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# Molecular Characterization of Hap Complex Components Responsible for Methanol-Inducible Gene Expression in the Methylophilic Yeast *Candida boidinii*

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**We identified genes encoding components of the Hap complex, *CbHAP2*, *CbHAP3*, and *CbHAP5*, as transcription factors regulating methanol-inducible gene expression in the methylophilic yeast *Candida boidinii*. We found that the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* gene-disrupted strains showed severe growth defects on methanol but not on glucose and nonfermentable carbon sources such as ethanol and glycerol. In these disruptants, the transcriptional activities of methanol-inducible promoters were significantly decreased compared to those of the wild-type strain, indicating that CbHap2p, CbHap3p, and CbHap5p play indispensable roles in methanol-inducible gene expression. Further molecular and biochemical analyses demonstrated that CbHap2p, CbHap3p, and CbHap5p localized to the nucleus and bound to the promoter regions of methanol-inducible genes regardless of the carbon source, and heterotrimer formation was suggested to be necessary for binding to DNA. Unexpectedly, distinct from *Saccharomyces cerevisiae*, the Hap complex functioned in methanol-specific induction rather than glucose derepression in *C. boidinii*. Our results shed light on a novel function of the Hap complex in methanol-inducible gene expression in methylophilic yeasts.**

Methylophilic yeasts, such as *Pichia pastoris*, *Hansenula polymorpha*, and *Candida boidinii*, have been used as heterologous hosts for protein production (1–4). Efficient production has been achieved with their strong and tightly regulated methanol-inducible gene promoters in combination with high-cell-density cultures in methanol-containing medium. The genes encoding methanol-metabolic enzymes are highly induced in the presence of methanol, i.e., alcohol oxidase (AOD), dihydroxyacetone synthase (DAS), glutathione-dependent formaldehyde dehydrogenase (FLD), and formate dehydrogenase (FDH), and these promoter regions have been used commercially for heterologous gene expression (5–7).

Methanol-inducible gene expression is assumed to be controlled under three distinct modes of regulation: glucose repression, glucose derepression, and methanol-specific induction (6, 8). In *C. boidinii*, the promoter of the AOD-encoding gene (*P<sub>AOD1</sub>*) is completely repressed in the presence of glucose and derepressed upon exhaustion of glucose or in the presence of a nonfermentable carbon source without methanol, exhibiting ~10% of the maximum AOD expression level. Maximum activation of *P<sub>AOD1</sub>* is achieved with methanol as the inducer by methanol-specific induction. While this mechanism of methanol-inducible gene expression seems to be generally conserved among yeast species, the mode of carbon source regulation has some differences among yeast species, i.e., susceptibility to glucose repression and glycerol repression (3, 8). For example, while glycerol represses methanol-inducible gene expression in *P. pastoris*, glycerol supports high-cell-density cultivation and methanol-inducible gene expression in *H. polymorpha* and *C. boidinii*. In addition, susceptibility to glucose repression in *H. polymorpha* is less tightly controlled than in the other two strains. As such, the culturing methods and conditions for protein production are significantly

affected by the carbon source and the regulatory mode of gene expression in a particular yeast.

In previous studies, we identified several transcription factors responsible for methanol-inducible gene expression in *C. boidinii* and revealed the specific function of each transcription factor, i.e., CbMig1p in glucose repression, CbTrm2p in glucose derepression, and CbTrm1p in methanol-specific induction (8–11). Among these three modes of regulation, the molecular mechanism of glucose repression/derepression has been extensively studied in the budding yeast *Saccharomyces cerevisiae* after a shift of the carbon source from glucose to the nonfermentable carbon source ethanol or glycerol (12, 13). *S. cerevisiae* preferentially ferments glucose with conversion to ethanol, and after glucose is depleted, cells use ethanol as a carbon source by respiration. This reprogramming of metabolism, called the diauxic shift, is achieved by the derepression of glucose-repressed genes and the induction of genes necessary for nonfermentable carbon source

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metabolism (e.g., gluconeogenesis, respiration, and mitochondrial development) (13).

