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Effect of Nrg1 Repressor on NTH1 Transcription and Molecular Docking of Nrg1 on NTH1 Promoter

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Abstract

The amount of intracellular trehalose increases in response to environmental stress in yeast (Saccharomyces cerevisiae). When that stress is terminated, the accumulated trehalose rapidly degrades into glucose rapidly. Synthesis of trehalose is fulfilled by the Trehalose Phosphate Synthase (TPS) enzyme complex, whereas the degradation of trehalose is done by the neutral trehalase enzyme. Under different stress conditions, transcription of the NTH1 gene is activated and Stress Response Elements (STRE) are required for this activation. Nrg1 protein can bind promoters including STRE and PDS elements. Because of the presence of three possible Nrg1 repressor binding sites on the NTH1 promoter, the NTH1 gene may be regulated by the Nrg1 repressor. In order to test this hypothesis, Nrg1 mutant yeast and its isogenic wild-type yeast strain were used to analyze the transcriptional activation of the NTH1 gene under nitrogen starving conditions. Nth1 transcription of the mutant yeast was seven-fold higher than that of the wild-type under growth conditions, and was not changed during nitrogen starvation. The protein-DNA docking analysis also supported the possibility of Nrg1 binding to the NTH1 promoter. These results revealed that NTH1 gene expression is constitutive in the absence of the Nrg1 repressor protein, hence the transcription of NTH1 is repressed by the Nrg1 protein.

Keywords: Nrg1, NTH1, Nitrogen starvation, Protein-DNA docking, Saccharomyces cerevisiae

Introduction

Saccharomyces cerevisiae regulates its genetic and metabolic processes to adapt itself to newly changing environments as do all living organisms. Changing gene expression levels in response to environmental stresses, except food deficiency, are rapid and transient [1]. S. cerevisiae accumulates α-1,1 trehalose under unfavorable environmental conditions. When these stress conditions ease, yeast cells convert trehalose into glucose and use it as an energy source. In addition to these functions, trehalase also regulates the glycolytic pathway in yeast cells [2-4]. The level of trehalase in the cell changes depending on the stress factors and growth stages. Therefore, the genes and proteins involved in the synthesis and degradation of trehalose must be tightly controlled. Synthesis of trehalose is catalyzed by the Trehalose Phosphate Synthase (TPS) enzyme complex that is composed of four subunits (Tps1p, Tps2p, Tps3p, and Tsl3p) [5, 6]. Stress-accumulated trehalose degradation is activated by the neutral trehalase enzyme encoded by the NTH1 gene. Neutral trehalase is localized in the cytoplasm and has an optimum pH value of 7.0 [7, 8]. The trehalase enzyme, Nth1, regulates trehalose metabolism by keeping intracellular trehalose at a constant level, and regulates the glycolytic pathway by affecting hexokinase activity [6, 9].

The NTH1 gene has a 2079 bp long intron-free coding region and is located on chromosome IV as a single copy. Nth1 protein is an 80 kDa protein and consists of 693 amino acids [10, 11]. NTH1 gene transcription and the enzymatic activity of the Nth1 protein are increased by heat, oxidative, and metal stress, and by nitrogen starvation [12, 13, 14]. The NTH1 promoter includes STRE (Stress Response Element) (5'-CCCCT-3') sequences which are necessary for transcriptional activation of the NTH1 gene [9, 12, 15]. The zinc-finger DNA-binding transcription factors, Msn2 and Msn4, bind to STRE sequences and activate gene expression in response to stress [16]. Activity of the Msn2/4 proteins is controlled by the SWI/SNF1 complex, and the rapamycin (TOR) and cAMP/PKA signaling pathways [17, 18]. The target of (the TOR) pathway controls...
subcellular localization of Msn2/4 by regulating its interaction with cytoplasmic anchor protein, Bmh1 [19]. Activity of the TOR complex is regulated depending on the quality of the nitrogen source [20]. Transcription of the \NTHI gene increases under different stress conditions but trehalase activity has not been observed under the same conditions. Whenever stress conditions are eased, Nth1 protein is activated by cAMP-dependent PKA phosphorylation.

The \NRG1 gene encodes the transcriptional repressor protein, Nrg1 (Negative Regulator of Glucose-repressed genes) in \S. cerevisiae. Nrg1 is a zinc-finger DNA-binding protein like Msn2/4, and localizes in the nucleus, nucleolus, and anti-nucleolar nucleus within the cell [21]. Nrg1 represses the \STAI gene by interacting with the Snf6-Tup1 corepressor complex in the presence of glucose [22]. Nrg1 plays a role in the repression of \DOG2, \SUC2, \GAL, and \GAL10 genes [23, 24]. In addition, Nrg-mediated repression of the FLO11 gene is overcome by the activity of Snf1 kinase, which means Nrg1 is working antagonistically with Snf1 kinase [25]. Genome-wide location analysis has revealed the consensus binding sequence of Nrg1 as GGaCCCT [26]. Analysis of 150 gene promoters repressed by Nrg1/2 showed that Nrg1/2 proteins bind to the STRE-like sequences as in Msn2/4. It has also been found that most of these genes are targets of Msn2/4 and are regulated against oxidative, osmotic, and heat stress, nitrogen starvation, and many other stresses. Therefore, in our research, we investigated the effect of Nrg1 protein on \NTHI gene expression under nitrogen starvation conditions.

