Unique C-terminal region of Hap3 is required for methanol-regulated gene expression in the methylotrophic yeast *Candida boidinii*

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The Hap complex of the methylotrophic yeast *Candida boidinii* was found to be required for methanol-regulated gene expression. In this study, we performed functional characterization of CbHap3p, one of the Hap complex components in *C. boidinii*. Sequence alignment of Hap3 proteins revealed the presence of a unique extended C-terminal region, which is not present in Hap3p from *Saccharomyces cerevisiae* (ScHap3p), but is found in Hap3p proteins of methylotrophic yeasts. Deletion of the C-terminal region of CbHap3p (Δ256–292 or Δ107–237) diminished activation of methanol-regulated genes and abolished the ability to grow on methanol, but did not affect nuclear localization or DNA-binding ability. However, deletion of the N-terminal region of CbHap3p (Δ1–20) led to not only a growth defect on methanol and a decreased level of methanol-regulated gene expression, but also impaired nuclear localization and binding to methanol-regulated gene promoters. We also revealed that CbHap3p could complement the growth defect of the *Schap3Δ* strain on glycerol, although ScHap3p could not complement the growth defect of a *Cbhap3Δ* strain on methanol. We conclude that the unique C-terminal region of CbHap3p contributes to maximum activation of methanol-regulated genes, whilst the N-terminal region is required for nuclear localization and binding to DNA.

### INTRODUCTION

Methylotrophic yeasts, such as *Hansenula polymorpha*, *Pichia pastoris* and *Candida boidinii*, are unique yeasts that can utilize methanol as a sole carbon and energy source. The promoters of genes encoding methanol-metabolizing enzymes, including alcohol oxidase (AOD), dihydroxyacetone synthase (DAS), glutathione-dependent formaldehyde dehydrogenase and formate dehydrogenase, are highly induced by methanol, and tightly regulated by the presence of alternative carbon sources. Therefore, these promoters have been used for industrial protein production with methylotrophic yeasts as hosts (Gellissen, 2000; Daly & Hearn, 2005; Yurimoto et al., 2011; Vogl & Glieder, 2013). Methanol-regulated gene expression is presumed to be conducted by three distinct pathways. Methanol-regulated genes are completely repressed in the presence of glucose, which requires CbMig1p (glucose repression). Exhaustion of glucose releases glucose repression, resulting in activation of methanol-regulated genes by CbTrm2p, which does not require methanol for gene activation (derepression). In addition, the presence of methanol induces maximum activation of methanol-regulated genes via CbTrm1p (methanol induction; methanol-specific induction) (Hartner & Glieder, 2006; Sasano et al., 2008; Yurimoto, 2009; Sasano et al., 2010; Zhai et al., 2012).

In a previous study, we identified a multimeric transcription factor, the CbHap complex, which is involved in methanol-regulated gene expression, specifically methanol induction (Oda et al., 2015). The Hap complex is highly conserved amongst all eukaryotes, from yeasts to humans (Ramil et al., 2000; McNabb & Pinto, 2005; Sybirna et al., 2005; Singh et al., 2011; Ridenour & Bluhm, 2014). It consists of a stable heterotrimer (Hap2p/3p/5p), which binds to a CCAAT consensus sequence and the activator protein Hap4p. In *Saccharomyces cerevisiae*, whilst ScHap3p and ScHap5p are constitutively expressed, ScHap4p is regulated in a carbon-source-dependent manner at the transcriptional level; it is repressed in the presence of glucose and induced by exhaustion of glucose or in the presence of non-fermentable carbon sources such...
as ethanol or glycerol. The Schap2p/3p/4p/5p complex activates genes involved in respiratory metabolism and mitochondria biogenesis, and is indispensable for respiratory growth on non-fermentable carbon sources. Although CbHap2p/3p/5p proteins were found to be necessary for maximum activation of methanol-regulated genes and growth on methanol in C. boidinii, the CbHap complex was not necessary for growth on non-fermentable carbon sources or for derepression (Oda et al., 2015).

In this study, we further characterized CbHap3p, which was found to contain a unique C-terminal region specific to methylotrophic yeasts, but not present in S. cerevisiae. We revealed that the N-terminal and C-terminal regions of CbHap3p have distinct roles during methanol induction in C. boidinii.

