

Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene *BAP3* of *Saccharomyces cerevisiae*

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ABSTRACT

Expression of the *BAP3* gene of *Saccharomyces cerevisiae*, encoding a branched chain amino acid permease, is induced in response to the availability of several naturally occurring amino acids in the medium. This induction is mediated via an upstream activating sequence (called UAS_{aa}) in the *BAP3* promoter, and dependent on Stp1p, a nuclear protein with zinc finger domains, suggesting that Stp1p is a transcription factor involved in *BAP3* expression. In this paper, we show that Stp2p, a protein with considerable similarity to Stp1p, is also involved in the induction of *BAP3* expression. To gain more insight into the roles of *STP1* and *STP2*, we have overexpressed both Stp1p and Stp2p in yeast cells. Gel shift assays with the UAS_{aa} as a probe show that the UAS_{aa} can form two major complexes. One complex is dependent on Stp2p overexpression and the other is formed independently of *STP1* or *STP2*, suggesting that the UAS_{aa} is also bound by another factor. Here we show that the other factor is Abf1p, which binds specifically to the UAS_{aa} of *BAP3*.

INTRODUCTION

Saccharomyces cerevisiae can respond to the availability of several naturally occurring amino acids in its environment by inducing the expression of the corresponding amino acid uptake systems. It does so by sensing of the extracellular amino acids via a sensor, Ssy1p (1,2). The addition of amino acids results in a drastic induction of expression of several amino acid permease genes, e.g. the *AGP1* gene, encoding a broad specificity amino acid permease (3), the *TAT1* and *TAT2* genes encoding the tyrosine and tryptophan permeases (4), the *BAP2* gene encoding a branched chain amino acid permease (5) and the *BAP3* (*PAP1*) gene, encoding a protein with high similarity to Bap2p (6). We have identified an element in the promoter of *BAP3* that we called UAS_{aa} (amino acid-dependent upstream activator sequence), which is responsible

for this induction (7). We have shown that this UAS_{aa} is both necessary and sufficient for induction of *BAP3* expression by most L-amino acids found in proteins. Furthermore, we showed that Stp1p, a nuclear protein with zinc finger domains, plays an essential role in induction via the UAS_{aa}. Gel shift analysis showed that the UAS_{aa} can form a specific DNA–protein complex when incubated with total yeast extract. This complex is formed irrespective of whether the extract is isolated from cells grown in the presence or absence of amino acids in the medium, or from cells that contain or lack Stp1p. A limited mutational analysis of the UAS_{aa} showed that there is a strong correlation between the ability of the mutated UAS_{aa} to form a complex *in vitro* and its ability to function as an amino acid-dependent promoter element when fused to a *CYC1–LacZ* reporter. These observations led to the conclusion that the UAS_{aa} is constitutively bound by a factor different from Stp1p, and that this factor is also involved in the induction of transcription in response to amino acids in the medium.

In this paper we show that the factor that *in vitro* binds to the UAS_{aa} is Abf1p. This global transcription factor, encoded by *ABF1* (also known as *OBF*, *BAF1*, *TAF* and *GFI*; 8), was originally identified as a protein binding to autonomously replicating sequences. Abf1p has been shown to take part also in the regulation of expression of a large number of genes involved in various cellular processes and with a variety of expression patterns. For example, Abf1p has been shown to be involved in the regulation of transcription of some of the ribosomal protein genes, as well as the *COX6* gene (9), the *ARO3* gene (10), the *FOX3* gene (11), the *CHAI* gene (12) and the *HIS7* gene (13). We show that the UAS_{aa} harbours an Abf1p-binding site and that mutations within the UAS_{aa} that obliterate Abf1p binding also lead to a loss of the capacity of this DNA element to function *in vivo* as an amino acid-dependent UAS. However, we also show that the mere binding of Abf1p to a DNA element placed in front of a *CYC1–LacZ* reporter is not sufficient for induction of that reporter by amino acids.

Furthermore, we provide evidence that in addition to Stp1p, Stp2p, a protein with considerable similarity to Stp1p and having the same number and arrangement of zinc finger domains as Stp1p, is also involved in amino acid-induced transcription via the UAS_{aa}. Whereas amino acid-induced

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expression of *BAP3* is severely compromised in $\Delta stp1$ as well as in $\Delta stp2$ mutant cells, induction is completely lost in a $\Delta stp1/\Delta stp2$ double mutant. To test whether Stp1p or Stp2p can bind to the UAS_{aa}, we have overexpressed both proteins in yeast cells. Using extracts from cells overexpressing Stp2p in band shift assays, we found formation of an Stp2p-dependent complex.

Taken together, we have shown that induction of *BAP3* transcription in response to amino acids requires Stp1p and/or Stp2p, and that Abf1p is involved in the response.

MATERIALS AND METHODS

Strains, media and genetic methods

The *S. cerevisiae* strains used in this study are derived from M4054 (MAT α , *gap1*- $\Delta 101$, *ura3*, *gal2*) as described by Gauslund *et al.* (5). For the extraction of protein extracts used in the band shift assays we used the protease minus strain BJ1991 (MAT α , *leu2*, *trp1*, *ura3*-251, *pbr1*-1122, *pep4*-3, *gal2*; 14). Yeast cells were grown at 30°C, either in SD medium (2% glucose, 0.67% YNB with or without amino acids; Difco) or non-selectively in YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose). Yeast transformation was performed as described by Chen *et al.* (15). The *Escherichia coli* strain used in this study was SURETM {*el4*-(*mcrA*), Δ (*mcrCB*-*hsdSMR*-*mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn5 (*kan^r*), *uvrC*, *supE44*, *lac*, *gyrA96*, *relA1*, *thi-1*, *end A1* [F' *proAB*, *lacIqZDM15*, Tn10, (*tet^r*)]}. Bacterial cells were grown in 2YT medium (1% yeast extract, 1.6% bacto-tryptone, 0.5% NaCl), to which ampicillin (100 μ g/ml) was added if necessary. Bacterial transformation and plasmid DNA isolation were performed according to procedures described elsewhere (16,17).