Computational approaches have become important understanding the protein-DNA interactions involved in many important biological processes such as gene regulation. In the last two decades, experimental results have been complemented with numerous computational approaches to predict the three-dimensional (3D) structural model of interacting macromolecules. Protein-protein and protein-nucleic acid complexes are the most commonly-attempted targets of molecular docking processes [27-32]. \NTHI promoter analysis revealed that three Nrg1 binding sites present in the promoter region. Therefore, in our research, we attempted to predict the three-dimensional structural model of the interaction between the zinc-finger domain of Nrg1 protein and Nrg binding sites on the \NTHI promoter.

In this research we investigated the molecular function of Nrg1p in the transcriptional regulation of the \NTHI gene under nitrogen starvation conditions. In order to validate our experimental results and to determine which of the three Nrg1 binding sequences on the \NTHI promoter is involved in transcriptional regulation, we tried to form an \emph{in silico} binding model of Nrg1 protein to the \NTHI promoter. Our experimental results clearly indicated that transcription of \NTHI is repressed by Nrg1 protein depending on growth conditions, and our computational results showed that the predicted target sequence of the Nrg1 repressor protein on the \NTHI promoter region is the third CCCT box located –349 bp upstream of the transcriptional start site.

Materials and Methods

\textbf{Yeast Strains and Plasmids.} \textit{S. cerevisiae} strains BY4741 (\textit{Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0}) and their mutant derivative \Delta\nrg1 (\textit{Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, YDR043C::KanMX4}) were used in this study [33]. All strains were obtained from EUROSCARF (Frankfurt, Germany). \NRG1 gene was completely replaced with the geneticin resistance-codifying \KanMX4 module in the \Delta\nrg1 strain. BY4741 strain of \textit{S. cerevisiae} has no known mutations relevant to trehalose metabolism.

Plasmid used in this research, pNL1, was provided by Professor Jean Marie François (Institut National des Sciences Appliquées, Toulouse, France). pNL1 plasmid containing \NTHI::lacZ gene fusion was used to quantitate promoter activity of the \NTHI gene in response to varying growth conditions. In this expression vector, the 770bp promoter region, upstream of the translation start site, the NTHI gene fused in-frame to the lacZ gene. It has been shown that this promoter region contains all of the regulatory sites required for \NTHI gene expression [34]. pNL1 is a Yep353-based expression vector stably maintained in selective growth conditions in \textit{S. cerevisiae} transformants [35]. Escherichia coli strain, DH5α, was used to amplify both plasmid DNAs.

\textit{S. cerevisiae} strains were cultured in YPD medium (1% Yeast Extract, 2% Bactopeptone and 2% glucose) for plasmid transformation. The plasmids were transformed into the wild-type and \Delta\nrg1 mutant yeast strains using lithium acetate-polyethylene glycol procedure as described previously [36]. Selection of transformants was done on the yeast synthetic drop-out medium without uracil (YSD w/o URA), supplemented with 2% glucose and grown at 30 °C in an incubator to get well-grown colonies. Yeast colonies were patched to fresh YSD plates and grown for 2–3 days at 30 °C. These yeast patches were used in liquid culture inoculations.

\textbf{Growth Conditions.} To determine the doubling times (dt) and specific growth rates (\mu) of the yeast strains, yeast cells were grown in a minimal medium containing 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, and the appropriate amino acids. Optical densities of yeast cultures (OD600) were measured spectrophotometrically every 2 h and used for calculation.

Wild type and \Delta\nrg1 mutant yeast transformants were grown overnight in YSD culture supplemented with 2%
glucose at 120 rpm and 30 °C to get saturated yeast cultures. Saturated overnight cultures were refreshed in the same culture and grown to an optical density of 0.8–1.0 (A600) at 30 °C with constant shaking. At the end of this growth period, the yeast cultures were divided into two parts, and half was directly used for enzyme assays (non-treated culture). The second part of the culture was harvested and washed twice with sterile distilled water, the resuspended in fresh YSD culture supplemented with 0.1% proline instead of ammonium sulfate, and further incubated 4 h at 30 °C. At the end of the incubation periods, yeast cells were harvested and used for the measurements of β-galactosidase activities and the trehalose content of the yeast transformants.

**β-galactosidase Activity Assay.** β-galactosidase activity of the yeast transformants was determined as described previously [37]. After completion of incubation, the harvested yeast cells were washed and resuspended in 200 µL of the breaking buffer (100 mM Tris HCl pH:8.0, 1 mM DTT, 20% glycerol, 4 mM PMSF). Cells were permeabilized with 20 µL of 0.1% SDS and 20 µL of chloroform in Z-Buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol, pH 7.0). Beta galactosidase units were given in nanomoles of o-nitrophenyl-β-D-galactopyranoside (ONPG), cleaved per minute, per milligram of protein in permeabilized yeast cells. Protein concentrations were determined by Lowry assay and BSA was used as a standard [38]. Yeast transformants were grown in triplicate and β-galactosidase assays repeated twice. Standard deviations for β-galactosidase units were <15%.