**METHODS**

Strains, media and cultivation conditions. The haploid strain C. boidinii S2 was used as the WT strain (Tani et al., 1985). C. boidinii strain TK62 (ura3) was used as a host for transformation (Sakai et al., 1991). S. cerevisiae strain BY4741 (MATa his3A1 leu2Δ0 met15Δ0 ura3Δ0) was used as a host for transformation (Brachmann et al., 1998).

C. boidinii strains were grown on either YPD medium (2 % glucose, 2 % Bacto peptone, 1 % Bacto yeast extract) or YNB medium (0.17 % yeast nitrogen base without amino acids and ammonium sulphate, 0.5 % ammonium sulphate). One of the following was used as the carbon source in YNB medium: 2 % (w/v) glucose (YNM) or 0.7 % (v/v) methanol (YNM). S. cerevisiae strains were grown on either YPD medium or YPGly medium (2 % glycerol, 2 % Bacto peptone, 1 % Bacto yeast extract). The initial pH of the medium was adjusted to 6.0. All yeasts were cultured aerobically at 28 °C.

Construction of strains expressing domain-deleted protein of CbHap3p-YFP (yellow fluorescent protein) proteins. C. boidinii strains expressing domain-deleted protein of CbHap3p-YFP proteins were constructed as follows. PCR was performed with the primer pairs listed in Table S1 (available in the online Supplementary Material) using pCbHAP3-YFP as a template. Amplified fragments were self-ligated to produce pCbHAP3–YFPΔ256–292, pCbHAP3–YFPΔ107–224, pCbHAP3–YFPΔ107–237, pCbHAP3–YFPΔ107–241, pCbHAP3–YFPΔ107–256, pCbHAP3–YFPΔ1–15, pCbHAP3–YFPΔ1–20, pCbHAP3–YFPΔ256–292, pCbHAP3–YFPΔ107–224, pCbHAP3–YFPΔ98–224 and pCbHAP3–YFPΔ86–224. The constructed plasmids were linearized with EcoT22I and used to transform C. boidinii strain Cbhap3Δura3. The plasmids were integrated into the ara3 locus in the genome of C. boidinii.

Fluorescence microscopy and nuclear staining. CbHAP3–YFP/ Cbhap3Δ cells grown to mid-exponential phase in YND, YNE, YNG, YNM or YNO medium were harvested, washed once and fixed in 1 ml of 70 % ethanol for 30 min at room temperature. Fixed cells were then washed twice, resuspended in 150 μl sterilized water and stained with 150 μl DAPI solution (0.125 μg ml⁻¹). After 10 min of incubation, fluorescence was observed using a fluorescence microscope (IX81; Olympus).

Western blot analysis. Yeast cells grown in 5 ml YNM medium to OD₆₀₀ 1.0 were collected and resuspended in 1 ml lysis buffer (1 % NaOH, 1 % mercaptoethanol), and kept on ice for 10 min. Then, 120 μl 10 % trichloroacetic acid was added and samples were kept on ice for 10 min. Samples were centrifuged at 14 000 g for 10 min at 4 °C. After washing twice with cold acetone, pellets were dissolved in distilled water.

Samples were separated by 12 % SDS-PAGE and blotted onto a nitrocellulose membrane. Detection was performed using anti-AOD or anti-DAS polyclonal antibody and horseradish peroxidase-linked anti-rabbit antibody.