STP1 and STP2 disruption

For the disruption of *STP2* we used 5 Δ Xho3 (a plasmid encoding the *STP2* gene, kindly provided by A. Hopper, Seattle, WA). First, the *STP2* gene was subcloned into pUC19. Subsequently, a 626 bp *HindII*-*SphI* fragment encoding amino acids 13–225 of Stp2p was replaced by the *KanMX4* gene (18), resulting in a *STP2* disruption cassette. The disruption cassette was isolated and transformed to strain M4054. After 6 h of growth in liquid non-selective YPD medium, transformants were plated onto YPD containing G418 (200 mg/l; Calbiochem) in order to select for disruptants. To make an *STP1* disruption mutant we made use of the *URA3*-based *STP1* disruption cassette pBR*stp1*::*URA3* as described by Wang and Hopper (19). Correct disruption of both *STP1* and *STP2* was confirmed by Southern blot analysis. The *stp2* $\Delta 10$ mutant M4270 and *stp1* $\Delta 10$ /*stp2* $\Delta 10$ double mutant M4272 were constructed as follows. From plasmid Yep24 containing the *STP2* ORF in a 2.8 kb *SspI* fragment 1150 bp were removed by digestion with *BstXI* followed by religation. The remaining ORF was inserted into plasmid pRS306. This construct was linearised with *HindIII* and introduced into strains M4054 and M4173. Subsequent selection on FOA plates yielded clones having lost *STP2*. Two clones, M4270 and M4272, were identified by PCR on chromosomal DNA to contain only *stp2* $\Delta 10$.

Overexpression of Stp1p and a HA epitope-tagged version of Stp2p

To overexpress Stp1p and Stp2p we placed the *STP1* and *STP2* genes under the control of the *PGK1* promoter. The 850 bp upstream region of the *PGK1* gene was isolated as a *HindIII*-*EcoRI* fragment from YEpR5 (a plasmid encoding the *PGK1* gene and flanking regions; 20) and subcloned (*EcoRI*-*HindIII*) into pBluescript (pBSKS; Stratagene), resulting in pMB15. The *STP1* gene was amplified from genomic DNA by PCR using the primers *STP1*-forward (5'-AAAGAATTCTGACATGAAGTGTACTACTATTGACG-3') and *STP1*-reverse (5'-TTTCTGCAGCTACGAATCGACTCTATGCGCTG-3'). The *STP2* gene was amplified from pRS426 (a plasmid encoding the *STP2* gene, generously supplied by A. Hopper, Seattle, WA) using the primers *STP2*-forward (5'-AAAGAATTCATCATGGGCGGCCGCCCTATCTTACTACT-ATCTTCAACACGG-3') and *STP2*-reverse (5'-TTTCTGCAGGAGGAGAGGAGTTTTGGGG-3'). The forward primers introduced an *EcoRI* site (bold) just in front of the ATG start codon (underlined) and in the case of *STP2* also a *NotI* site just after the ATG start codon (italic). The reverse primers introduced a *PstI* site at position +1987 relative to the ATG start codon for *STP1* (255 bp after the coding region) and a *PstI* site at position +1877 relative to the ATG start codon for *STP2* (250 bp after the open reading frame). The amplified genes were fused to the *PGK1* promoter in pMB15 making use of the *EcoRI* site. The *PGK1*-*STP1* fusion was cloned into the expression vectors YCplac33 (CEN, *URA3*) and YEplac195 (2 μ , *URA3*) (21) resulting in the single and multiple copy vectors pMB70 (SCPGK1-*STP1*) and pMB71 (MCPGK1-*STP1*), respectively. The *NotI* site introduced into the *STP2* gene was used to insert a DNA fragment encoding a triple HA epitope tag [(HA)₃] (22). After this insertion, the *PGK1*-(HA)₃-*STP2* fusion was cloned into YCplac33 (CEN, *URA3*) and YEplac195 (2 μ , *URA3*) resulting in the single and multiple copy vectors pMB73 [SCPGK1-(HA)₃-*STP2*] and pMB74 [MCPGK1-(HA)₃-*STP2*], respectively. To combine Stp1p and Stp2p overexpression, the *PGK1*-(HA)₃-*STP2* fusion was cloned into the expression vectors YCplac111 (CEN, *LEU2*) and YEplac181 (2 μ , *LEU2*), resulting in the single and multiple copy vectors pMB79 [*LEU2*, SCPGK1-(HA)₃-*STP2*] and pMB80 [*LEU2*, MCPGK1-(HA)₃-*STP2*].

Expression of Abf1p in *Escherichia coli*

For the expression of Abf1p in *E. coli* we made use of the constructs described by Halfter *et al.* (23). These constructs encode the wild-type Abf1p and an Abf1p mutant lacking its N-terminal DNA-binding domain (Abf1p $\Delta 33/240$), both under the control of the isopropyl- β -D-1-thiogalactopyranoside (IPTG; Fluka)-inducible *LacZ* promoter. Both constructs were transformed into *E. coli* and pregrown overnight in 2YT with ampicillin. Subsequently, the cells were inoculated into fresh 2YT medium with ampicillin at an OD_{660 nm} = 0.05 and grown to an OD_{660 nm} = 0.4–0.5. Then IPTG was added to a final concentration of 1 mM and aliquots were taken at 0, 1, 2 and 3 h after IPTG addition. Protein extracts were made as described by Halfter *et al.* (23) and expression of the yeast proteins was monitored by subjecting the protein extracts to SDS-PAGE (10%); subsequently the separated proteins were electroblotted onto nitrocellulose membranes (as recommended by the

manufacturer). The Abf1 proteins were visualised using a commercially available affinity-purified goat polyclonal primary antibody, yC-20 (diluted 1:500), raised against a peptide corresponding to an amino acid sequence mapping at the C-terminus of Abf1p, and with a horseradish peroxidase-conjugated secondary anti-goat antibody (diluted 1:5000). Both antibodies were purchased from Santa Cruz Biotechnology. Subsequently, proteins were visualised using the ECL detection kit from Amersham.