The Hap complex, which is known as the CCAAT-binding factor (CBF), is one of the key transcription factors involved in the diauxic shift (14–16). The Hap complex consists of four components, Hap2p, Hap3p, Hap4p, and Hap5p. Hap2p, Hap3p, and Hap5p form a heterotrimer that is sufficient for binding to CCAAT elements within promoters (17). Another activator, Hap4p, is necessary to drive the transcription of target genes (18). Because methanol is considered to be a nonfermentable carbon source, it was expected that the Hap complex functioned in methanol-induced gene expression, especially during glucose derepression, in methylotrophic yeasts. Here, we identified three genes, *CbHAP2*, *CbHAP3*, and *CbHAP5*, encoding homologs of three components of the Hap complex in *C. boidinii*. Unexpectedly, CbHap2p, CbHap3p, and CbHap5p were found to be responsible for methanol-specific induction rather than glucose derepression. To our knowledge, this is the first report of a Hap complex involved in methanol-inducible gene expression in methylotrophic yeasts.

## MATERIALS AND METHODS

**Strains, media, and cultivation conditions.** The haploid strain *C. boidinii* AOU1 (19) was used as the wild-type strain. The *C. boidinii* TK62 (*ura3*) (20), *Cbhap2Δura3*, *Cbhap3Δura3*, and *Cbhap5Δura3* strains were used as hosts for transformation. *Escherichia coli* DH10B (TaKaRa Bio, Otsu, Japan) and SA116 (20) were used for plasmid propagation.

*C. boidinii* strains were grown on either yeast extract-peptone-dextrose (YPD) medium (2% glucose, 2% Bacto peptone, 1% Bacto yeast extract) or yeast nitrogen base (YNB) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate). One of the following components was used as the carbon source in YNB medium: 2% (wt/vol) glucose (YND), 2% (vol/vol) glycerol (YNG), 0.7% (vol/vol) methanol (YNM), 1% (vol/vol) ethanol (YNE), or 0.5% (vol/vol) oleate (YNO). Tween 80 was added to the oleate medium at a concentration of 0.05% (vol/vol). The initial pH of the medium was adjusted to 6.0. Cultivation was performed at 28°C under aerobic conditions with reciprocal shaking, and the growth of the yeast was monitored by measuring the optical density at 610 nm (OD<sub>610</sub>).

*E. coli* was grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented, when required, with ampicillin (50 μg/ml) or zeocin (50 μg/ml).

**Disruption of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes.** Oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. The upstream regions of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes were amplified by PCR with the primer pairs KpnI-HAP2up and PstI-HAP2up, KpnI-HAP3up and PstI-HAP3up, and KpnI-HAP5up and PstI-HAP5up, respectively, using *C. boidinii* genomic DNA as a template. The 0.6-, 0.5-, and 0.7-kb PCR-amplified fragments were inserted into pGEM-T Easy (Promega, Madison, WI), yielding pGEM-HAP2up, pGEM-HAP3up, and pGEM-HAP5up, respectively. The downstream regions of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes were amplified by PCR with the primer pairs BglII-HAP2down and PstI-HAP2down, BglII-HAP3down and PstI-HAP3down, and BglII-HAP5down and PstI-HAP5down, respectively, using genomic DNA as a template. The 0.6-, 0.5-, and 0.7-kb PCR-amplified fragments were inserted into pGEM-T Easy, yielding pGEM-HAP2down, pGEM-HAP3down, and pGEM-HAP5down, respectively. First, three fragments [3.0-kb NotI-EcoRI-digested pBluescript II SK(+), the NotI-PstI-digested upstream region fragment, and the EcoRI-PstI-digested downstream region fragment] were ligated, yielding the vectors, SK<sup>+</sup>HAP2up-down, SK<sup>+</sup>HAP3up-down, and SK<sup>+</sup>HAP5up-down, respectively. The 4.3-kb SacI-XhoI fragment from pSPR harboring the *C. boidinii* *URA3* gene (21) and the 4.2-, 4.0-, and 4.4-kb PstI fragments from SK<sup>+</sup>HAP2up-down, SK<sup>+</sup>HAP3up-down,

