normally associated with nutrient deprivation (Thevelein and de Winde, 1999). PKA also regulates the activity of trehalase enzyme (Schepers *et al.,* 2012).

The aim of this study to analyze the role of cAMP dependent PKA and TOR signaling pathways in the transcriptional regulation of *NTH1* gene and reserve carbohydrate metabolism during nutrient limitation and nutrient replenishment periods in *S. cerevisiae*.

Materials and methods

Yeast strains and Expression vector

Saccharomyces cerevisiae strains BY4741 (MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and Σ1278b (MATa, ura3-52) were used in this study (Brachmann *et al.,* 1998; Zurita-Martinez and Cardenas, 2005). *S. cerevisiae* BY4741 strain has no known mutations relevant to trehalose metabolism. It is known that *S. cerevisiae* Σ1278b strain has an intrinsically high levels of cAMP and high levels of cAMP dependent

PKA activity (Stanhill *et al.,* 1999).

pNL1 plasmid (gift from JM. Francois) that contains NTH1-lacZ gene fusion was used to quantitate the promoter activity of *NTH1* gene in response to varying growth conditions (Parrou *et al.,* 1997). In this expression vector, 770 bp promoter region, upstream of translation start site, of *NTH1* gene fused in-frame to lacZ gene. It has been shown that this promoter region contains all of the regulatory sites required for NTH1 gene expression (Parrou *et al.,* 1997). pNL1 is a YEp353 based expression vector and stably maintained in selective growth conditions in *S. cerevisiae* transformants (Liao *et al.,* 1987).

S. cerevisiae strains were cultured in YPD (2% yeast extract, 1% peptone, 2% glucose) medium for plasmid transformation. pNL1 plasmid was transformed into the yeast strains using lithium acetate-polyethylene glycol procedure as described previously (Rose *et al.,* 1990). Transformants were plated on synthetic complete medium without uracil (Sc-ura, 2% glucose) and grown at 30 C incubator till to get well-grown yeast colonies. Yeast colonies patched to fresh Sc-uracil +2% glucose plates and grown for 2 days at 30C. These yeast patches were used in liquid culture inoculations.

Growth Conditions

First, yeast transformants were grown in two sets of 30 mL Sc-ura + 2% glucose medium at 30 °C incubator shaker for 18 hours to get saturated yeast pre-cultures first. At the end of first growth period, one sets of stationary stage yeast cultures were harvested and stored at -70 °C freezer to analyze the β-galactosidase activities and trehalose contents of stationary stage cultures. The second sets of liquid pre-cultures were harvested and washed twice with sterile distilled water and then resuspended in fresh 30 mL Sc-ura + 2% glucose medium w/o caffeine. Yeast cultures were further incubated at 30 C for either 90 min (for short recovery period) or 5 hours (for long recovery period). At the end of incubation periods, yeast cells were harvested and used for the measurements of β-galactosidase activities, trehalose and glycogen contents of the yeasts.

To analyze the effects of TOR pathway on the regulation of *NTH1* expression, caffeine (8 mM) that inhibits the TOR pathway, was added to yeast cultures as indicated (Reinke *et al.,* 2006). Yeast cultures were further incubated with caffeine for either 90 min or 5 hours, and then harvested for analysis of β-galactosidase activities and for determination of trehalose and glycogen contents.

Enzyme Assays

β-galactosidase activities of yeast transformants were determined essentially as described (Rose *et al.,* 1990; Guarante, 1983). Briefly, Yeast transformants were harvested at the end of growth periods and washed with 1 mL of distilled sterile water twice. yeast pellets were suspended in 200 µL of breaking buffer and stored at -70 C freezer till assay times. Yeast transformants were thawed at room temperature and permeabilized with 20 µL of 0.1% SDS and 20 µL chloroform to obtain cell lysates. β -galactosidase activities of yeast transformants were determined by using ONPG (2-nitrophenyl β-D-Galactopyranoside) in Tris/HCl buffer. Protein contents of cells were determined by Lowry assay (Lowry *et al.,* 1951). β-Galactosidase units are expressed in nmol of ONPG hydrolyzed per minute per mg of protein (nmol ONPG/mg protein/min).in permeabilized yeast cells. Yeast cultures were grown in duplicates in all experiments. β-galactosidase assays were done in triplicates and all experiments were repeated at least twice under same experimental conditions. Hence the β-galactosidase activities given in tables are the mean values of 12 independent assays. Standard deviations for β-galactosidase units were approximately 10% in triplicate assays**.**

Trehalose and glycogen contents of the yeast transformants, that were grown as explained in aforementioned manner, were determined by enzymatic methods as explained (Parrou and François, 1997). Trehalose and glycogen concentrations were expressed in µg glucose equivalent per g wet mass of the yeast cells.

Results and discussions

NTH1 transcription is activated by nutrient pulse at early stage

In order to analyze the effects of nutrient signals on the promoter activity of *NTH1* gene, stationary stage yeast cells transferred to nutrient rich environment. NTH1-lacZ gene fusion yielded 1879 units of β-galactosidase activity at stationary stage yeast cells (Table 1). Transcription of *NTH1* is activated by 60% when the yeast cells transferred to fresh growth medium. Unexpectedly, transcription of *NTH1* rapidly declined to 500 units at the end of 5 hrs incubation period. This result indicates that activation of *NTH1* in response to nutrient signals is transient (Table 1).

Table 1. Promoter activity of *NTH1* gene in response to nutrient pulses.