STP2 expression in *E.coli*

The *STP2* gene was amplified from total *S.cerevisiae* DNA by PCR using primers 5'-CCCGCTAGCCCTATCTTATCAC-TATCTTCAACACG-3' and 5'-CCCGAATTCTTAAAATTC TATCCATAAGC-3'. The PCR product was digested with *NheI* and *EcoRI* and subcloned into the *E.coli* expression vector pET24a(+) (Novagen), which had been opened with *NheI* and *EcoRI*. The resulting plasmid was termed pVDH22. The cloned *STP2* PCR product in pVDH22 was sequenced, and the sequence proved to be identical to the published *STP2* sequence. For expression of the *STP2* gene in *E.coli*, plasmids pVDH22 and pET24a(+) (as control) were transformed into strain BL21 (Novagen). Transformants were grown at 37°C in 100 ml LB cultures containing 50 µg/ml kanamycin to an OD_{600 nm} of 0.5. IPTG was then added to a final concentration of 100 mM and the cultures were incubated overnight at 20°C. Subsequently, the cultures were centrifuged (15 min, 6000 r.p.m.) and the cells were resuspended in 4 ml binding buffer (20 mM Tris-HCl pH 8, 200 mM KCl, 5 mM MgCl₂, 0.5 mM CaCl₂, 0.1 mM EDTA, 0.5 mM DTT, 11% glycerol). PMSF was added to the suspensions to a concentration of 1 mM and, subsequently, the suspensions were sonicated five times for 30 s with cooling on ice. The resulting lysates were centrifuged (15 min, 15 000 g, 6°C) and the supernatants of this centrifugation were used for band shift assays.

Gel shift assays

Gel shift analyses using the double-stranded oligonucleotides described in Table 2 were carried out essentially as described earlier (7). As a control for Abf1p binding we used the *S28A* oligonucleotide. This oligonucleotide encodes part of the *rpS28A* promoter harbouring the Abf1p-binding consensus as described by Dorsman *et al.* (24). The putative Abf1p-binding site found in the UAS_{aa} of *BAP3* is located in the reverse direction. Therefore, we also designed a double-stranded oligonucleotide harbouring the Abf1p-binding consensus in the reversed direction (*S28A^{rev}*). As a negative control for Abf1p binding we used the *S28Aⁿ* oligonucleotide, which contains a point mutation that is known to abolish Abf1p interaction (see Table 2, shown in bold; 24). Labelling, annealing and purification of oligonucleotides used for gel shift analyses were carried out as described earlier (7). Gel shift analyses using extracts from Abf1p-expressing *E.coli* mutants were performed as described by Halfter *et al.* (23). The Abf1p-DNA complexes were supershifted by adding the yC-20 anti-Abf1p antibody to the binding reaction after incubation of the binding reactions for 20 min on ice. The antibody-containing reactions were subsequently incubated for 10 min at room temperature.

Miscellaneous techniques

Expression of *LacZ* fusions was measured by liquid β-galactosidase assay and *BAP3* transcription was monitored by northern blot analysis as described earlier (7).

RESULTS

Abf1p binds *in vitro* to the UAS_{aa}

A thorough analysis of the previously identified UAS_{aa} (7) shows that it comprises a sequence having considerable similarity to the Abf1p-binding site present in, for example, the *FOX3* promoter (11). This Abf1p-binding site does not completely conform to the consensus Abf1p-binding sequence as published by Dorsman *et al.* (24). Since then, however, several reports have been published in which it was shown that Abf1p can bind to alternative sequences found in the promoters of genes involved in distinct biochemical processes (see Table 1). Table 1 also illustrates that the putative Abf1p-binding site in the *BAP3* promoter contains the fully conserved C nucleotide at position 3 and ACG nucleotides at positions 11–13. Based on the observation that the UAS_{aa} may comprise an Abf1p-binding site we set out to test whether indeed Abf1p is the protein that does bind to the UAS_{aa} *in vitro* in gel shift analyses. First, we carried out a gel shift experiment with a labelled double-stranded oligonucleotide containing the UAS_{aa} (Δ BAP3-D) and using excess of an unlabelled double-stranded oligonucleotide harbouring an Abf1p-binding site (i.e. the Abf1p-binding site as present in the promoter of the *rpS28A* gene) as competitor. We found that this latter oligonucleotide is an efficient competitor for protein binding to the UAS_{aa}, suggesting that both the UAS_{aa} and the Abf1p-binding site bind the same protein (data not shown). Next, we made mutations at two of the four fully conserved nucleotides present in the consensus Abf1p-binding sequence, i.e. at the conserved C at position 3 (Δ BAP3-L) or at the conserved C at position 12 (Δ BAP3-K; see also Table 2) and used these oligonucleotides as probes in a gel shift assay. From Figure 1 it is clear that the mutations in both Δ BAP3-L and K lead to a severe decrease in protein binding. We also found that a mutation at position 14 (Δ BAP3-M), which is less conserved, reduces protein binding. Point mutations as present in Δ BAP3-N, -P and -R which do not affect the Abf1p-binding consensus, have no effect on the formation of complex Y. Then we asked whether bacterially produced Abf1p is able to bind to the UAS_{aa}. Abf1p has been shown to contain two DNA-binding domains. The first is a zinc finger motif in the N-terminal region (amino acids 40–91) and the second a DNA-binding region located in the middle of the protein (25). Using two constructs described by Halfter and co-workers (23) we expressed both a full-length Abf1p and a shorter version from which the N-terminal zinc finger domain was deleted and which therefore could no longer bind to Abf1p-binding sites in *E.coli*. Production of *bona fide* Abf1p and Abf1p Δ 33/240 was established by western blotting using an antiserum against Abf1p (results not shown). We then used extracts from *E.coli* producing these two proteins in gel shift analyses. Figure 2A illustrates that the complete recombinant Abf1p can form a complex with the UAS_{aa} with the same mobility as the complex it forms with the *S28A* probe (a double-stranded oligonucleotide harbouring an Abf1p-binding site as present in