and SK<sup>+</sup>HAP5up-down, respectively, were then blunt ended and ligated by using a Blunting High kit (Toyobo, Osaka, Japan), yielding the *CbHAP2* disruption vector pCbHAP2-D, the *CbHAP3* disruption vector pCbHAP3-D, and the *CbHAP5* disruption vector pCbHAP5-D, respectively. After pCbHAP2-D, pCbHAP3-D, and pCbHAP5-D were digested with KpnI and BglII, the 5.5-, 5.3-, and 5.7-kb fragments were used to transform the *C. boidinii* TK62 strain to uracil prototrophy by using a modified version of the lithium acetate method. The disruption of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes was confirmed by Southern analysis using BglII-digested genomic DNA of transformants. The 0.5-kb PCR-amplified fragments obtained with the primer pairs HAP2probe-Fw and HAP2probe-Rv, HAP3probe-Fw and HAP3probe-Rv, and HAP5probe-Fw and HAP5probe-Rv were used as the probes. The *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains were converted to uracil auxotrophy by 5-fluoroorotic acid (5-FOA) selection, yielding the *Cbhap2Δura3*, *Cbhap3Δura3*, and *Cbhap5Δura3* strains.

**Construction of strains expressing the *ScPHO5* gene under the control of various methanol-inducible promoters.** Plasmids harboring the *S. cerevisiae* acid phosphatase (APase) gene *ScPHO5* under the control of various methanol-inducible promoters (pAPU, pDPU, and pFPU) (22) were used to transform the *Cbhap2Δura3*, *Cbhap3Δura3*, and *Cbhap5Δura3* strains. The integrative events in the transformants were analyzed by Southern analysis with EcoRI-digested chromosomal DNA, using the 0.5-kb PCR-amplified fragment obtained with primers URA3probe-Fw and URA3probe-Rv as a probe (data not shown). Transformants that showed a single integration event for pAPU, pDPU, and pFPU at the *ura3* locus of the chromosomal DNA in the *C. boidinii* *Cbhap2Δura3*, *Cbhap3Δura3*, or *Cbhap5Δura3* strain were isolated. APase activity was measured as described previously (8).

**Construction of strains expressing the CbHap2p-YFP, CbHap3p-YFP, and CbHap5p-YFP fusion proteins.** The *C. boidinii* strains expressing the CbHap2p-yellow fluorescent protein (YFP), CbHap3p-YFP, and CbHap5p-YFP fusion proteins were constructed as follows. First, fragments including the promoter and coding regions of *CbHAP2*, *CbHAP3*, and *CbHAP5* were amplified by PCR with the primer pairs SacI-CbHAP2-Pro and PstI-CbHAP2-Rv, SacI-CbHAP3-Pro and PstI-CbHAP3-Rv, and SacI-CbHAP5-Pro and PstI-CbHAP5-Rv, respectively, using genomic DNA as a template. The 6.1-kb SacI-PstI fragment of pTRM2-YFP (11) and the 1.2-, 1.4-, and 1.5-kb SacI-PstI fragments of the promoter and coding regions of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes were then ligated to yield pCbHAP2-YFP, pCbHAP3-YFP, and pCbHAP5-YFP, respectively. The resulting plasmids were linearized with EcoT22I and used to transform the *Cbhap2Δura3*, *Cbhap3Δura3*, and *Cbhap5Δura3* strains, respectively. The resulting strains were named the *CbHAP2-YFP/Cbhap2Δ*, *CbHAP3-YFP/Cbhap3Δ*, and *CbHAP5-YFP/Cbhap5Δ* strains, respectively. The *CbHAP3-YFP/Cbhap2Δ*, *CbHAP3-YFP/Cbhap5Δ*, *CbHAP2-YFP/Cbhap5Δ*, and *CbHAP5-YFP/Cbhap2Δ* strains were also constructed.

Fluorescence microscopy and nuclear staining were performed as follows. Cells grown to mid-log phase on YND, YNE, YNG, YNM, or YNO medium were harvested, washed once with water, and fixed with 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 of a 0.125-μg/ml 4',6'-diamidino-2-phenylindole (DAPI) solution. After 10 min of incubation, fluorescence was observed under an inverted fluorescence microscope (IX81; Olympus, Tokyo, Japan).

