Stb3 Binds to Ribosomal RNA Processing Element Motifs That Control Transcriptional Responses to Growth in *Saccharomyces cerevisiae*  

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Transfer of quiescent *Saccharomyces cerevisiae* cells to fresh medium rapidly induces hundreds of genes needed for growth. A large subset of these genes is regulated via a DNA sequence motif known as the ribosomal RNA processing element (RRPE). However, no RRPE-binding proteins have been identified. We screened a panel of 6144 glutathione S-transferase-open reading frame fusions for RRPE-binding proteins and identified Stb3 as a specific RRPE-binding protein, both *in vitro* and *in vivo*. Chromatin immunoprecipitation experiments showed that glucose increases Stb3 binding to RRPE-containing promoters. Microarray experiments demonstrated that the loss of Stb3 inhibits the transcriptional response to fresh glucose, especially for genes with RRPE motifs. However, these experiments also showed that not all genes containing RRPEs were dependent on Stb3 for expression. Overall, our data support a model in which Stb3 plays an important but not exclusive role in the transcriptional response to growth conditions.

Evolutionary pressures make it imperative that quiescent yeast cells respond rapidly to fresh medium. By the same token, the cell must cease growth with precision as nutrients become depleted (1, 2). *Saccharomyces cerevisiae* cells have developed elaborate and redundant pathways for sensing glucose (3, 4). Early microarray work established that specific gene sets are regulated as cells deplete glucose and pass through the diauxic shift (5). More recent work has focused on the transition from quiescence in poor or depleted medium to rapid growth in fresh glucose medium. Nutrient repletion produces a very rapid induction of genes involved in protein synthesis, mass accumulation, and cell division (6–9).

A significant portion of genes that are rapidly induced by fresh medium encode ribosomal proteins (RPs). 2 RP genes frequently contain binding sites for the Rap1 and Abf1 transcriptional regulators (10, 11). In addition, the TOR kinase and cAMP-dependent protein kinase nutrient signaling pathways converge on the Sfp1 and Crf1 transcriptional regulators to control RP gene transcription (12–14). The Rap1 cofactor Gcr1 appears to regulate the nuclear location and the expression of RP genes (15). The nutrient-controlled kinases Sch9 and Yak1 also play a role in RP gene regulation (14, 16).

However, many of the growth–related genes induced by fresh medium do not encode ribosomal proteins but instead encode other gene products needed for rapid growth, including transcription factors, components involved in ribosome assembly, and translation factors (6, 14, 17). We refer to this set as non-RP growth genes (9). Little is known about the control of this gene set. In addition to being induced by fresh medium, these genes are negatively regulated by a wide variety of stresses (8, 18–20). As with the RP genes, induction of the non-RP growth genes by fresh medium involves the cAMP-dependent protein kinase and TOR kinase pathways. However, it is clear that there are key differences between the regulation of the RP and non-RP growth genes. Induction of the non-RP growth genes does not appear to involve Rap1, Abf1, or Crf1 (21, 22). Furthermore, the kinetics of non-RP growth gene induction by nutrient addition are slightly different from kinetics for RP gene induction (6, 14, 23).

One clue to the mechanism underlying non-RP growth gene regulation has been the computational identification of two sequence motifs, termed ribosomal RNA processing element (RRPE) and polymerase A and C (PAC), that are overrepresented in non-RP growth promoters (6, 18, 24). The RRPE was initially identified via computational analysis of a set of growth-related genes (24), whereas PAC was identified in promoters of polymerase II transcribed genes that encode RNA polymerase I and III subunits (25). Work from independent laboratories has shown that RRPE and PAC can confer nutrient regulation to reporter genes (6, 9, 18, 26). Although these *cis*-acting elements are thought to play an important role in bringing quiescent cells into a state of rapid growth, the *trans*-acting regulatory proteins that bind to and act at these sequences have remained elusive.