Analysis of interaction between CbHap3p and CbHap5p. The interaction of CbHap3p and CbHap5p was investigated as described previously (Oda et al., 2015). C. boidinii strains expressing internal amino-acids-deleted CbHap3p-HA (haemagglutinin) were constructed as follows. PCR was performed with primers CbHAP3–100E/ CbHAP3–225E-down, CbHAP3–97K-up/CbHAP3–225E-down and CbHAP3–85E-up/CbHAP3–225E-down using pPACTI–CbHAP3– HA (Oda et al., 2015) as a template, yielding pCbHAP3–HA101–224, pCbHAP3–HA98–224 and pCbHAP3–HA86–224, respectively. The resulting plasmids were linearized with EcoT22I and used to transform the Cbhap3Δura3 strain. A C. boidinii strain expressing CbHap3p-His was constructed as follows. First, the coding region of the CbHAP5 gene was amplified by PCR with primers Nor1–CbHAP5–5W/nor1– CbHAP5–rv using genomic DNA as a template. The 7.4 kb Nor1 fragment of pNOTel (Sakai et al., 1996) and the 0.9 kb Nor1 fragment of the coding region of CbHAP5 were then ligated to yield pNOT– CbHAP5. Using the resulting plasmid as a template, PCR was performed with primers CbHAP5–C–His–up/pNOTel–His–down. The amplified fragment was then self-ligated to yield pNOT–CbHAP5–His. The plasmid was linearized with EcoT22I and used to transform strain TK62. The resulting strain was named the CbHAP5–His/TK62 strain. CbHAP3–HA101–224/Cbhap3Δ, CbHAP3–HA98–224/Cbhap3Δ, CbHAP3–HA86–224/Cbhap3Δ and CbHAP5–His/TK62 cells grown in 100 ml YNM medium to OD₆₀₀ 1.0 were harvested and used for immunoprecipitation.

Chromatin immunoprecipitation (ChIP) assay. CbHAP3–YFP/Cb hap3Δ and Schap3–GFP/Cbab3Δ cells grown to mid-exponential phase in YNM medium were cross-linked by using 1 % formaldehyde for 10 min. Immunoprecipitation was performed by using an anti-GFP antibody at a dilution of 1 : 400 with a MAGnify Chromatin Immunoprecipitation System (Invitrogen).

Quantitative real-time (qRT)-PCR. Yeast cells were pre-cultured in YND medium for 10 h, and washed twice with distilled water and transferred to YNM at OD₆₀₀ 1.0. After cultivation for 4 or 8 h, cells were harvested by centrifugation at 3000 r.p.m. for 5 min at 4 °C and treated with Yeast Processing Reagent (TaKaRa Bio). Total RNAs were extracted from cells using an RNasy Mini kit (Qiagen). In addition, to eliminate genomic DNA contaminating total RNA, total RNA was treated with DNase I (RNase-Free DNase Set; Qiagen). Reverse transcription was performed with Random primer (Promega) and ReverTra Ace (Toyobo). For reverse transcription, 1.0 μg total RNA was used.

qRT-PCR was performed with a LightCycler (Roche Diagnostics). The PCR was performed with SYBR Premix Ex Taq (TaKaRa Bio), and the primers for ACTI (actin), DASI and CbHAP3 listed in Table S1. The program was as follows: 10 s at 95 °C, 40 cycles of 5 s at 95 °C of 20 °C s⁻¹, 20 s at 60 °C of 20 °C s⁻¹. Amplification specificity was verified by melting curve analyses conducted at 65–95 °C (0 s at 95 °C of 20 °C s⁻¹, 15 s at 65 °C of 20 °C s⁻¹, 0 s at 95 °C of 0.1 °C s⁻¹). The number of copies of each sample was determined with LightCycler software.

Construction of yeast strains expressing heterologous HAP3 genes. Oligonucleotide primers are listed in Table S1. The Schap3Δ strain was generated by homologous recombination by replacing the coding region of CbHAP3 with the KanMX6 cassette (Wach, 1996).
following amplification by PCR with the primers ScHAP3del-Fw/ScHAP3del-Rv.

The *S. cerevisiae* strain expressing ChbHap3p (P*Sc*HAP3-ChbHAP3/ScHap3Δ) was constructed as follows. First, the ScHAP3 promoter region and the coding region of ChbHAP3 were amplified by PCR with the primers PRS-ScHAP3pro-Fw ScHAP3pro-CbHAP3-Rv using *S. cerevisiae* genomic DNA as a template and ScHAP3pro-CbHAP3-Fw/PSc-ScHAP3-Rv using *C. boidinii* genomic DNA as a template, respectively. Then, using the 0.3 kb fragment of P*Sc*HAP3 and the 0.9 kb fragment carrying full-length CbHAP3 as a template, the PCR was performed with primers PRS-ScHAP3pro-Fw/PRS-CbHAP3-Rv. Finally, PCR was performed with primers P8-up/P8-down using pRS316 as a template. The 4.9 kb fragment of pRS316 and the 1.2 kb fragment of P*Sc*HAP3-CbHAP3 for P*Sc*HAP3-CbHAP3/ScHap3Δ were used to transform strain Schap3Δ to uracil prototrophy using the lithium acetate method (Ito *et al.*, 1983).