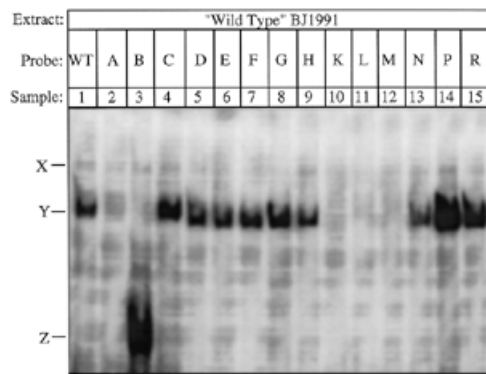


Figure 1. Gel shift analysis of the UAS_{aa} and mutated versions thereof. The oligonucleotides oBAP3-WT through ΔBAP3-R (see Table 2 for sequences) were radioactively labelled and used as probes in gel shift analysis. Each reaction contained 16 μg of yeast protein extract isolated from wild-type cells. The minor and major protein-DNA complexes are called X and Y, respectively. Z indicates the complex found using ΔBAP3-B as probe.

the promoter of the *rpS28A* gene). Furthermore, the complexes found with the recombinant Abf1p have similar mobility to the complexes observed using yeast extracts (Fig. 2A, compare lanes 2 and 4). When we use a protein extract from *E.coli* cells expressing Abf1pΔ33/240 no complex formation was observed (Fig. 2A, lanes 3 and 8). Finally, we studied the effect of antibodies directed against Abf1p in gel shift assays to reveal the nature of the protein(s) that binds to the UAS_{aa}. Using the double-stranded oligonucleotide oBAP3-WT, comprising the UAS_{aa}, and yeast extract in a gel shift assay we find two complexes, a weak complex X and a much stronger complex Y (Fig. 2B, lane 2). Addition of anti-Abf1p antibodies to such a gel shift experiment resulted in disappearance of the major complex, Y, and a concomitant increase in the intensity of complex X (Fig. 2B, lanes 3–6). This effect can either be explained by a supershift of the Y complex as a result of binding of the antibody to the complex or by the inability of antibody-bound Abf1p to bind to the Abf1p-binding site, which can then complex with another factor(s) to form complex X. To distinguish between these two possibilities we performed a similar series of gel shift assays using the *S28A* probe and yeast extract in the presence or absence of anti-Abf1p antibodies. Figure 2B (lanes 11–14) clearly shows that upon addition of anti-Abf1p antibodies the *S28A*-Abf1p complex is supershifted to a complex of similar mobility to that of complex X. Taken together, these experiments unambiguously demonstrate that complex Y is formed as a result of the interaction of oBAP3-WT with Abf1p, meaning that *in vitro* the UAS_{aa} can be bound by Abf1p. It must be noted, however, that this binding site is much weaker than the Abf1p-binding site in the *S28A* promoter (cf. Fig. 2A, lanes 4 and 9).

In addition to Stp1p, Stp2p is involved in the induction of BAP3

STP1 encodes a nuclear protein with zinc finger domains which is involved in pre-tRNA maturation (26) and which is also required for the induced expression of *BAP2* (27). We previously reported that Stp1p is also involved in the induction

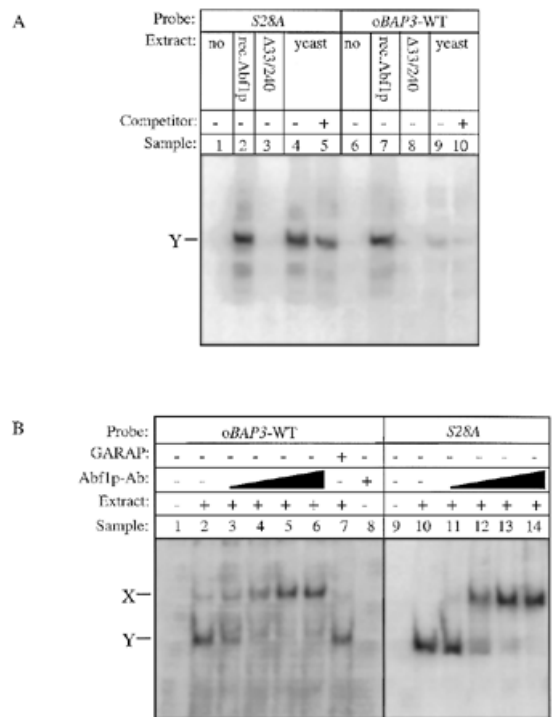


Figure 2. Identification of Abf1p complexes using recombinant Abf1p and Abf1p antibodies in gel shift assays. (A) The oBAP3-WT fragment and the *rpS28A* oligonucleotide were radioactively labelled and used as probes in gel shift assays. Whole cell protein extracts were prepared from wild-type yeast cells and from *E.coli* cells expressing the yeast Abf1 protein and a mutated Abf1 protein lacking the N-terminal DNA-binding domain (Abf1pΔ33/240) as described in Materials and Methods. Each reaction contained 16 μg of yeast protein extract (lanes 4, 5, 9 and 10) or 5 μg of total protein extract isolated from wild-type Abf1p-expressing *E.coli* cells (lanes 2 and 7) or from *E.coli* cells expressing the mutated Abf1p form (lanes 3 and 8). As negative controls the probes were incubated without protein extract (lanes 1 and 6). The position of the protein-DNA complex is marked Y. Complex formation was competed with 250-fold excess of unlabelled double-stranded oligonucleotide oBAP3-WT where indicated (lanes 5 and 10). (B) The oBAP3-WT and the *S28A* oligonucleotides were radioactively labelled and used as probes in gel shift assays. To show that Abf1p is involved in the formation of complex Y, we added increasing amounts of anti-Abf1p antibody to the binding reactions (in lanes 2–6 and 11–14, respectively). As negative control the probes were incubated without protein extract (lanes 1 and 9) or with only anti-Abf1p antibody in lane 8. As a negative control for the supershift we added a goat anti-rabbit antibody to the binding reaction (lane 7).