**Analysis of interactions between CbHap2, CbHap3, and CbHap5.** To investigate the interaction between CbHap3 and CbHap5, a *C. boidinii* strain coexpressing CbHap3p-hemagglutinin (HA) and CbHap5p-YFP was constructed as follows. First, the coding region of the *CbHAP3* gene was amplified by PCR with primers Sall-CbHAP3-Fw and PstI-CbHAP3-Rv, using genomic DNA as a template. The 7.4-kb Sall-PstI fragment of pGFP-PTS1 and the 0.9-kb Sall-PstI fragment of the coding region of *CbHAP3* were then ligated to yield pP<sub>ACT1</sub>-CbHAP3. Using the resulting plasmid as a template, PCR was performed with primers CbHAP3-

HA-up and HA-down. The amplified fragment was then self-ligated to yield pP<sub>ACT1</sub>-CbHAP3-HA. The plasmid was linearized with EcoT22I and used to transform the *Cbhap3Δura3* strain. The resulting strain was named the *CbHAP3-HA/Cbhap3Δ* strain. HA-tagged CbHap3p complemented the growth defect of the *Cbhap3Δ* strain on methanol (see Fig. S1 in the supplemental material). Next, the DNA fragment harboring the zeocin resistance gene expression cassette was amplified by PCR with primers Zeo-Hind-fw and Zeo-Hind-rv, using pREMI-Zc as a template. The amplified fragment was digested with HindIII and inserted into the HindIII site of pCbHAP5-YFP to yield pCbHAP5-YFP-Zeo. The resulting plasmid was linearized with EcoT22I and used to transform the *CbHAP3-HA/Cbhap3Δ* strain. The resulting strain was named the *CbHAP3-HA-CbHAP5-YFP/Cbhap3Δ* strain.

Immunoprecipitation was performed as follows. Cells grown in 100 ml YNM medium to an OD<sub>610</sub> of 1.0 were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by using a French press (Constant Cell Disruption System One Shot model; Constant Systems Ltd., United Kingdom). Fifty microliters of anti-HA-tagged monoclonal antibody (MAb) magnetic beads (MBL, Nagoya, Japan) was added to the crude extract (2 mg), and the mixture was incubated for 6 h at 4°C. Beads were washed three times with lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) and then heated for 5 min at 95°C in 30 μl Laemmli buffer. Fifteen-microliter samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

To investigate the interaction between CbHap2 and CbHap3, a *C. boidinii* strain expressing CbHap2-His was constructed as follows. First, the coding region of the *CbHAP2* gene was amplified by PCR with primers NotI-CbHAP2-Fw and NotI-CbHAP2-Rv, using genomic DNA as a template. The 7.4-kb NotI fragment of pNOTeI (23) and the 0.5-kb NotI fragment of the coding region of *CbHAP2* were then ligated to yield pNOT-CbHAP2. Using the resulting plasmid as a template, PCR was performed with primers CbHAP2-C-His-up and pNOTeI-His-down. The amplified fragment was then self-ligated to yield pNOT-CbHAP2-His. The plasmid was linearized with EcoT22I and used to transform strain TK62. The resulting strain was named the *CbHAP2-His/TK62* strain.

*CbHAP2-His/TK62* and *CbHAP3-HA/Cbhap3Δ* cells grown in 100 ml YNM medium to an OD<sub>610</sub> of 1.0 were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM PMSF. Cells were disrupted by using a French press. Each crude extract (200 μg) was mixed together and incubated at room temperature for 30 min. Fifty microliters of anti-HA-tagged MAb magnetic beads was then added, and the mixture was incubated for 1 h at 4°C. Beads were washed three times with lysis buffer and then heated for 5 min at 95°C in 30 μl Laemmli buffer. Fifteen-microliter samples were subjected to SDS-PAGE.

**SDS-PAGE and Western analysis.** SDS-PAGE was performed with 10% polyacrylamide slab gels. Western analysis was performed according to a method described previously by Towbin et al. (24). After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. Anti-green fluorescent protein (GFP) monoclonal antibody and horseradish peroxidase-linked anti-mouse antibody were used at dilutions of 1:3,000 and 1:10,000, respectively. Detection was performed with Western Lighting Chemiluminescence Reagent Plus (PerkinElmer, Waltham, MA).

**Chromatin immunoprecipitation assay.** A chromatin immunoprecipitation (ChIP) assay was performed as described previously (6, 8), and primers used for this assay are listed in Table S1 in the supplemental material. *CbHAP2-YFP/Cbhap2Δ*, *CbHAP3-YFP/Cbhap3Δ*, and *CbHAP5-YFP/Cbhap5Δ* cells grown to mid-log phase in YND or YNM medium were cross-linked with 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 (Life Technologies, Carlsbad, CA).

**Nucleotide sequence accession numbers.** The nucleotide sequences of *CbHAP2*, *CbHAP3*, and *CbHAP5* were deposited in DDBJ under accession numbers AB909501, AB909502, and AB909503, respectively.