**Trehalose Assay.** Trehalose assays of the transformant yeast cells were determined as described previously [34]. Yeast transformants were removed and washed with ice-cold water, resuspended in 250 µL of 0.25 M Na2CO3, and boiled for 2 h. 150 µL of 1 M acetic acid and 600 µL of 0.2 M sodium acetate pH 5.2 were then added. The cell mixture was incubated in the presence of 3 mM trehalase enzyme (Sigma, T8778) at 37 °C for 18 h. Amount of the liberated glucose was determined enzymatically via the glucose oxidase-peroxidase system (GOD-POD assay) using a commercial kit (Fluistest®- GLU, Biocon, Germany) [39]. Determined trehalose content of the yeast cells was given as micrograms of glucose equivalent per milligram of wet mass (µg glucose/mg cell wet weight) of the yeast cells.

**In silico Analysis.** NTH1 promoter region (1000 bp) and Nrg1 amino acid sequence were obtained from the Saccharomyces cerevisiae Genome Database. The potential Nrg1 binding site on NTH1 promoter was determined using the JASPAR CORE data base (ID:MA0347.1) and are given in Figure 1. 60 bp of DNA fragments, including Nrg1 binding site(s), were converted to .pdb format using the 3D-DART web server with default parameters [40]. The 3D-DART web server accepted the fasta formatted files that included the bases belonging to the 5‘–3’ template strand. The server generated an additional restraint file to maintain the DNA conformation during the flexible refinement stage of the docking. This case server added 60 bases on the 3’ site of the template strand. The 3D-DART server (3DNA-Driven DNA Analysis and Rebuilding Tool) provided a convenient means of generating custom3D structural models of DNA with control over local and global conformation. Visualization of the two ‘nrg1p_znf1.pdb’ and ‘nrg1p_znf2.pdb’ files was done in UCSF Chimera v.1.13.1 [41]. UCSF Chimera is a highly extensible program for interactive visualization and analysis of molecular structures and related data. Predicted 3D models of both Nrg1 protein (231 residues)

![Figure 1. NTH1 Promoter (1000 bp Upstream of Coding Region) and Amino acid Sequence of Nrg1 protein. (A) Potential Nrg1 Binding Sites Indicated in Italic; Two DNA Fragments used in Molecular Docking Analysis Underlined and Labeled as znf1 and znf2; the Powerful Site for Nrg1 Protein Binding Indicated in Bold; Transcription Initiation Site Labeled in Bold. (B) Zinc-finger DNA-binding Region Underlined; Amino acids Making Close Contact with DNA is Presented in Frames; Numbers in Superscripts Indicate Amino Acid Positions in Zinc-finger Domain](image-url)
and C2H2 domain (residues between 174 and 226) were created by means of the I-TASSER webserver with default parameters [42, 43, 44]. I-TASSER (Iterative Threading ASSEMBly Refinement) predicted protein structure and function in a hierarchical approach. It first identified structural templates from the PDB by the multiple threading approach LOMETS, with full-length atomic models constructed by repetitive template-based fragment assembly simulations. I-TASSER predicted the top five final models for both Nrg1 full protein and C2H2 domain according to C-score. Confidence of each model was quantitatively measured by C-score. C-score is typically in the range of ~5 to 2, where a C-score of a higher value signifies a model with a higher confidence. For molecular docking, the .pdb files of Nrg1 (full protein and C2H2 domain) obtained from the I-TASSER and the NTH1 promoter fragments (nrg1p_znf1.pdb and nrg1p_znf2.pdb) were uploaded to the NPDock web server with default parameters [45]. NPDock (Nucleic Acid-Protein Dock) is a web server for the modeling of RNA-protein and DNA-protein complex structures. UCSF Chimera v.1.13.1 was used for visualization of the NPDock files [41].

Results

Yeast strains used in this research, BY4741 and Y03979 (Δnrg1), were incubated in a minimal medium at 30 °C for 48 h. Optical densities of yeast cultures (OD_{600}), measured spectrophotometrically every 2 h, were used for calculation of doubling times (dt) and specific growth rates (µ). Doubling times of Δnrg1 and wild-type yeast cells were determined to be 217 min (3 h 37 min) and 156 min (2 h 36 min), respectively. Specific growth rate of Δnrg1 yeast cells (0.2003 h^{-1}) was slower than that of the wild-type (0.2848 h^{-1}). In order to determine the metabolic and genetic effects of environmental changes, yeast cells must complete at least one cell cycle. Cell cycles were completed in 3.5 and 2.5 hours in Δnrg1 mutant and wild-type yeast cells, respectively. For this reason, the incubation period performed in the next steps was 4 h for both yeast strains.

Effect of Nrg1 Protein on NTH1 Gene Expression and Trehalose Accumulation. Nrg1 represses some genes involved in carbohydrate metabolism such as STAI, DOG2, SUC2, GAL1, and GAL10 genes [22,23,24]. The NTH1 gene is also involved in carbohydrate metabolism and regulated by glucose level as in STAI and SUC2 genes. Therefore, NTH1 gene expression may be controlled by Nrg1 protein. NTH1-LacZ gene fusion includes 770 bp of the NTH1 promoter region, which contains STRE sequences and other cis-elements necessary for the regulation of NTH1 gene expression [34]. NTH1 gene expression level and trehalose amount were determined in exponentially growing yeast transformants.