of *BAP3* transcription by leucine (7). Since the absence of Stp1p leads to a strongly reduced induction of *BAP3* expression, rather than to a complete loss of induction, we looked for other proteins that might be involved in the induction process. Stp2p is such a protein. *STP2* was found in a screen for genes that in higher copy number would confer growth on minimal proline medium containing the inhibitor of branched chain amino acid synthesis, methylsulfonyl urea (MM), plus isoleucine (0.2 mM) and valine (20 μM). On this medium cells grow only when the uptake of valine is increased. This may for instance be the result of an increased activity or an increased expression of one or more of the permeases Bap2p, Bap3p or Tat1p. To investigate the role of *STP2* in *BAP3* expression, we used northern blot

Table 1. Comparison of the putative Abf1p-binding sites found in the UAS_{aa} of *BAP3* to known Abf1p-binding sites

Gene	Sequence	Reference
Abf1p-binding consensus	5'-RTCRYYYNNN ACG -3'	24
<i>RPS28A</i> (-164/-152) ^a	5'-GTCACTCTAG ACG -3'	24
<i>BAP3</i> (-414/-402) ^a	5'-AGCCGCATGC ACG -3'	This work
<i>BAP2</i> (-416/-404)	5'-GCCGCTTCTC ACG -3'	This work
<i>FOX3</i> (-213/-201) ^a	5'-AGCGCCTTAC ACG -3'	11
<i>HIS7</i> (-200/-188)	5'-CTCTCTCTCC ACG -3'	13
<i>HSP12</i> (-264/-252)	5'-AGCACTCTAG ACG -3'	29

^aSequence as present on the non-coding strand.

analysis and monitored *BAP3* expression in a Δ *stp2* as well as in a Δ *stp1*/ Δ *stp2* double disruption mutant in comparison to the *BAP3* expression in isogenic wild-type cells after addition of leucine. Figure 3A shows that as in a Δ *stp1* mutant, *BAP3* induced expression is severely diminished in a Δ *stp2* mutant.

Table 2. Oligonucleotides used in this study^a

Name	Sequence	Abf1p	Stp2p-dependent	β -Galactosidase activity ^b (U/mg)	
		interaction	complex formation	-Leucine	+Leucine (2 mM)
<i>oBAP3</i> -WT	AGCTTAGCCGTGCATGCGGGCTCCGCGAAAAGAGCTCTGCTATATTG	+	+	13	180
Δ <i>BAP3</i> -A	AGCTTAGAAA ATGCATGCGGGCT CCGCGAAAAGAGCTCTGCTATATTG	-	+	13	18
Δ <i>BAP3</i> -B	AGCTTAGCCGTGCATG AAACT CCGCGAAAAGAGCTCTGCTATATTG	-	-	7	10
Δ <i>BAP3</i> -C	AGCTTAGCCGTGCATGCGGGCT CAA AGAAAAGAGCTCTGCTATATTG	+	+	9	166
Δ <i>BAP3</i> -D	AGCTTAGCCGTGCATGCGGGCTCCGCGAAAAG	+	+	25	297
Δ <i>BAP3</i> -E	AGCTTAGCCGTGCATGCGGGCTG	+	-	8	17
Δ <i>BAP3</i> -F	AGCTTAGCCGTGCATGCGGGCTCCGCG	+	+	20	263
Δ <i>BAP3</i> -G	AGCTTAGCCGTGCATGCGGGCT AC GCGAAAAG	+	-	21	236
Δ <i>BAP3</i> -H	AGCTTAGCCGTGCATGCGGGCTCCG CA AAAAG	+	+	17	256
Δ <i>BAP3</i> -K	AGCTTAGCC ATGCATGCGGGCT CCGCGAAAAG	-	+	17	67
Δ <i>BAP3</i> -L	AGCTTAGCCGTGCATGCG ACT CCGCGAAAAG	-	-	10	11
Δ <i>BAP3</i> -M	AGCTTAG ACGTGCATGCGGGCT CCGCGAAAAG	-	+	19	25
Δ <i>BAP3</i> -N	AGCTTA ACCGTGCATGCGGGCT CCGCGAAAAG	+	+	30	83
Δ <i>BAP3</i> -P	AGCTTAGCCGTGCATGCG GA TCCGCGAAAAG	+	-	12	26
Δ <i>BAP3</i> -R	AGCTTAGCCGTGCAT ACGGCT CCGCGAAAAG	+	+	18	266
<i>S28A</i>	AGCTTTGCGTGGTCACTCTAG ACG GCCGCG	++	-	26	21
<i>S28A</i> ⁿ	AGCTTTGCGTGGTCACTCTAG AGG GCCGCG	-	-	9	8
<i>S28A</i> ^{ev}	AGCTTGCGGCCGTCTAGAGTG ACC ACGCG	++	-	25	21

^aOligonucleotide *oBAP3*-WT corresponds to the region between -418 and -376 of the *BAP3* promoter. The Abf1p-binding site in the oligonucleotides (see also Table 1) is underlined. Oligonucleotides Δ *BAP3*-A, B and C correspond to the same fragment but contain a number of mutations, indicated in bold. Oligonucleotides D, E and F correspond to the wild-type *BAP3* promoter sequence, but are shortened from the 3'-side in comparison to *oBAP3*-WT (7). Oligonucleotides G, H, K, L, M, N, P and R correspond to the Δ *BAP3*-D oligonucleotide but contain point mutations, indicated in bold. *S28A* and *S28A*^{ev} contain sequences that comply with the Abf1p-binding consensus (see Materials and Methods) and *S28A*ⁿ contains a point mutation in the Abf1p-binding consensus that abolishes Abf1p interaction (24). The ability to form *in vitro* Abf1p- or Stp2p-dependent complexes (as illustrated in Figs 1 and 4B) is indicated by: -, no; +, yes; ++, very strong. Only the coding strand of the fragments is shown and each fragment has at its 5'- and 3'-termini sequences which are complementary to the cohesive ends generated by the restriction enzymes *Hind*III and *Eco*RI, respectively (as described in ref. 7). This enabled easy insertion into the *LacZ* reporter construct.