Our preliminary work revealed a specific RRPE-binding protein in yeast extracts. We then used gel shift assays to screen a panel of 6144 ORF-GST fusions generated by Martzen *et al.* (27) roughly following the strategy reported by Hazbun and Fields (28). This approach successfully yielded an RRPE-binding protein, Stb3. Furthermore, we found that Stb3 binds to RRPE-containing promoters *in vivo*. Loss of Stb3 impedes the
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nutrient induction of many, but not all, non-RP growth genes. Taken together, our results establish a biological and biochemical role for Stb3 and identify an RRPE-binding protein involved in the induction of non-RP growth genes by glucose.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—The cells were grown in either YPD or SC medium with 2% glucose (29). The strains were: BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and 13527 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 stb3Δ::KanMX) (Research Genetics, Huntsville, AL); DLY6 (BY4742 in which Stb3 is chromosomally tagged with 3×Myc as described (30)). 64 GST-ORF fusion pools, containing 96 strains each, were a generous gift from Eric Phizicky (27). YDR169C-TAP (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 HIS3 STB3-Tap::HIS3) Open Biosystems (Huntsville, AL). (A more detailed description of the experimental procedures is provided in the supplemental materials.)

Screening of GST Fusion Proteins and Gel Shift Assays—Purification and gel shift assays of these pools were as described (28, 31, 32). The probe used for pool screening contained a triple repeat of RRPEs (AAAAATTT). For other experiments probes correspond to a NSR1 promoter segment containing a single PAC element flanked by RRPEs: 5′-GG TGG TGA AAA ATT TGC AAG GGC AG 3′ (RRPEs are underlined; PAC is underlined italics). When mutated, RRPE sequences were AagAagT and PAC sequences were CcagaC. In addition, a double-stranded oligonucleotide from the CLN3 promoter was used as a control competitor. This oligonucleotide is referred to as UR, unrelated.

Microarray Hybridization and Data Analysis—Labeled cRNA was mixed with hybridization controls, hybridized to Yeast Genome S98 Arrays (Affymetrix), washed, and stained with streptavidin-phycocerythin in a GeneChip Fluidics Station 400. The arrays were scanned using an Agilent GeneArray Scanner and Microarray Suite 5.0, and .CEL files were analyzed using DCHIP 1.3 (33). The intensity values were normalized across 12 independent microarrays using the invariant set normalization method of DCHIP (34). Model-based analysis including log2 transformation of expression indexes using the perfect match only model was performed using values from two independent microarrays for each time point/condition. The microarray data are shown in detail in Excel files (supplemental Table S1). Microarray data has been deposited at the GEO website (www.ncbi.nlm.nih.gov/geo) with accession number GSE8379.

RNA Preparation and Northern Blotting—RNA was separated by formaldehyde gel electrophoresis, blotted, and probed as described previously (35). Northern blots were probed with ATCI (+161 to +720), NOP14 (+100 to +621), YMR244W (+121 to +661), and a 600-bp SacI fragment from U2 to confirm uniform loading.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were conducted as described in Ref. 36. Samples were collected from quiescent cells grown for 3 days in YPD and also at 10 min after glucose addition and fixed in 1% formaldehyde for 15 min. After extract preparation, the clarified sonicated samples were incubated with IgG-Sepharose beads (Amersham Biosciences) in 50-μl suspensions for 16 h. Cross-links were reversed by incubating overnight at 65 °C, and the samples were treated with proteinase K and DNase-free RNase A for 2 h at 37 °C. DNA was extracted using QiAquick PCR purification kit (Qiagen). PCR primers were: NSR1, −400/+50 upstream, 5′-GAA GAA AGA CGA CAG CAA TTC-3′, and downstream, 5′-CAAGAAGGAAGTTAAGGCTTC-3′; DBP10, −400/+50 upstream, 5′-TGC CAC TAT CAC GTT GTC AAA-3′; and downstream, 5′-TCT CGA AGA TCA AGA CGA CAA-3′; ADH4, +582/+899 upstream, 5′-GGC TAC TAA CGG TGG GGA AAT CGG AGA C-3′; and downstream, 5′-GCA CAG GCA TCG GTG ATT GGG TTA GAG GC-3′.