Beta galactosidase enzyme activity in wild-type yeast cells was calculated as 76.8±8.6 Units, and in Δnrg1 mutant yeast cells 555.7 ± 34.2 Units (Figure 2). NTH1 expression in Δnrg1 mutant yeast cells was observed to be about 7x greater than in wild-type yeast strain. The high level of NTH1 transcription in the absence of Nrg1 protein suggested that Nrg1 protein was involved in the repression of NTH1 gene. It is known that Nrg1 protein acts as a negative regulator of carbon-repressed genes in S. cerevisiae yeast cells [24]. Similarly, our results showed that Nrg1 protein was a negative regulator of the NTH1 gene.

In order to determine the effect of Nrg1 protein on trehalose accumulation, trehalose content of the yeast cells was enzymatically hydrolyzed into glucose. Released glucose was then measured. The amount of trehalose was given as micrograms of glucose measured in mg cell wet weight of the yeast cells. The amount of trehalose measured in the wild-type yeast cells (277.4 ± 57.1 µg glucose/mg cell wet weight) was 2x the amount of trehalose measured in the Δnrg1 mutant yeast cells (100 ± 14.2 µg glucose/mg cell wet weight) (Figure 3). The high NTH1 gene expression in Δnrg1 mutant yeast cells may have resulted in a breakdown of trehalose. However, newly synthesized trehalase enzyme was inactivated. It was activated by PKA-mediated phosphorylation after environmental stress terminated. This means that, the high level of NTH1 transcription was not directly proportional to trehalase activity and could not cause that amount of trehalose accumulation in Δnrg1 mutant yeast cells. The strong probability was that Nrg1 protein may have been the repressor of genes involved in trehalose synthesis. Therefore, TPS1 gene expression in Δnrg1 mutant yeast cells needs to be determined.

Effect of Nitrogen Starvation on NTH1 Gene Expression and Trehalose Level. Glutamine, glutamate, asparagine and ammonium are good and preferred nitrogen sources for S. cerevisiae yeast cells, while proline and urea are weak and poor nitrogen sources. Expression of genes involved in the uptake and utilization of poor nitrogen sources is repressed in the presence of strong nitrogen sources by activity of the nitrogen catabolite repression mechanism [46, 47]. The Tor signaling pathway, which regulates NTH1 gene expression via Msn2/4 transcription factors, is regulated depending on the quantity and quality of nitrogen. We therefore analyzed the effects of poor nitrogen sources on NTH1 gene expression and trehalose accumulation, both in the wild-type and Δnrg1 mutant yeast cells. First, yeast cells were grown to logarithmic stage in the preferred nitrogen source, ammonium, then washed and transferred to a poor nitrogen source, proline. After four-hour incubation, yeast cells were harvested and used for determination of beta galactosidase activity and trehalose content.
β-galactosidase activities of wild-type and Δnrg1 mutant yeast strains were measured at 626.0 ± 42.6 Units and 553.6±55.5 Units, respectively, when transferred into the poor nitrogen source (Fig. 2). As shown, NTH1 transcription increased about 8-fold in the wild–type yeast strains, while transcription levels did not change much in the Δnrg1 mutant yeast cells under nitrogen starvation conditions.

The presence of proline in the growth condition of the yeast cells produced low nitrogen signaling and caused a rapamycin-like repression of the TOR signaling system. The repression of Tor signaling resulted in localization of Msn2/Msn4 in the nucleus and bound to STRE elements to activate NTH1 transcription. If we assume that Nrg1 protein is not involved in starvation conditions, due to the occupation STRE by Msn2/4

Figure 2. Transcription Levels of NTH1::LacZ Gene in Wild Type and Δnrg1 Mutant Yeast Cells. ‘No Stress’ Indicates that Yeast Cells were Grown in a Good Nitrogen Source. ‘Nitrogen Starvation’ Indicates that Yeast Cells were Grown in Proline. Beta Galactosidase Activities are Given in Nanomoles of ONPG Cleaved per min per mg of Protein

Figure 3. Transcription Levels of Wild Type and Δnrg1 Mutant Yeast Cells Before and After Nitrogen Starvation. Trehalose Content is Given as Micrograms of Glucose per Milligram of Wet Weight of the Yeast Cells
factors, the similar levels of NTH1 transcription in both Δnrg1 and wild-type yeast cells can be expected. Transcription of NTH1 in Δnrg1 mutant yeast cells did not change before and during starvation conditions. That meant that Nrg1 repressor was essential for NTH1 transcription under normal growth conditions, where NTH1 gene expression was repressed.

It has been reported that the accumulation of trehalose starts as a diauxic shift and continues until the stationary phase of growth in S. cerevisiae yeast cells [48]. Similarly, the trehalase activity is low in the exponential phase and begins to increase during the stationary phase [49]. Also, yeast cells accumulate trehalose in response to stress irrespective of growth phase. The trehalose contents of starved wild-type and Δnrg1 mutant yeast cells were determined to be 656.1 ± 68.4 and 1298 ± 72.4 μg glucose/ng cell wet weight, respectively (Figure 3). It was determined that the amount of trehalose increased two-fold in wild-type yeast cells and 13-fold in Δnrg1 mutant yeast cells after exposure to nitrogen starvation. It was shown that the trehalose level in mutant yeast cells under nitrogen starvation was 2x that of the wild type.