*C. boidinii* strains expressing ScHap3p (ScHAP3/CbHAP3Δ), the ScHap3p-GFP fusion protein (ScHAP3-GFP/CbHAP3Δ) or the chimeric protein comprising the full length of ScHap3 and aa 106–292 of CbHap3p (Sc-CbHAP3/CbHAP3Δ) were constructed as follows. For the ScHAP3/CbHAP3 strain, the coding region of ScHAP3 was amplified by PCR with the primer SalI-ScHAP3-Fw/PstI-ScHAP3-Rv using *S. cerevisiae* genomic DNA as a template. For the ScHAP3-GFP/CbHAP3Δ strain, the coding region eliminating the stop codon of *Cbhap3* and bp 316–876 of *ScHAP3* were amplified by PCR with the primers SalI-ScHAP3-Fw/PstI-CbHAP3-Rv using *S. cerevisiae* genomic DNA as a template and ScHAP3-GFP/CbHAP3Δ as a template. The 4.9 kb fragment of pRS316 and the 0.9 kb fragment were ligated to yield pPACT1-ScHAP3, pPACT1-Sc-HAP3-end or pPACT1-Sc-CbHAP3, respectively. Then, the 7.8 kb PstI fragment of pPACT1-Sc-HAP3-end and the 1.7 kb PstI fragment of the coding region of GFP were ligated to yield pPACT1-ScHAP3-GFP, pPACT1-ScHAP3, pPACT1-ScHAP3-GFP and pPACT1-Sc-CbHAP3 were linearized with EcoT22I, and used to transform strain Cbhap3Δura3 (Oda *et al.*, 2015). The resulting strains were named ScHAP3/CbHap3Δ, ScHAP3-GFP/CbHap3Δ and Sc-CbHAP3/CbHAP3Δ, respectively.

**RESULTS**

**CbHap3p has a unique C-terminal region**

Hap3p has a core region that is highly conserved in eukaryotes, including yeasts, fungi, plants and animals. The core region contains the histone fold motif of histone H2B

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**Fig. 1.** (a) Schematic model of CbHap3p and ScHap3p. The core regions showing high similarity (grey) and the DNA-binding motifs (black) are represented. (b) Alignment of amino acid sequences of CbHap3p and ScHap3p.

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In *S. cerevisiae*, the core region was reported to be responsible for formation of the Hap complex and binding to DNA (McNabb *et al.*, 1997).

Sequence alignment of Hap3p from *C. boidinii* and *S. cerevisiae* revealed that, in addition to the conserved N-terminal region, CbHap3p had an extended structure of ∼190 aa at the C terminus (Fig. 1). We also found that, in addition to CbHap3p, Hap3p proteins in *P. pastoris* and *H. polymorpha* had similar extended structures at their C termini. In particular, a sequence of ∼40 aa at their C termini (aa 256–292 of CbHap3p) showed high similarity amongst methylotrophic yeasts (Fig. S1). Based on this information, we speculated that the unique C-terminal region of Hap3p in methylotrophic yeasts has a specific function in methanol-regulated gene expression.

**Unique C-terminal region of CbHap3p is essential for specific induction by methanol**

In a previous study, we showed that strain *Cbhap3Δ* was impaired in methanol induction during methanol-regulated gene expression (Oda *et al.*, 2015). In order to elucidate the functional role of the C-terminal region of CbHap3p in methanol induction, we first identified the essential regions necessary for gene activation by expressing CbHap3p-YFP mutant proteins in *Cbhap3Δ* cells (Fig. 2). WT CbHap3p-YFP (CbHap3pFL) complemented the growth defect on methanol and showed induced production of DAS (Fig. 3). Deletion of aa 256–292 (CbHap3p Δ256–292) abolished the ability to grow on methanol (Fig. 3a), and the DAS1 transcript (Table S2) and protein (Fig. 3d) levels were reduced.