RESULTS

Evidence for an RRPE-binding Protein—If RRPEs act as regulatory elements controlling the transcription of genes in response to glucose, then there should be at least one yeast protein that specifically interacts with RRPE sequences. To test this, we used gel shift assays to detect protein binding to a radiolabeled double-stranded oligonucleotide probe chosen from the NSR1 promoter sequence. We chose this sequence as a test oligonucleotide because NSR1 is a non-RP growth gene that is induced ~8-fold within 10 min of fresh medium addition (9, 37, 38). In addition, this region contains a single PAC sequence flanked by two RRPE sequences.

We found that this probe produced several nonspecific bands along with a single RRPE-specific band when added to a wild type yeast extract (Fig. 1, arrow). This band was judged specific because it was competed by an excess of cold competitor but was not competed when the RRPEs in the cold oligo-
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Screening within Pool 53 identified Stb3 as the RRPE-interacting protein. Stb3 binds to a complex containing Sin3 and Rpd3 (39, 40). Purified GST-Stb3 produced a very strong, specific gel shift band using either the tandem repeat RRPE probe used in our screen (not shown) or with the NSR1 promoter probe containing the pair of RRPEs flanking a PAC element (Fig. 2B).

Binding of GST-Stb3 to the NSR1 DNA probe was completely dependent on RRPE sequences but did not require the PAC sequences (Fig. 2B). Adding a 100-fold excess of unlabeled NSR1 probe effectively competed away the prominent gel shift band produced by purified GST-Stb3. However, the cold oligonucleotide was unable to bind and compete if it contained point mutations within the RRPE sequence (ΔRRPE). In contrast, mutations in the PAC sequence (ΔPAC) did not affect competition.

In addition to testing the ability of GST-Stb3 to bind to RRPEs, we examined the ability of the normal Stb3 in yeast protein extracts to form complexes with the RRPE-containing probe from NSR1 (Fig. 3). As shown in Fig. 1, this probe produced several complexes with yeast cell extracts; however, only one of them appeared to be specific. This specific Stb3-dependent band, indicated by the arrow, was consistently absent with stb3Δ extracts (Fig. 3A). This experiment also showed that addition of glucose to post-log cells did not alter the intensity of this band.

When we tested extracts from a strain expressing a chromosomally Myc-tagged Stb3, we found that the gel shift band had slightly reduced mobility (Fig. 3B, lower shaded arrow) and was...
dramatically super shifted in the presence of Myc antibodies (upper shaded arrow). We conclude that the Stb3 protein is directly associated with the protein-DNA complex.

**Stb3 in Vivo**—ChIP assays demonstrated in vivo binding of Stb3 at two different non-RP growth promoters that contain RRPEs: NSR1 and DBP10 (Fig. 4). This binding was affected by nutrients, being higher in the presence of glucose. A fragment from the open reading frame of ADH4, used as a control, was not immunoprecipitated, nor did we see enrichment of NSR1 or DBP10 promoter DNA in immunoprecipitates from untagged control extracts.

If Stb3 plays a role in the transcriptional response to glucose, then the loss of Stb3 should diminish this response. To test this we used Northern blots to follow the glucose induction of NSR1 and DBP10 (Fig. 5). This showed that loss of Stb3 reduced the glucose induction of both genes, with DBP10 induction being more Stb3-dependent than induction of NSR1. These two probes suggest that Stb3 plays a role in glucose induction but cannot account for all of the response.

To obtain a large scale view of the effect that STB3 deletion has on gene induction, we used Affymetrix microarrays to compare the induction of transcripts in wild type and stb3Δ cells as glucose was added to quiescent 3-day cultures. Within 10 min of glucose addition, a set of 1355 genes were induced by at least 2-fold over post-log expression levels (Fig. 6A and supplemental Table S1). This corresponds well to previously reported gene sets induced by nutrient repletion (6, 7, 9, 20). As can be seen by the heat map, the induction of some transcripts was little affected by loss of Stb3, whereas for others, STB3 deletion had a substantial impact. To further confirm that STB3 deletion blocks glucose induction of some transcripts, we probed the Northern blot shown in Fig. 5 with probes for YMR244w, ATC1, and NOP14. Our microarray experiment showed that glucose induction of these transcripts was Stb3-dependent; the Northern blots confirmed this (Fig. 5).