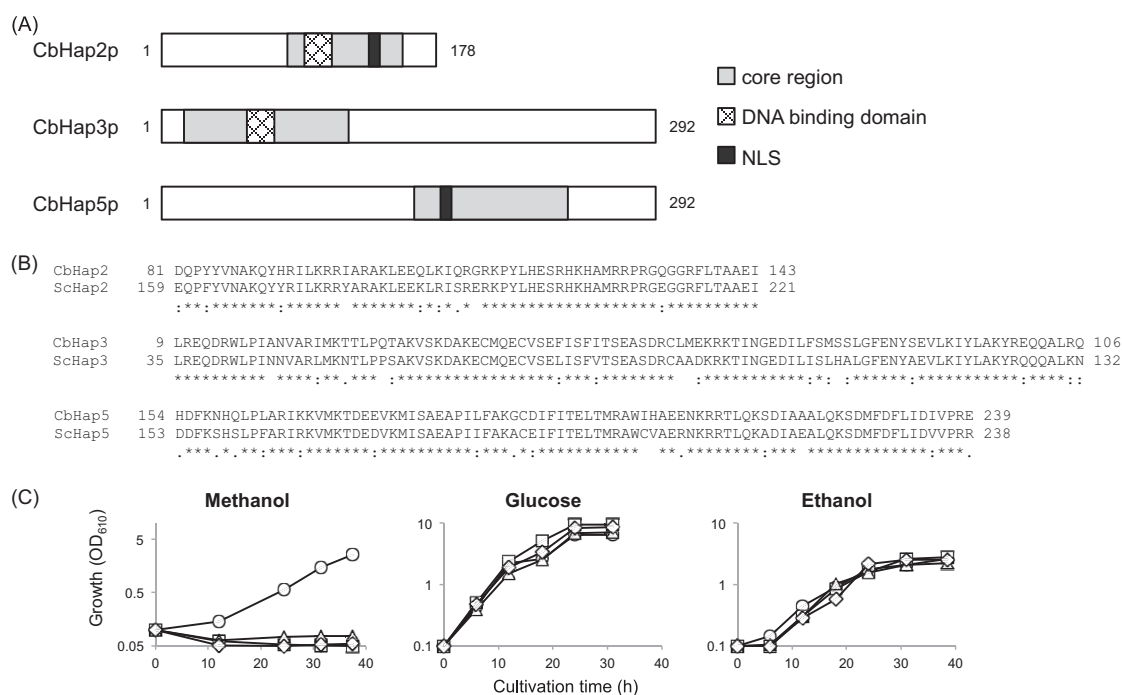
## RESULTS

**Cloning and primary structures of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes.** In our previous study, we performed a genetic screen for *C. boidinii* gene-tagging mutants with reduced levels of methanol-inducible gene expression, using strain FM as the host (9, 11). Strain FM expresses the *S. cerevisiae* *PHO5* gene under the control of the modified *FDH1* promoter fused with six copies of UAS-FM, which is a promoter element that responds to both methanol and formate. We obtained mutant strain FM31, which showed a reduced level of reporter enzyme activity, and found that the deduced amino acid sequence for the disrupted gene in the FM31 mutant strain exhibited a high degree of similarity to that for the *S. cerevisiae* gene *ScHAP3*. We termed the disrupted gene *CbHAP3*. The *CbHAP3* gene encodes an 876-bp open reading frame (ORF) corresponding to 292 amino acid residues (Fig. 1A). The similarity and identity of the amino acid sequence of CbHap3p to that of ScHap3p are 38.0% and 30.1%, respectively. In particular, the N-terminal amino acid sequences of CbHap3p, including the DNA binding domain, show a high degree of similarity to those of ScHap3p (Fig. 1B).

A further search of the draft genome sequence of *C. boidinii* revealed the presence of putative homologs of *S. cerevisiae* *HAP2* and *HAP5*, designated *CbHAP2* and *CbHAP5*, respectively. The *CbHAP2* and *CbHAP5* genes encode 534- and 876-bp ORFs corresponding to 178 and 292 amino acid residues, respectively (Fig. 1A). The deduced amino acid sequence of CbHap2p is 47.2% identical and 78.0% similar to that of ScHap2p, and the deduced amino acid sequence of CbHap5p is 38.7% identical and 61.6% similar to that of ScHap5p. Hap2p and Hap5p each have a core domain that is highly conserved among eukaryotes (25, 26). Also, each region of CbHap2p and CbHap5p, including the core domain, shows a high degree of similarity to that of ScHap2p and ScHap5p, respectively (Fig. 1B).