**In silico Binding of Nrg1 Protein to NTH1 Promoter.** Computational techniques were used for elucidating a theoretical model of protein-DNA interactions. The three-dimensional structure of protein and target DNA sequences must be selected for making a good protein-DNA docking model that is close to its native interaction. Our experimental results showed that Nrg1 protein had a negative role in NTH1 transcription. We assumed that Nrg1 binds to the NTH1 promoter as in the STA1 gene [22]. From this perspective, we attempted to determine in silico binding of Nrg1 repressor protein to the NTH1 promoter region and to determine which Nrg1 binding sequence motif was involved in this interaction. Predicted 3D models of the full Nrg1 protein and zinc-finger-C2H2 domain were created by means of I-TASSER [42, 43, 44]. I-TASSER predicted the top five final models for both Nrg1 full protein and C2H2 domain according to C-score. The protein models having highest C-scores are seen in Figure 4A and 4B for Nrg1 protein and C2H2 domain, respectively. Nrg1 protein was used for molecular docking to DNA fragments (nrg1p_znf1.pdb and nrg1p_znf2.pdb) using NPDock with a default value for RMSD threshold set to 10 Å since values up to 10 Å gave the most reasonable results [45]. Within the RMSD threshold values, no simulation was obtained for the first DNA fragment (nrg1p_znf1.pdb). However, the second DNA fragment (nrg1p_znf2.pdb) yielded a reasonable simulation (Figure 5). The second DNA fragment included two Nrg1 binding sites so one or both of them could be used for interaction. In order to determine close contacts between atoms of amino acid residues and nucleotides, molecular interaction of the C2H2 domain with the second DNA fragment (nrg1p_znf2.pdb) was formed using NPDock with the same parameters [45]. Short contacts between the atoms of C2H2 residues and the nucleotides localized in the Nrg1 binding site were determined and 3D structure of each interaction is given in Figure 6. Results showed the Nrg1 binding site localized on the non-template strand (5'-GGA(GA)G(A)G(A)TGG(A)GGGGA-3'). Amino acid residues in the C2H2 domain, threonine (T15), histidine (H19) and arginine (R18), showed a close interaction with A190, G289 (or G297), and G299, respectively (Figure 6). Computational analysis revealed that the potential binding site of the Nrg1 protein to the NTH1 promoter was localized ~349 bp upstream of the transcriptional start site.

![Figure 4. Modeling of Nrg1 Protein and Zinc-finger Domain.](image)

(A) Nrg1 Protein Modeled via I-TASSER Showing C-score −0.85, Estimated RMSD 7.5 ± 4.3Å and Estimated TM-score 0.61 ± 0.14Å; (B) Zinc-finger Domain of Nrg1 Protein Modeled via I-TASSER Showing C-score 0.19, Estimated RMSD 2.8 ± 2.0Å, and Estimated TM-Score 0.74 ± 0.11Å

![Figure 5. Simulation of Nrg1 Binding to NTH1 Promoter (image)](image)

Using NPDock. Default Value for RMSD Threshold 10Å. Blue Represents NTH1 Promoter; Cyan Circles Represent the Two Zinc (Zn) Ions; C2H2 Region is Colored in Orange and Regulatory Region Colored in Green
Discussion

In *S. cerevisiae* yeast cells, the TOR signaling pathway responded to nutrient availability by regulating related genes. This was accomplished by controlling the nuclear localization of some transcription factors involved in regulation of the genes. The zinc-finger DNA-binding transcription factors, Msn2/4, bound to STRE sequences in the *NTH1* promoter and activated gene expression. Like Msn2/4, Nrg1 protein also contained a zinc-finger motif and bound to DNA. Binding site of Nrg1 protein on the *NTH1* promoter was the same as that of the Msn2/4 binding site. Nrg1 protein represses *ENA1* gene expression by binding to STRE sequences [50]. In addition, Nrg1 interacts with the Cyc8/Tup1 corepressor complex in the repression of *SUC2, STA1*, and *GAL* genes [22,24], since the Cyc8/Tup1 complex cannot bind to DNA and interacts with a connectible protein such as Nrg1 and Mig1.

In our study, it was determined that *NTH1* gene expression increased seven-fold in Δ*nrg1* mutant yeast cells under normal growth conditions and did not change in nitrogen starvation. This suggested that Nrg1 protein was required for repression of the *NTH1* gene. Nrg1 protein acted as a negative regulator of glucose-repressible genes, but the binding sequences of Nrg1 and Mig1 proteins on DNA were completely different. While the repression mechanism of Mig1 protein, which was mediated by Snf1 kinase-dependent phosphorylation,
as was clearly revealed, there was no sufficient information about the mechanism of Nrg1 repression. It has been suggested that Nrg1 and Nrg2 proteins may be directly or indirectly targeted by Snf1 kinase [23]. However, the appropriate phosphorylation sites on Nrg1 protein for Snf1 kinase have not yet been identified. For this reason, it is thought that the repression mechanism of Nrg1 is different from Mig1 [51].