^b*LacZ* expression was measured in cells grown with or without 2 mM leucine (see Materials and Methods for further details). The specific β -galactosidase activity is given in U/mg protein extract (1 U = 1 nmol *o*-nitrophenol/min). All assays were carried out in triplicate using single copy integrants and individual measurements differed by <10%.

However, in either single disruption mutant we still find a low level of leucine-induced *BAP3* expression. In a Δ *stp1*/ Δ *stp2* double mutant, however, leucine-induced *BAP3* expression is completely lost. We have also monitored expression of *BAP2* in a similar experiment and found that *BAP2* expression follows a pattern similar to *BAP3* (data not shown). These results indicate that both Stp1p and Stp2p are involved in the amino acid-induced expression of *BAP2* and *BAP3*.

Overexpression of Stp1p and Stp2p

To gain more insight into the functions of Stp1p and Stp2p, we sought to overexpress either or both these proteins by placing the corresponding genes under control of the *PGK1* promoter and to express them from a multiple copy vector (see Materials and Methods). Stp1p was overexpressed as the wild-type protein, whereas the overexpressed Stp2p carried a triple HA epitope tag [(HA)₃] at its N-terminus. Strong overexpression of either protein was clearly demonstrated in a western blot analysis using either an anti-Stp1p antibody (a kind gift of Dr A. Hopper, Seattle, WA) or the 12CA5 antibody directed against the HA epitope in Stp2p (results not shown). To find out whether the overproduced Stp1p or (HA)₃-Stp2p is functional in leucine-induced *BAP3* expression we tested the recovery of *BAP3*

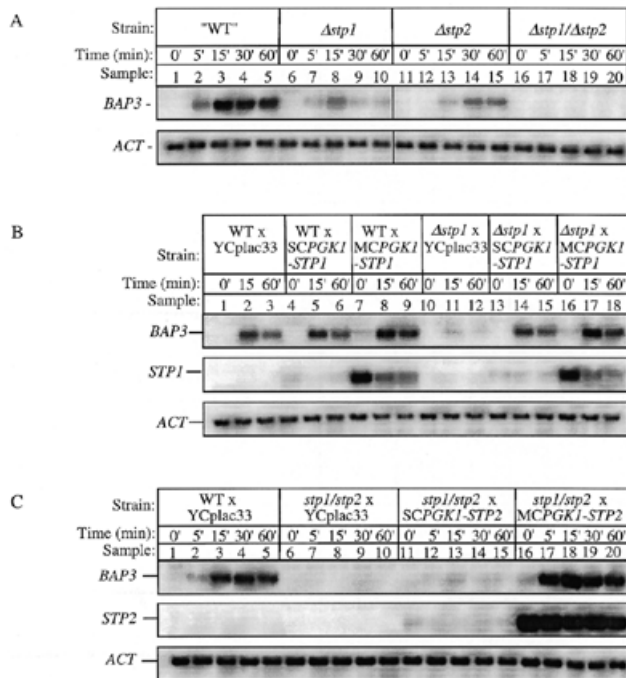


Figure 3. The induction of *BAP3* is dependent on both *STP1* and *STP2*. (A) *BAP3* expression was analysed by northern blot analysis in $\Delta stp1$, $\Delta stp2$ and $\Delta stp1/\Delta stp2$ mutants and in their isogenic wild-type (WT) after addition of leucine to a final concentration of 2 mM. Total RNA was isolated at 0, 5, 15, 30 and 60 min after leucine addition. Probes were used corresponding to parts of the genes encoding Bap3p and actin. The actin mRNA signal was used as a loading control. (B) Functionality of the *STP1*-overexpressing constructs was tested by monitoring *BAP3* induction in wild-type and $\Delta stp1$ cells transformed with the corresponding *STP1*-overexpression constructs after addition of leucine to a final concentration of 2 mM. As a negative control the cells were transformed with the empty expression vector YCplac33. Total RNA was isolated at 0, 15 and 60 min after leucine addition. Probes were used corresponding to parts of the genes encoding Bap3p, Stp1p and actin. The actin mRNA signal was used as a loading control. (C) Functionality of the *STP2*-overexpressing constructs was monitored by northern blot analysis as described above using part of the gene encoding *STP2* as probe.

induction in $\Delta stp1$ and $\Delta stp1/\Delta stp2$ disruption mutants with the respective overexpression constructs by northern blot analysis. Figure 3B shows that upon transformation of the $\Delta stp1$ mutant with either the single copy *STP1* overexpression construct (*SCPGK1-STP1*) or the multiple copy *STP1* overexpression vector (*MCPGK1-STP1*) leucine-induced *BAP3* induction was restored. Figure 3C shows, likewise, that upon transformation of the $\Delta stp1/\Delta stp2$ mutant with either the single copy (*HA*)₃-*STP2* overexpression construct (*SCPGK1-STP2*) or the multiple copy (*HA*)₃-*STP2* overexpression vector (*MCPGK1-STP2*) leucine-induced *BAP3* induction was restored. We find that overexpression of either *STP1* or *STP2* leads to an increase in *BAP3* induction as compared to untransformed wild-type cells. Furthermore, strong overexpression of *STP1* or *STP2* leads to *BAP3* expression without the addition of leucine (compare lanes 1 and 7 of Fig. 3B and lanes 1 and 16 of Fig. 3C). We conclude that we have successfully constructed vectors that enable us to overexpress functional Stp1p or Stp2p in yeast.