Yeast strains	Stationary stage	Fresh Culture	
		90 min incubation	5 hr incubation
BY4741	1879±347*	3108±104	501±38
Σ1278b	427±27	613±65	75±6

*Numbers indicates β-galactosidase activities (in nmol ONPG/min/mg protein) of yeast transformants.

It is known that the Σ1278b strain of *S. cerevisiae* has a high level of cAMP-PKA activity (Stanhill *et al.*, 1999). Hence, we have also analyzed the effects of nutrient signals on the transcriptional regulation of *NTH1* in Σ1278b strain. *NTH1* transcription was determined as 427 units at the stationary stage yeast cultures of Σ1278b strain (Table 1). This expression level is 4.4-fold lower than the BY4741 strain of *S. cerevisiae*, which implies that the transcription of *NTH1* is inversely correlated to PKA activity in yeast. Shifting of Σ1278b yeast transformants to fresh culture also resulted with transient activation of *NTH1* transcription (from 427 units to 613 units in 1.5 hrs). However, transcription of *NTH1* significantly decreased (from 613 units to 75 units) at the end of 5 hours (Table 1). It was previously shown that trehalase activity is controlled by PKA dependent phosphorylation in yeast (Veisova *et al.,* 2012; Panni *et al.*, 2007). It appears that while PKA activates neutral trehalase enzyme by post translational modification, it negatively regulates the transcription of *NTH1* gene.

Table 2. TOR signaling pathway may involve in the regulation of *NTH1* gene promoter activities.

Yeast strains	No caffeine (90 min)	With caffeine (90 min)	No caffeine (5 hr)	With caffeine (5 hr)
BY4741	3108±104	1111±43	501±38	904±91
Σ1278b	613±65	261±36	75±6	105±12

*Numbers indicates β-galactosidase activities (in nmol ONPG/min/mg protein) of yeast transformants.

These results clearly indicate that shifting yeast cells from nutrient limited conditions to nutrient replenished fresh medium rapidly but transiently activates *NTH1* transcription.

TOR signaling pathway is essential for transcriptional activation of NTH1 gene

TOR1 complex in *S. cerevisiae* involves in the activation of several pathways when the yeast cells grown in well fed, nutritionally replenished medium (Loewith and Hall, 2011). It is known that the caffeine can inhibit the TOR signaling in yeast cells (Reinke *et al.,*2006). To investigate the role of TOR signaling in the activation of *NTH1* transcription, we have included caffeine in our nutrient replenishment experiment. Our results indicate that caffeine, which inhibits TOR kinase signaling, also interferes with the activation of *NTH1* gene transcription in response to nutrient replenishment. *NTH1* transcription was measured as 1111 units at the end of 1.5 hrs incubation

period. This expression level was 3-fold lower than the normal nutrient pulse (3108 units vs 1111 units) (Table 2). *NTH1* transcription remained essentially at same levels at the end of 5 hrs of incubation periods in caffeine treated BY4741 strain of *S. cerevisiae*. Caffeine treatment also interfered with the activation of *NTH1* transcription in Σ1278b strain of *S. cerevisiae*. When caffeine was added to growth medium, transcription of *NTH1* decreased to 261 units in fresh cultures (Table 2). This expression level was 2,4-fold lower than the expression level that of without caffeine. Transcription of *NTH1* further decreased to 106 units in Σ1278b strain at the end of 5 hrs incubation periods (Table 2).

Table 3. Trehalose levels of yeast cultures before and after nutrient replenishments.

Yeast strains	Stationary stage	Fresh culture-90 min		Fresh Culture-5 hr	
		No caffeine	With caffeine	No caffeine	With caffeine
BY4741	3300* ±15	770 ±44	1380 ±97	1250 ±50	340±2
Σ1278b	1600 ±89	200 ±12	1200 ±12	2800 ±147	2500 ±112

*Numbers indicates trehalose contents (in µg glucose equivalent/g wet mass) of yeast transformants.

Trehalose degraded by nutrient replenishment and partially depends on TOR signaling

It is well known fact that reserve carbohydrates rapidly degraded to glucose when the yeast cells transferred from poor to nutritionally rich medium (Attfield, 1987). Especially, trehalose rapidly recycled to glucose by neutral trehalase when stress conditions relieved (Nwaka *et al.,* 1995).

To see that if the trehalose contents of the yeast cells will respond to nutrient signal, trehalose contents of the yeast cells were also determined. Our results clearly indicated that TOR signaling involves in the activation of *NTH1* gene transcription. To see the effects of TOR signaling on the trehalase activities, we have also determined the trehalose contents of the caffeine treated yeast cells.

Trehalose amounts of stationary stage BY4741 yeast cells were determined as 3300 µg/g (Table 3). As expected, shifting of yeast cells to fresh medium resulted with rapid degradation of trehalose. Its amount decrease to 770 µg/g at the end of 1.5 hrs incubation period. However, it seems that caffeine prevents trehalose degradation at certain level. In the presence of caffeine in the nutrient replenishment medium, trehalose content decreased to 1380 µg/g and 340 µg/g at the end of 1.5 hrs and 5 hrs incubation period, respectively. Similarly, trehalose accumulation of Σ1278b yeast cells was decreased to 200 µg/g in response to nutrient replenishment. As in the BY4741 yeast cells, caffeine treatment of Σ1278b yeast cells caused to prevent degradation of trehalose that its level became 1200 µg/g at the end of 1.5 hrs incubation period (Table 3).

In conclusion, it was shown that the transcription of *NTH1* gene is not constitutive and it is also regulated in response to metabolic signals. Moreover, it appears that the TOR signaling pathway involves both in the regulation of *NTH1* transcription and also in the regulation of neutral trehalase activity in the yeast *S cerevisiae*.

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