**Disruption of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes causes a severe growth defect on methanol but not on other carbon sources.** The *CbHAP2*, *CbHAP3*, and *CbHAP5* genes were disrupted by replacing the open reading frame with the *C. boidinii* *URA3* gene as a selective marker. Disruption of each gene was confirmed by Southern analysis (data not shown). In order to restore the selective marker, we isolated the *Cbhap2Δura3*, *Cbhap3Δura3*, and *Cbhap5Δura3* strains by popping out the *URA3* sequence after 5-FOA selection, as described in Materials and Methods.

We cultivated the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains in liquid media containing various carbon sources and compared their growth with that of the wild-type strain (Fig. 1C). All disruptants showed a severe growth defect on methanol but no growth defect on glucose or the nonfermentable carbon sources glycerol, ethanol, and oleate (Fig. 1C; see also Fig. S2 in the supplemental material). These results were unexpected because the *Schap3Δ* strain showed growth defects on nonfermentable carbon sources such as ethanol and glycerol (17, 27). These results suggest that CbHap2p, CbHap3p, and CbHap5p play indispensable roles in methanol metabolism that are specific to growth on methanol and distinct from the role of the Hap complex in *S. cerevisiae*.



**FIG 1** Primary structure of CbHap2, CbHap3, and CbHap5 and growth of the gene disruptants. (A) Domain structures of CbHap2, CbHap3, and CbHap5. (B) Alignment of amino acid sequences of the core regions of each subunit of the Hap complex. (C) Growth of the wild-type, *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains on various carbon sources. Open circles, open squares, open triangles, and open rhombuses represent the wild-type, *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains, respectively.

**The *CbHAP2*, *CbHAP3*, and *CbHAP5* genes positively regulate the transcriptional activity of methanol-inducible promoters.** In order to investigate the influence of *HAP* gene deletions on the activity of methanol-inducible promoters ( $P_{AOD1}$ ,  $P_{DAS1}$ , and  $P_{FDH1}$ ), we constructed reporter strains expressing the *ScPHO5* gene under the control of these promoters in the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains and assayed the reporter activity. In all disruptant strains, the activities of all promoters decreased significantly compared to that of the wild-type strain (Table 1), indicating that CbHap2, CbHap3p, and CbHap5p are involved in the transcriptional activation of methanol-inducible promoters. Quantitative reverse transcription-PCR (RT-PCR) analysis also revealed that transcripts of methanol-inducible genes were at low levels in all disruptant strains (see Fig. S3 in the supplemental material). Among the tested promoters, the activities of  $P_{AOD1}$  in all disruptant strains remained at 16 to 28% of the levels of the wild-type strain, whereas the activities of  $P_{DAS1}$  and  $P_{FDH1}$  decreased to levels below 10% of those of the wild-type strain. Be-

**TABLE 1** Levels of APase activity in the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains grown on methanol<sup>a</sup>

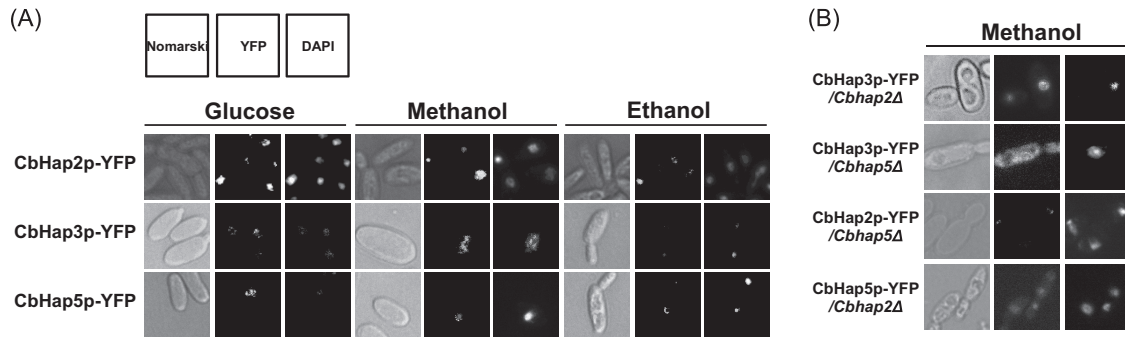
Strain	Mean APase activity (U/OD <sub>660</sub> ) ± SD		
	$P_{AOD1}$	$P_{DAS1}$	$P_{FDH1}$
Wild type	70.1 ± 8.00	335 ± 31.2	76.0 ± 7.72
<i>Cbhap2Δ</i>	11.3 ± 0.80	4.25 ± 1.86	6.18 ± 1.29
<i>Cbhap3Δ</i>	19.6 ± 2.90	36.1 ± 1.81	0.77 ± 0.03
<i>Cbhap5Δ</i>	18.8 ± 3.58	18.7 ± 2.47	8.79 ± 1.55