To get a better picture of this effect, the ratio of glucose induction in stb3Δ versus glucose induction in wild type cells was determined for each of the 1355 transcripts and plotted as a histogram (Fig. 6B). If glucose induction of a transcript was unaffected by loss of Stb3, then the log2 ratio is zero. The values falling to the left of zero reflect reduced glucose induction in stb3Δ mutants, whereas positive ratios reflect increased glucose induction in the stb3Δ mutants. As can be seen, the mode falls at a log2 value of approximately 0.5, which corresponds to a 1.4-fold reduction in the glucose response in the stb3Δ mutant. For ~550 of the genes, deletion of STB3 reduced glucose induction by 1.5-fold or more (supplemental Table S1).

The list of 1355 genes induced by glucose contains genes with and without RRPEs. If Stb3 regulates transcription by binding to RRPE elements, then there should be an association between
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Stb3 dependence and the presence of RRPEs in the promoters of these genes. We compared sets of genes that were strongly affected by STB3 deletion to sets of genes that were relatively Stb3-independent (Fig. 6C). Consistent with our model, the percentage of genes having at least one promoter RRPE jumped from 22% for Stb3-independent genes to 61% for the genes that showed the greatest Stb3 effect.

DISCUSSION

Stb3 as an RRPE-binding Protein—The addition of fresh medium to quiescent yeast cells causes the rapid induction of a very large gene set of genes (6–9). A large fraction of the genes induced by nutrients tend to play a role in protein synthesis and cellular growth. One set of these genes encodes RPs themselves, whereas another set is involved in other aspects of protein synthesis. These have sometimes been referred to as the ribosome biogenesis genes, but they actually cover a broader range of functions; we have referred to this latter set as the non-RP growth genes (9). It is not known how glucose induces the non-RP growth genes, but several groups have identified the RRPE and PAC elements in the promoters of these genes.

In this work, we initially demonstrated the presence of an RRPE binding activity in yeast cell extracts. We then went on to clearly identify Stb3 as an RRPE-binding protein. Loss of Stb3 affects the glucose induction of a large group of RRPE-containing genes.

Stb3 and Gene Regulation—Our results show that Stb3 plays a role in the transcriptional response to glucose repletion, but they also raise questions about the regulatory mechanism. The ChIP experiments indicate that glucose causes binding of Stb3 to the promoter regions of Stb3-dependent genes, a process that correlates with glucose activation of these genes. This suggests that glucose addition permits Stb3 to become associated with RRPEs to turn on gene expression.

Stb3 was originally identified in a two-hybrid screen for Sin Three Binding proteins (39, 40). This is significant because the Sin3-Rpd3 histone deacetylase has been implicated in the repression of non-RP growth genes in response to stresses including nutrient depletion (41–45). Thus, the discovery of Stb3 as an RRPE-binding protein may provide a link with nutrient sensing pathways already being investigated.

Although genes affected by Stb3 were more likely to have RRPEs in their promoters than Stb3-independent genes, a substantial fraction of the Stb3-dependent genes had no identifiable RRPE sequences, and the glucose induction of some genes with multiple promoter RRPE sequences were not affected by the loss of Stb3. These results are most easily explained by proposing that Stb3 plays a part in a process that involves multiple, redundant components. For example, some genes may be regulated by glucose through the actions of multiple pathways and/or transcription factors. For these, the loss of Stb3 alone might have little impact. It is also possible that Stb3 binds to sites that are not readily identified as RRPEs. In this case, a gene might be regulated via Stb3 and yet have no identifiable RRPEs.

Although it is clear that proteins such as Sfp1 play a role in regulating the non-RP growth genes containing RRPEs and PACs, direct interaction between Sfp1 and the PAC or RRPE motifs has not been found, despite numerous attempts (26). This has led to the speculation that Sfp1 regulates these genes indirectly through another DNA-binding protein. Thus, it is possible that Sfp1 lies downstream of Stb3. This might be regulated via Stb3 and yet have no identifiable RRPEs.

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