Mig1 is not sufficient for repression of some glucose-repressible genes. It is known that some genes can be repressed in the absence of Mig1, or that some other genes cannot completely be repressed, even if the Mig1 protein binds to their promoters. More than one repressor protein can bind to a promoter to ensure complete glucose repression [52]. It was shown that the Nrg1 protein specifically bound to two regions in the upstream activation sequence of the STA1 gene; deletion of the NRG1 gene caused an increase in STA1 transcription in the presence of glucose [22]. Nrg1 acted as a DNA-binding repressor and mediated glucose repression of the STA1 gene expression by recruiting the Ssn6-Tup1 complex. Expression of SUC2, GAL1, and GAL10 genes in Amig1, Amrg1, and Amig2 mutant yeast strains was lower than in the Amrg1Amig1Amig2 triple-mutant yeast strain [24]. Therefore, as in the STA1 gene, Nrg1 protein may be necessary for ‘complete repression’ of NTH1 gene expression.

Trehalose synthesis is accomplished by the TPS enzyme complex. Stress-dependent trehalose degradation is carried out by Nth1. Breakdown of trehalose is controlled by a number of transcriptional and posttranslational mechanisms. In our study, the amount of trehalose increased two-fold during nitrogen starvation conditions in the wild-type yeast cells. However, deletion of the NRG1 gene caused a 13-fold increase in NTH1 transcription during nitrogen starvation. This suggested that the Nrg1 protein may also be the repressor of the TPS complex.

Our computational analysis revealed that Nrg1 protein can bind to NTH1 promoter for regulating transcription. NTH1 promoter includes three putative binding site for Nrg1 protein. We showed that the third Nrg1 binding site was localized –349 bp upstream from the transcription initiation site, and is a powerful binding site for Nrg1 protein. A similar molecular approach may be applied to TPS1 promoter.

**Conclusion**

In conclusion, results of this study show that Nrg1 protein repress the transcription of the NTH1 gene. Nrg1 protein may interact with other repressor proteins and may be involved in the complete repression of the NTH1 gene. Our computational modeling of protein-DNA docking supports the possibility of Nrg1 binding to the NTH1 promoter region. Nrg1 protein may also be involved in the repression of genes involved in TPS complex formation. To support these data, further genetic and biochemical analysis must be conducted in the future using Amig1, Amig2, and Amig1Amig2 double-mutant yeast strains.

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**References**

[1] Vyas, V.K., Berkey, C.D., Miyao, T., Carlson, M. 2005. Repressors Nrg1 and Nrg2 regulate a set of stress-responsive genes in *Saccharomyces cerevisiae*. Eukaryotic Cell. 4(11): 1882-1891, doi: 10.1128/EC.4.11.1882-1891.2005.

[2] Singer, M.A. and Lindquist S. 1998. Thermotolerance in *Saccharomyces cerevisiae*: The Yin and Yang of trehalose. Trends Biotechnol. 16: 460-468, https://doi.org/10.1016/S0167-7799(98)01251-7.

[3] François, J. and Parrou, J.L. 2001. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 25: 125-145, https://doi.org/10.1111/j.1574-6976.2001.tb00574.

[4] Trevisol, E.T., Panek, A.D., De Mesquita, J.F., Eleutherio, E.C. 2014. Regulation of the yeast trehalose-synthase complex by cyclic AMP-dependent phosphorylation. Biochim. Biophys. Acta. 1840(6): 1646-1650, doi: 10.1016/j.bbagen.2013.12.010.

[5] Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., Wiemken, A., Thevelein, J.M. 1998. Composition and functional analysis of the *Saccharomyces cerevisiae* trehalose synthase complex. J. Biol. Chem. 273: 33311-33319, doi: 10.1074/jbc.273.50.33311.

[6] Elbein, A.D., Pan, Y.T., Pastuszak, I., Carroll, D. 2003. New insights on trehalose: A multifunctional molecule. Glycobiology. 13(4): 17-27, https://doi.org/10.1093/glycob/cwg047.

[7] Londesborough, J. and Varimko, K. 1984. Characterization of two trehalases in Baker’s yeast. Biochem. J. 219: 511-518, doi:10.1014/bj2190511.

[8] App, H. and Holzer, H. 1989. Purification and characterization of neutral trehalase from the yeast ABYS1 Mutant. J. Biol. Chem. 264(29): 17583-17588.

[9] Nwaka, S., Kopp, M., Holzer, H. 1995. Expression and function of the trehalase genes NTH1 and YBR0106 in *Saccharomyces cerevisiae*. J. Biol.
Effect of Nrg1 Repressor on NTH1 Transcription and Molecular

[10] Kopp, M., Müller, H., Holzer, H. 1993. Molecular analysis of the neutral trehalase gene from Saccharomyces cerevisiae. J. Biol. Chem. 268(7): 4766-4774, http://www.jbc.org/content/268/7/4766.long.

[11] Wolfe, K.H. and Lohan, A.J. 1994. Sequence Around the Centromere of Saccharomyces cerevisiae Chromosome II: Similarity of Cen2 to Cen4. Yeast. 10: 41-46, doi: 10.1002/yea.32010006.