<sup>a</sup> The level of APase activity is expressed as U per OD<sub>660</sub>. The mean of data from three independent experiments are shown. The wild-type, *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains were incubated in the presence of 0.7% methanol for 8 h.

cause  $P_{AOD1}$  is partly activated by glucose derepression, which is independent of methanol-specific induction (6, 8), CbHap2p, CbHap3p, and CbHap5p are assumed to be more involved in methanol-specific induction.

**CbHap2p, CbHap3p, and CbHap5p are constitutively expressed and localized to the nucleus regardless of the carbon source.** In order to investigate the expression of the *CbHAP2*, *CbHAP3*, and *CbHAP5* genes and the subcellular localization of CbHap2p, CbHap3p, and CbHap5p, we constructed strains expressing proteins fused with YFP under the control of their native promoters. YFP-tagged CbHap2p, CbHap3p, and CbHap5p complemented the growth defects of the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains, respectively, on methanol (see Fig. S4 in the supplemental material). Subcellular localization of CbHap2p-YFP, CbHap3p-YFP, and CbHap5p-YFP was observed by fluorescence microscopy. In all media containing various carbon sources, all YFP-tagged proteins were localized to the nucleus (Fig. 2A). This result suggests that CbHap2p, CbHap3p, and CbHap5p are constitutively expressed regardless of the carbon source and localized to the nucleus.

CbHap2p and CbHap5p have putative nuclear localization signal (NLS) sequences, while CbHap3p does not (Fig. 2A). In *Aspergillus nidulans*, only HapB (corresponding to Hap2) has a NLS, and the localization of HapC/E (corresponding to Hap3 and Hap5, respectively) to the nucleus depended on HapB (28). Therefore, we investigated the localization of CbHap3p in a *CbHAP2* or *CbHAP5* deletion mutant. As shown in Fig. 2B, CbHap3p was localized to the nucleus in the *Cbhap2Δ* strain as in the wild-type strain. On the other hand, CbHap3p was diffuse in the cytosol in the *Cbhap5Δ* strain. These results suggest that the



**FIG 2** Subcellular localization of YFP-fused CbHap2p, CbHap3p, and CbHap5p. Each YFP fusion protein was expressed under the control of its native promoter. (A) Subcellular localization of YFP-fused CbHap2p, CbHap3p, and CbHap5p in the wild-type background. Cells were grown on YND, YNM, or YNE medium. (B) Subcellular localization of YFP-fused CbHap2p, CbHap3p, and CbHap5p in the *Cbhap2Δ* or *Cbhap5Δ* background. Cells were grown on YNM medium.

localization of CbHap3p to the nucleus is dependent on CbHap5p but not on CbHap2p. CbHap2p in the *Cbhap5Δ* strain and CbHap5p in the *Cbhap2Δ* strain were localized to the nucleus (Fig. 2B), suggesting that these two proteins could be imported into the nucleus in an independent manner.

To confirm a direct interaction between CbHap3p and CbHap5p, we performed coimmunoprecipitation assays using cells coexpressing CbHap3p-HA and CbHap5p-YFP. In the sample immunoprecipitated with anti-HA antibody, the presence of CbHap5p-YFP was detected by Western analysis using anti-GFP antibody (Fig. 3A). These results indicated that CbHap3p localized to the nucleus through its direct interaction with CbHap5p. We also confirmed an interaction between CbHap2p and CbHap3p by coimmunoprecipitation analysis (Fig. 3B). Judging from these results, it was suggested that CbHap2p, CbHap3p, and CbHap5p form a heterotrimer.