We deleted the C-terminal region of CbHap3p-YFP from the opposite side, yielding CbHap3p (Δ107–224), CbHap3p (Δ107–237), CbHap3p (Δ107–241) and CbHap3p (Δ107–256) (Fig. 2). CbHap3p (Δ107–237) and CbHap3 (Δ107–241) showed severe growth defects on methanol (Fig. 3a) and decreased amounts of DAS (Fig. 3d). CbHap3p (Δ107–224) caused retarded growth on methanol, but the amount of DAS protein in this strain was comparable to that in CbHap3pFL (Fig. 3a, d). As a result, we concluded that the C-terminal aa 225–292 were necessary for methanol induction.

CbHap3p needs to be localized to the nucleus and bind specifically to methanol-regulated promoters for gene activation to occur. We observed localization of CbHap3p-YFP proteins under methanol-induced conditions and performed ChIP assays with *Cbhap3Δ* cells producing each
CbHap3p-YFP-mutant. Fig. 2 summarizes the results of complementation experiments examining growth on methanol, and localization and binding activity of each mutant CbHap3p-YFP protein to P_DAS1. As with CbHap3pFL, CbHap3p (Δ256–292) localized to the nucleus (Fig. 4a) and bound to P_DAS1 (Fig. 4b). Similarly, both nuclear localization and DNA binding were normal for all of the above tested CbHap3p proteins (Fig. 4). These results indicated that the C-terminal region of CbHap3p (aa 256–292) was not required for nuclear localization and DNA binding, but was required for activation of methanol-regulated gene expression.

N-terminal region of CbHap3p is necessary for binding to DNA

Next, we performed deletions of the N-terminal region of CbHap3p in order to determine whether the conserved N-terminal region of CbHap3p was responsible for nuclear localization and binding to P_DAS1. Similar to CbHap3pFL, the CbHap3p (Δ1–15) deletion mutant missing the N-terminal 15 aa showed nuclear localization and DNA-binding activity (Fig. 4). In contrast, deletion of the N-terminal 20 or 25 aa [CbHap3p (Δ1–20) and CbHap3p (Δ1–25), respectively] caused a severe growth defect on methanol (Fig. 3b) and DAS protein was not produced under methanol-induced conditions (Fig. 3d, Table S2). We confirmed that expression levels of CbHap3p (Δ1–20) and CbHap3p (Δ1–25) were comparable to that of CbHap3p (Δ1–15) (Fig. S2). Both CbHap3p (Δ1–20) and CbHap3p (Δ1–25) were diffused in the cytosol and did not bind to P_DAS1 (Fig. 4). These results indicate that the N-terminal region (from aa 16 including the putative DNA-binding motif) is essential for nuclear localization and DNA binding. Our previous study suggested that nuclear localization of CbHap3p depended on CbHap5p (Oda et al., 2015); therefore, the N-terminal region of CbHap3p might also be involved in interacting with CbHap5p.

Further internal deletions in CbHap3p-YFP proteins, i.e. CbHap3p (Δ101–224), CbHap3p (Δ98–224) and CbHap3p (Δ86–224), were analysed in Cbhap3Δ cells. The function of CbHap3p (Δ101–224) was similar to that of the CbHap3p (Δ107–224) mutant (Figs 3a, c, d and 4). However, the deletion of aa 98–224 [CbHap3p (Δ98–224)] and aa 86–224 [CbHap3p (Δ86–224)] caused growth impairment on methanol (Fig. 3c) and a low level of DAS protein (Fig. 3d). Interestingly, the mutant proteins CbHap3p (Δ98–224) and CbHap3p (Δ86–224) could bind weakly to DNA, but were mostly diffused in the cytosol.