[12] Zähringer, H., Burgert, B.M., Holzer, H., Nwaka, S. 1997. Neutral trehalase Nhlp1 of Saccharomyces cerevisiae encoded by the NTH1 gene is a multiple stress responsive protein. FEBS Lett. 412(3): 615-620, https://core.ac.uk/download/pdf/82232615.pdf.

[13] Zähringer, H., Thevelein, J.M., Nwaka, S. 2000. Induction of neutral trehalase Nhl1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: Variations of PKA effect during stress and growth. Mol. Microbiol. 35(2): 397-406, https://doi.org/10.1046/j.1365-2958.2000.01706.x.

[14] Genç, T.T. and Akmen, N. 2018. The Role of SWI/SNF Chromatin Remodelling Complex in the metabolism of Trehalose. IJRSR 9: 29024-29029, http://dx.doi.org/10.24327/ijrsr.2018.0909.2780.

[15] Winderickx, J., De W.J., Crauwels, M., Hino, A., Hohmann, S., Van Dijck, P., Thevelein, J. M. 1996. Regulation of genes encoding subunits of the trehalose synthase complex in Saccharomyces cerevisiae: Novel variations of stre-mediated transcription control. Mol. Gen. Genet. 252(4): 470-482, doi: 10.1007/bf02173013.

[16] Martinez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., Estruch, F., 1996. The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. 15(9): 2227-2235, https://doi.org/10.1002/j.1460-2075.1996.tb00576.x.

[17] Inoki, K. and Guan, K.L. 2006. Complexity of the TOR signaling network. Trends Cell Biol. 16(4): 206-212, https://doi.org/10.1016/j.tcb.2006.02.002.

[18] Veisova, D., Macakova, E., Rezabkova, L., Sulc, M., Vacha, P., Sychrova, H., Obsil, T., Obsilova, V. 2012. Role of individual phosphorylation sites for the 14-3-3-protein-dependent activation of yeast neutral trehalase Nhl1. Biochem. J. 443(3): 663-670, doi: 10.1042/Bj20111615.

[19] Beck, T and Hall, M.N. 1999. The TOR signaling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402: 689-692, doi: 10.1038/45287.

[20] Stracka, D., Jozefczuk, S., Rudroff, F., Sauer, U. and Hall, M.N. 2014. Nitrogen source activates TOR (Target of Rapamycin) complex1 via glutamine and independently of Gtr/Rag proteins. J. Biol. Chem. 289(36): 25010-25020, doi:10.1074/jbc.M114.574335.

[21] Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., O’Shea, E.K. 2003. Global Analysis of Protein Localization in Budding Yeast. Nature. 425: 686-691, doi: 10.1038/nature02026.

[22] Park, S.H., Koh, S.S., Chun, J.H., Hwang, H.J., Kang, H.S. 1999. Nrg1 is a transcriptional repressor for glucose repression of STAL gene expression in Saccharomyces cerevisiae. Mol. Cell. Biol. 19(3): 2044-2050, doi: 10.1128/MCB.19.3.2044.

[23] Vyas, V.K., Kuchin, S., Carlson, M. 2001. Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. Genetics. 158(2): 563-572, doi: 10.1128/mcb.19.3.2044.

[24] Zhou, H. and Winston, F. 2001. NRG1 is required for glucose repression of the SUC2 and GAL genes of Saccharomyces cerevisiae. BMC Genet. 2: 5, doi: 10.1186/1471-2156-2-5.

[25] Kuchin, S., Vyas, V.K., Carlson, M. 2002. Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate FLO11, haploid invasive growth and diploid pseudohyphal differentiation. Mol. Cell. Biol. 22(12): 3994-4000, doi: 10.1128/mcb.22.12.3994-4000.2002.

[26] Harbison, C.T., Gordon, D.B., Lee, T.I., Rinaldi, N.J., Macisaac, K.D., Danford, T.W., Hannett, N.M., Tagne, J.B., Reynolds, D.B., Yoo, J., Jennings, E.G., Zeitlinger, J., Pokholok, D.K., Kellis, M., Rolfe, P.A., Takusagawa, K.T., Lander, E.S., Gifford, D.K., Fraenkel, E., Young, R.A. 2004. Transcriptional regulatory code of a eukaryotic genome. Nature. 431: 99-104, doi: 10.1038/nature02800.

[27] Rodrigues, J.P. and Bonvin, A.M. 2003. Integrative computational modeling of protein interactions. FEBS J. 281: 1988-2003, https://doi.org/10.1111/febs.12771.

[28] Huang, S.Y. 2014. Search strategies and evaluation in protein-protein docking: Principles, advances and challenges. Drug Discov. Today 19: 1081–1096, doi: 10.1016/j.drudis.2014.02.005.

[29] Huang, S.Y. 2015. Exploring the potential of global protein-protein docking: An overview and critical assessment of current programs for automatic ab initio docking. Drug Discov. Today 20(8): 969-977, doi: 10.1016/j.drudis.2015.03.007.

[30] Van Dijk, M. and Bonvin, A.M. 2008. A protein-DNA docking benchmark. Nucleic Acids Res. 36 (14): e88, doi: 10.1093/nar/gkn386.