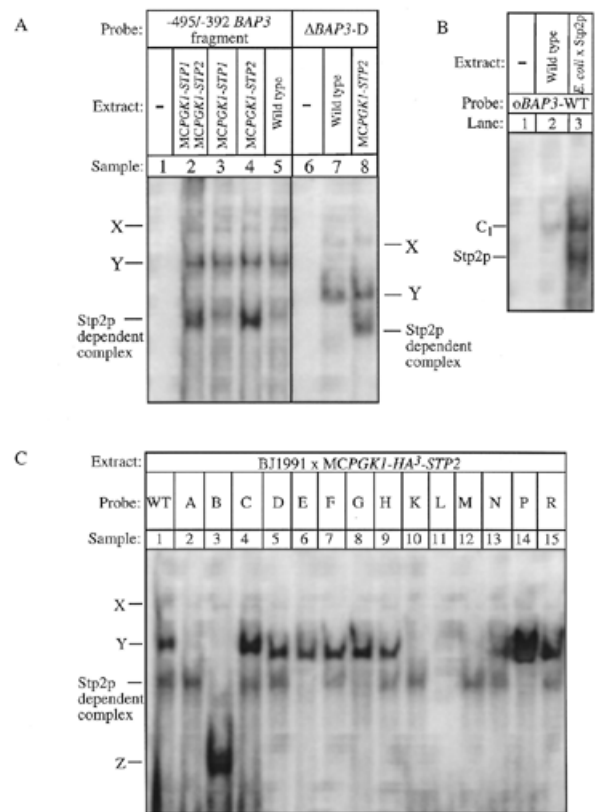


Figure 4. Gel shift analysis with extracts of cells overexpressing Stp1p and Stp2p. (A) The -495/-392 *BAP3* promoter fragment and the double-stranded oligonucleotide $\Delta BAP3$ -D were radioactively labelled and used as a probe in gel shift assays. Each reaction contained 16 μ g of yeast protein extract (except for lanes 1 and 6) isolated from either wild-type cells (lanes 5 and 7), cells overexpressing both Stp1p and Stp2p (lane 2), cells overexpressing Stp1p (lane 3) or from cells overexpressing Stp2p (lanes 4 and 8). (B) The radioactively labelled *oBAP3*-WT fragment was used as a probe in gel shift assays. Each reaction contains 5 μ g of protein extract isolated from either wild-type *E. coli* cells (lane 2) or from *E. coli* cells expressing Stp2p (lane 3). As a negative control no protein extract was added in lane 1. (C) Gel shift analysis using the *UAS*_{aa} and mutated versions thereof (see Table 2) and protein extracts from cells overexpressing Stp2p (see also legend to Fig. 1).

To see whether Stp1p or Stp2p directly interacts with the *BAP3* promoter, we performed gel shift analysis with the -495 to -392 region of the *BAP3* promoter involved in induction by amino acids (7). Figure 4A shows that the *BAP3* promoter fragment forms several DNA-protein complexes when extracts from wild-type cells are used (lane 5). The weakest complexes with the slowest mobility (collectively called X) appear as one or two bands depending on the size of the *UAS*_{aa} fragment. Previously we have shown that these X complexes are formed by non-specific protein binding since their formation cannot be competed out by unlabelled fragments (7). The strongest complex, Y, is, as we have shown, formed by Abf1p. Finally, we found a very weak complex with a higher mobility than the Abf1p complex. Stp2p overexpression led to a significant increase in the formation of this weak DNA-protein complex, which we therefore called the Stp2p-dependent complex. The Stp2p-dependent complex is also formed using the $\Delta BAP3$ -D

fragment as probe and using yeast extracts from cells overexpressing Stp2p (Fig. 4A, lane 8). Overexpression of Stp1p has no apparent effect on the gel shift pattern. We have monitored the sequence requirements for Stp2p-dependent complex formation by testing various mutated versions of the *BAP3* UAS_{aa} in gel shift analyses (Fig. 4C; see Table 2 for the specific sequences). Stp2p-dependent complex formation is highly sequence specific; the complex is formed using the non-mutagenised UAS_{aa} version Δ BAP3-D (cf. Fig. 4A), but there is no complex formation with the mutagenised versions Δ BAP3-B, E, G, L and P. Loss of Stp2p-dependent complex formation with these latter oligonucleotides suggests that the sequence 5'-CGGCTC-3' in the UAS_{aa} is important for protein binding.

To show that the Stp2p-dependent complex involves Stp2p, we have expressed Stp2p in *E. coli* and used such extracts in gel shift assays. Using these protein extracts in band shift analysis, an Stp2p-dependent complex is formed with the oBAP3-WT promoter fragment, which is absent when protein extracts from wild-type *E. coli* cells are used (Fig. 4B, compare lanes 2 and 3). We have also tested binding of the Stp2p protein, expressed in *E. coli*, to the mutated *BAP3* fragments used in Figure 4C. We found a similar pattern of complex formation, indicating that *in vitro* produced Stp2p binds to the UAS_{aa} with the same sequence specificity as found for the tagged Stp2p version expressed in yeast (results not shown).

Expression of the *LacZ* fusions

To test whether the ability of a DNA fragment to bind Abf1p or/and to form the Stp2p-dependent complex *in vitro* correlates with its capacity to render a promoter inducible by amino acids we cloned the UAS_{aa} and several mutated versions thereof or the *rpS28A* elements (Table 2) in front of a *LacZ* reporter gene. The reporter constructs were integrated at the *URA3* locus and single copy integrants were tested for induction of the fusion gene by measuring β -galactosidase activity after growing the mutants with or without 2 mM leucine in the medium.

Table 2 shows that mutations in the Abf1p-binding site of the UAS_{aa} result in a loss of induced *LacZ* expression in response to leucine (Δ BAP3-A, -B, -K, -L and -M). This loss of induction correlates well with the results obtained from the gel retardation assays. In other words, binding of Abf1p correlates with induction in response to leucine via the UAS_{aa}. However, when the Abf1p-binding site as found in the *rpS28A* promoter was fused to the *LacZ* reporter (*S28A* and *S28A*^{rev}), no induction by the addition of leucine was observed. This means that Abf1p interaction is not enough for induction in response to leucine and that other elements and/or factors are required.