Fig. 3. (a–c) Growth of C. boidinii strains expressing CbHap3p-YFP variants in YNM medium. (d) Western blot analysis. Cells were incubated in YNM medium for 8 h. Western blot analysis was performed with anti-DAS antibody. Lane 1, Cbhap3Δ; 2, Δ86–224; 3, Δ98–224; 4, Δ101–224; 5, Δ1–15; 6, Δ1–20; 7, Δ1–25; 8, Δ107–224; 9, Δ107–237; 10, Δ107–241; 11, Δ107–256; 12, Δ256–292; 13, CbHap3pFL.
Therefore, the region from aa 86 to 100 may be partially involved in nuclear localization and DNA binding. As the DNA-binding motif (aa 41–62) is distant from this deleted region (Romier et al., 2003), the region from aa 86 to 100 may be involved in complex formation with CbHap2p and CbHap5p. To confirm this hypothesis, we performed co-immunoprecipitation analysis to investigate the interaction between CbHap3p and CbHap5p. As a result, the presence of His-tagged CbHap5p was detected only from the sample containing HA-tagged CbHap3p (Δ101–224) (Fig. 4c), indicating that CbHap3p (Δ101–224) interacts with CbHap5p, but CbHap3p (Δ98–224) and CbHap3p (Δ86–224) do not.

Taken together, the deletion analyses indicated that the N-terminal region from aa 16 to 100 and the C-terminal region from aa 225 to 292 were essential for methanol induction.

**Functional complementation of Hap3 proteins between C. boidinii and S. cerevisiae**

In order to determine whether CbHap3p functions in *S. cerevisiae*, we constructed an *S. cerevisiae* strain expressing CbHap3p in the Schap3Δ background and tested growth on glycerol medium. As shown in Fig. 5(a), the
Schap3Δ strain harbouring empty vector pRS316 did not grow on glycerol, but expression of ScHap3p or CbHap3p in the Schap3Δ strain restored the ability to grow. These results suggested that CbHap3p has conserved roles and could function as a transcription factor in S. cerevisiae, complementing the respiratory growth defect of the Schap3Δ strain. Next, we investigated whether ScHap3p could restore the growth defect of the Cbhap3Δ strain on methanol by transforming the Cbhap3Δ strain with the ScHap3p-GFP expression plasmid. The ScHAP3-GFP/Cbhap3Δ strain was unable to grow on methanol (Fig. 5c, d), although complementation of Cbhap3Δ with CbHAP3-YFP restored its ability to grow. The ScHAP3/Cbhap3Δ strain also could not grow on methanol (Fig. S3). These results indicated that ScHap3p could not restore the growth defect of the Cbhap3Δ strain.

We performed ChIP assays with ScHAP3-GFP/Cbhap3Δ cells that had been induced by methanol (Fig. 5c). As a result, all tested promoter regions of methanol-inducible genes could be amplified from the template DNA, whereas PACT1 was not amplified. These results indicate that ScHap3p could bind to methanol-inducible promoters in C. boidinii, but was unable to function as a transcription factor to restore the growth defect of the Cbhap3Δ strain on methanol.

These results support the model where the N-terminal region of CbHap3p is involved in DNA binding and the C-terminal extended region plays a unique role in methanol induction. To confirm this hypothesis, we constructed a chimeric Hap3 protein that consisted of the full-length ScHap3p and the region of CbHap3p from aa 106 to 292. Production of the chimeric protein Sc-CbHAP3 in the Cbhap3Δ mutant partially restored the ability to grow on methanol (Fig. 5d), indicating that the C-terminal region of CbHap3p functions in methanol induction.

In conclusion, our results revealed that the unique C-terminal region of CbHap3p was required for activation of methanol-regulated genes, but not for nuclear localization and DNA binding, whilst the N-terminal region was responsible for nuclear localization and binding of CbHap3p to methanol-regulated promoters (Fig. 6).
CbHap3p in methanol-regulated gene expression

DISCUSSION

The Hap complex is highly conserved amongst all eukaryotes, and is known to activate genes involved in gluconeogenesis, respiration and mitochondria biogenesis, and contributes to glucose repression/derepression (Buschlen et al., 2003; McNabb & Pinto, 2005). In a previous study, we demonstrated that the C. boidinii Hap complex is involved in methanol-regulated gene expression via methanol induction (Oda et al., 2015), revealing a unique role of the Hap complex in the methylo trophic yeast. It has been of great interest to understand how the Hap complex is able to execute such a specialized function in methanol-regulated gene expression in methylotrophic yeasts.