[31] Van Dijk, M., Visscher, K.M., Kastritis, P.L. and Bonvin, A.M. 2013. Solvated protein-DNA docking using HADDOCK. J. Biomol. NMR 56: 51–63, doi: 10.1007/s10858-013-9734-x.
[32] Tuszynska, I., Matelska, D., Magnus, M., Chojnowski, G., Kasprzak, J.M., Kozlowski, L.P., Dunin-Horkawicz, S. and Bujnicki, J.M. 2014. Computational modeling of protein-RNA complex structures. Methods 65: 310–319, doi: 10.1016/j.ymeth.2013.09.014.

[33] Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., Boeke, J.D. 1998. Designer Deletion Strains Derived from Saccharomyces cerevisiae S288C: A Useful Set of Strains and Plasmids for PCR-Mediated Gene Disruption and Other Applications. Yeast. 14: 115–132, doi: 10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2.

[34] Parrou, J.L. and Francois J. 1997. A Simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal. Biochem. 248(1): 186-188, doi: 10.1006/abio.1997.2138.

[35] Liao, X.B., Clare, J.J., Farabaugh, P.J. 1987. The upstream activation site of a Ty2 element of yeast is necessary but not sufficient to promote maximal transcription of the element. Proc. Natl. Acad. Sci. USA. 84(23): 8520-8524, doi: 10.1073/pnas.84.23.8520.

[36] Rose, M.D., Winston, F., Heiter, P. 1990. Methods in Yeast Genetics. A Laboratory Course Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, https://doi.org/10.1016/0307-4412 (91)90039-B.

[37] Rose, M. and Botstein, D. 1983. Construction and use of gene fusions to lacZ (β-galactosidase) that are expressed in yeast. Meth. Enzymol. 101: 167–180, doi:10.1016/0076-6879(83)01012-5.

[38] Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193(1): 265-275, http://www.jbc.org/content/193/1/265.long.

[39] Goldstein, A. and Lampen, J.O. 1975. Beta-D-fructofuranoside fructohydrolase from yeast. Meth. Enzymol. 42: 504-511, doi:10.1016/0076-6879(75)42159-0.

[40] Van Dijk, M and Bonvin, A.M.J.J. 2009. 3D-DART: a DNA structure modelling server. Nucleic Acids Res. 37: 235-239, doi: 10.1093/nar/gkp287. (Retrieved April 2017)

[41] Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. and Ferrin, T.E. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25: 1605–1612, doi: 10.1002/jcc.20084. (Retrieved August 2019)

[42] Yang J, Yan R., Roy, A., Xu, D., Poisson J. and Zhang, Y. 2015. The I-TASSER Suite: Protein structure and function prediction. Nature Methods, 12: 7-8, doi: 10.1038/nmeth.3213.

[43] Yang, J. and Zhang, Y. 2015. I-TASSER server: new development for protein structure and function predictions, Nucleic Acids Res. 43: 174-181, doi: 10.1093/nar/gkv342.

[44] Zhang, C., Freddolino, P.L. and Zhang, Y. 2017. COFACTOR: improved protein function prediction by combining structure, sequence and protein–protein interaction information. Nucleic Acids Res. 45: 291-299, doi: 10.1093/nar/gkx366.

[45] Tuszynska, I., Magnus, M., Jonak, K, Dawson, W. and Bujnicki, J.M. 2015. NPDock: a web server for protein-nucleic acid docking. Nucleic Acids Res. 43(1):425-430, doi:10.1093/nar/gkv493. (Retrieved April 2017)

[46] Ter Schure, E.G., Van Riel, N.A., Verrips, C.T. 2000. The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. FEMS Microbiol. Rev. 24(1): 67-83, doi: 10.1111/j.1574-6976.2000.tb00533.x.

[47] Magasanik, B. and Kaiser, C.A. 2002. Nitrogen regulation in Saccharomyces cerevisiae. Gene. 290(1-2): 1-18, doi:10.1016/s0378-1119(02)00558-9.

[48] Werner-Washburne, M., Braun, E., Johnston, G.C. and Singer, R.A. 1993. Stationary phase in the yeast Saccharomyces cerevisiae. Microbiol. Rev. 57(2): 383-401, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC372915/.

[49] Winkler, K., Kienle, I., Burgert M., Wagner J.C., Holzer H. 1991. Metabolic regulation of the trehalose content of vegetative yeast. FEBS Lett. 291: 269-272, https://doi.org/10.1016/0014-5793(91)81299-N.

[50] Lamb, T.M. and Mitchell, A.P. 2003. The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes NRGI and SMP1 in Saccharomyces cerevisiae. Mol. Cell. Biol. 23(2): 677-686, doi: 10.1128/mcb.23.2.677-686.2003.

[51] Devit, M.J., Waddle, J.A., Johnston, M. 1997. Regulated nuclear translocation of Mig1 glucose repressor. Mol. Biol. Cell. 8(8): 1603-1618, doi: 10.1091/mbc.8.8.1603.

[52] Lutfiyya, L.L. and Johnston M. 1996. Two zinc-finger-containing repressors are responsible for glucose repression of SUC2 expression. Mol. Cell. Biol. 16: 4790-4797, doi: 10.1128/mcb.16.9.4790.