Since Stp2p overexpression results in the formation of an additional complex in the gel shift assay, it is possible that Stp2p plays a direct role in *BAP3* transcription. For example, oligonucleotides E and P are still bound by Abf1p, but they do not form the Stp2p-dependent complex and have lost their capacity to induce the *LacZ* reporter in response to leucine. On the other hand, oligonucleotide Δ BAP3-G, which is still bound by Abf1p but does not form the Stp2p-dependent complex, is still able to induce the *LacZ* reporter in response to leucine. This would imply that, in addition to Abf1p and Stp2p, another protein(s) is also involved in the induction of *BAP3* in response to amino acids (e.g. Stp1p).

DISCUSSION

We previously identified an upstream activating sequence in the *BAP3* promoter that is responsible for the induced expression of *BAP3* in response to the availability of amino acids in the medium (7). This paper describes the identification of *trans*-acting factors interacting with this *cis*-acting element.

We have found that the UAS_{aa} constitutes a weak binding site for the global transcription factor Abf1p. This Abf1p-binding site does not completely comply with the consensus sequence, but has considerable similarity to the Abf1p-binding site as found in the promoters of other yeast genes. We tested several mutated versions of the UAS_{aa} to establish the sequence specificity of the Abf1p interaction and found that point mutations in nucleotides known to be essential for Abf1p binding result in a severe decrease in complex formation. We previously showed that the UAS_{aa} is both necessary and sufficient for induction of a *LacZ* reporter in response to addition of amino acids. We also tested mutated UAS_{aa} versions that can no longer be bound by Abf1p and found that there is a correlation between Abf1p complex formation and ability to induce the *LacZ* reporter. In other words, binding of Abf1p to the UAS_{aa} may be involved in the induced expression in response to amino acids. To investigate whether Abf1p binding alone is sufficient to induce the *LacZ* reporter, we fused an Abf1p-binding site as present in the *rpS28A* promoter to the *LacZ* reporter. We found that this fusion does not induce induction of the *LacZ* reporter in response to amino acids. This implies that the binding of Abf1p to its cognate site by itself is not sufficient to support induction of the gene concerned in response to amino acids and that other elements and factors are involved in the specific induction of expression of UAS_{aa}-controlled genes.

One candidate involved in the transcriptional regulation of *BAP3* might be the *STP1* gene product (7). We found that deleting the *STP1* gene leads to a severe decrease in *BAP3* induction; however, there is still some remaining residual *BAP3* induction. This implies that another factor(s) might play a role in leucine-induced *BAP3* transcription. *STP2* was found in a screen for genes that in higher copy number would confer increased activity of one or both of the permeases Bap2p and Bap3p. Comparison of *STP1* and *STP2* reveals that the *STP2* gene encodes a protein with considerable similarity to the *STP1* gene product. We constructed a Δ *stp2* mutant and found that this mutant, like Δ *stp1*, also had a decreased level of leucine-induced *BAP3* transcription. Not unexpectedly, upon deletion of both *STP1* and *STP2*, expression of *BAP3* was completely lost. Since the double deletion mutant had an aggravating effect in comparison to the single deletion mutants, we propose that the *STP1* and *STP2* gene products have similar functions in the regulation of *BAP3* expression. To confirm this, we overexpressed Stp2p in a Δ *stp1*/ Δ *stp2* deletion mutant. We found that overexpression of Stp2p could restore *BAP3* induction, even to higher levels than in wild-type cells. This means that indeed Stp1p and Stp2p have similar functions in *BAP3* expression and can function independently of each other.

Stp1p and Stp2p are the most likely candidates for being the transcription factors that directly interact with the UAS_{aa}. Overexpression of Stp2p does result in formation of an additional UAS_{aa}-protein interaction in an *in vitro* gel shift experiment. We have also used protein extracts from yeast

cells overexpressing Stp1p. However, we could not find any interaction of Stp1p with the *BAP3* promoter. Furthermore, attempts to express Stp1p in *E.coli* have been unsuccessful. Both negative findings could result from differences in protein stability and sequence specificity between Stp1p and Stp2p. We found that formation of the Stp2p-dependent complex requires the sequence 5'-CGGCTC-3' in the UAS_{aa}, which partially overlaps with the Abf1p-binding consensus. This suggests that Abf1p is either required to recruit other transcription factors, like Stp2p, for amino acid-induced transcription or that Abf1p competes with these factors for binding to the UAS_{aa}. Similar sequences appear to be present in the *BAP2* and *TAT1* promoters (see also ref. 7), which makes it interesting to note that we have found that the $\Delta stp1$ and $\Delta stp2$ mutations resulted in a similar expression pattern of *BAP2* as reported here for *BAP3* (data not shown).

Since overexpression of Stp2p does not lead to complexes that involve both Stp2p and Abf1p, it is not likely that there is concomitant binding of both Abf1p and Stp2p to the UAS_{aa}. For leucine-induced *BAP3* expression we propose a model in which Abf1p acts as a general transcription factor interacting with the UAS_{aa} irrespective of whether leucine is present or not. Several reports have been published in which it has been shown that Abf1p is responsible for transcriptional regulation of genes involved in distinct processes. This functional diversity can only be explained if Abf1p plays a role in a general mechanism such as, for example, chromatin (re)modelling (28). Recent data from our laboratory show that Abf1p is indeed involved in bending of DNA and nucleosome positioning, thereby regulating expression of the *rpS28* gene (Lascaris *et al.*, submitted for publication). In the case of the *BAP3* gene, Abf1p may also function by excluding nucleosomes or by bending the DNA, thereby rendering the UAS_{aa} accessible to the specific transcription factors Stp1p and Stp2p.

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