In this study, the unique C-terminal extended region of CbHap3p, which is not present in ScHap3p, was found to play a critical role in methanol induction. Interestingly, this C-terminal extended region is also present in other methylotrophic yeast strains, i.e. P. pastoris and H. polymorpha (Fig. S1). However, BLAST searches did not find sequences homologous to this C-terminal region in any eukaryotes other than methylotrophic yeasts. In particular, the 37 aa sequence identified within the C-terminal region (aa 256–292) was critical for methanol induction and was highly conserved amongst Hap3 proteins from methylotrophic yeasts, suggesting the functional importance of this region. We speculated that the C-terminal region of CbHap3p is responsible for methanol induction after binding to methanol-regulated promoters. This notion was supported by the demonstration that deletion of the C-terminal region abolished induction of methanol-regulated genes, but did not affect nuclear localization and binding to DNA. The identified N-terminal region of CbHap3p that is necessary for growth on methanol corresponds to the ScHap3p region required for growth on lactate (Xing et al., 1993). Moreover, the region of human NF-YB (corresponding to Hap3p) that is necessary for complex formation with NF-YC (corresponding to Hap5p) is also comparable to the identified N-terminal region of CbHap3p (Romier et al., 2003). CbHap3p was shown to interact with CbHap5p and localize to nucleus. The core regions of CbHap5p are also highly conserved amongst various eukaryotes (Oda et al., 2015). Therefore, it is strongly suggested that the CbHap3p N-terminal region also participates in complex formation with CbHap2p and CbHap5p (Fig. 6). Although we showed that the CbHap3p N-terminal region functions in S. cerevisiae, the growth defect of Sch3pΔ on glycerol was not recovered by expressing the first 121 aa of CbHap3p (data not shown). One possible reason is that the difference of the theoretical pI of Hap3 proteins (ScHap3p is 4.78 whilst CbHap3p 1–121 is 8.8) altered the specificity of DNA binding.

In our previous study, we showed that the CbHap complex localized to the nucleus regardless of the carbon source (Oda et al., 2015) and we confirmed that the transcript level of CbHAP3 was not increased by methanol (Table S3). Therefore, in addition to the Hap complex, induction of methanol-regulated genes seems to require other some activation factors. In S. cerevisiae and other yeasts, Hap4p interacts with the Hap2p/3p/5p heterotrimer (Forsburg & Guarente, 1989; Bourgarel et al., 1999; Sybirna et al., 2005, 2010). Indeed, a domain required for recruiting Hap4p to the Hap2p/3p/5p complex (Hap4p-recruiting domain) has been identified in Hap5p (McNabb et al., 1997) and this domain is also conserved in CbHap5p. However, this domain is not always required for the function of the Hap complex (Tanoue et al., 2006), indicating that other activators/repressors may interact with other regions of Hap2p/3p/5p. However, Hap4p-recruiting domain is absent in CBF-A/CFB-B/CFB-C, which corresponds to Hap3p/2p/5p in rat. However, the CBF-A/CFB-B/CFB-C complex can activate transcription of target genes without any other activators like Hap4p (McNabb et al., 1997). So far we have not been able to find a gene highly homologous to ScHAP4 in the C. boidinii draft genome sequence (Oda et al., 2015), but the...
hypothetical CbHap4p might interact with a putative Hap4p-recruiting domain in CbHap5p. Indeed, a constructed strain expressing CbHap5p deleted for the putative Hap4p-recruiting domain still grew normally on methanol (data not shown). This observation suggests that, in the case of C. boidinii, some methanol-specific transcriptional activators interact with other regions of the Hap complex, including the C-terminal region of CbHap3p.

Hap3p has been reported to be functionally interchangeable between yeast and human cells (Chodosh et al., 1988), indicating that Hap3p function has been highly conserved throughout evolution. Originally, Hap3p in methylotrophic yeasts was speculated to have some function as an activator for derepression during growth on non-fermentable carbon sources. However, during evolution, these Hap3 proteins may have acquired the specific function of activating methanol induction with their C-terminal region and lost the derepression function in methylotrophic yeasts. The identified features of CbHap3p suggest a mechanism for methanol induction by the C-terminal region. To the best of our knowledge, this is the first report identifying the function of the C-terminal region in Hap3p and indicates the presence of conserved machinery for methanol-regulated gene expression mediated by Hap3p in methylotrophic yeasts. This knowledge should contribute to the elucidation of a detailed molecular mechanism of methanol-regulated gene expression.

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