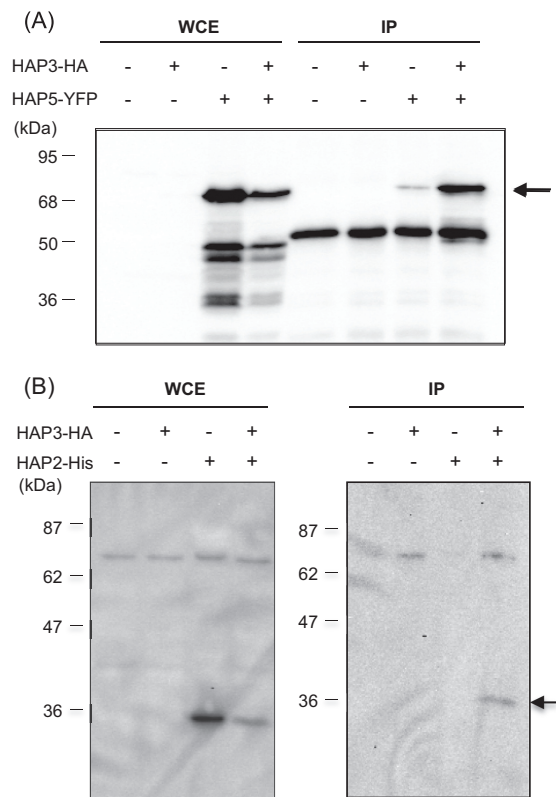
**CbHap2p, CbHap3p, and CbHap5p specifically bind to multiple methanol-inducible promoters in cells grown on methanol or glucose.** In order to investigate whether CbHap2p, CbHap3p, and CbHap5p bind to methanol-inducible promoters *in vivo*, ChIP assays were performed with methanol-grown and glucose-grown cells expressing CbHap2p-YFP, CbHap3p-YFP, or CbHap5p-YFP. In all strains, after immunoprecipitation using anti-GFP antibody, the promoter regions of three methanol-inducible genes ( $P_{AOD1}$ ,  $P_{DAS1}$ , and  $P_{FDH1}$ ) could be amplified from the template DNA of both methanol-grown and glucose-grown cells, whereas  $P_{ACT1}$  could not (Fig. 4A). These results indicate the association of CbHap2p, CbHap3p, and CbHap5p to the promoter regions of four methanol-inducible promoters regardless of the carbon source.

Next, we investigated the binding of CbHap3p to the promoter regions in the *Cbhap2Δ* or *Cbhap5Δ* deletion mutant. As a result, the bands observed with the wild type were almost abolished in the *Cbhap2Δ* and *Cbhap5Δ* mutants (Fig. 4B), indicating that the binding of CbHap3p to DNA is dependent not only on CbHap5p for nuclear localization but also on CbHap2p for binding to the DNA.

## DISCUSSION

Methylotrophic yeasts have a unique metabolic pathway for methanol utilization. The key enzymes of methanol metabolism in methylotrophic yeasts are highly induced by methanol and are virtually absent when cells are growing on glucose. We have studied the regulation of the methanol-inducible genes necessary for

methanol metabolism and characterized several transcription factors involved in their regulation in *C. boidinii* (8–11). Based on our previous results, we have proposed three modes of regulation during methanol-inducible gene expression, that is, CbMig1p-



**FIG 3** Interactions between CbHap2p, CbHap3p, and CbHap5p. (A) Interaction between CbHap3p and CbHap5p. Coimmunoprecipitation was performed with cells expressing CbHap3p-HA (+) or native CbHap3p (-) and CbHap5p-YFP (+) or native CbHap5p (-) grown on methanol. Immunoprecipitation (IP) was performed with anti-HA-tagged MAb magnetic beads, and Western blotting was subsequently performed with anti-GFP antibody. The arrow indicates the position of CbHap5p-YFP. (B) Interaction between CbHap2p and CbHap3p. Cells expressing CbHap2p-His (+) or native CbHap2p (-) and CbHap3p-HA (+) or native CbHap3p (-) were grown on methanol. Lysates were immunoprecipitated with anti-HA-tagged MAb magnetic beads, and Western blotting was subsequently performed with anti-His antibody. The arrow indicates the position of CbHap2p-His. WCE, whole-cell extract.

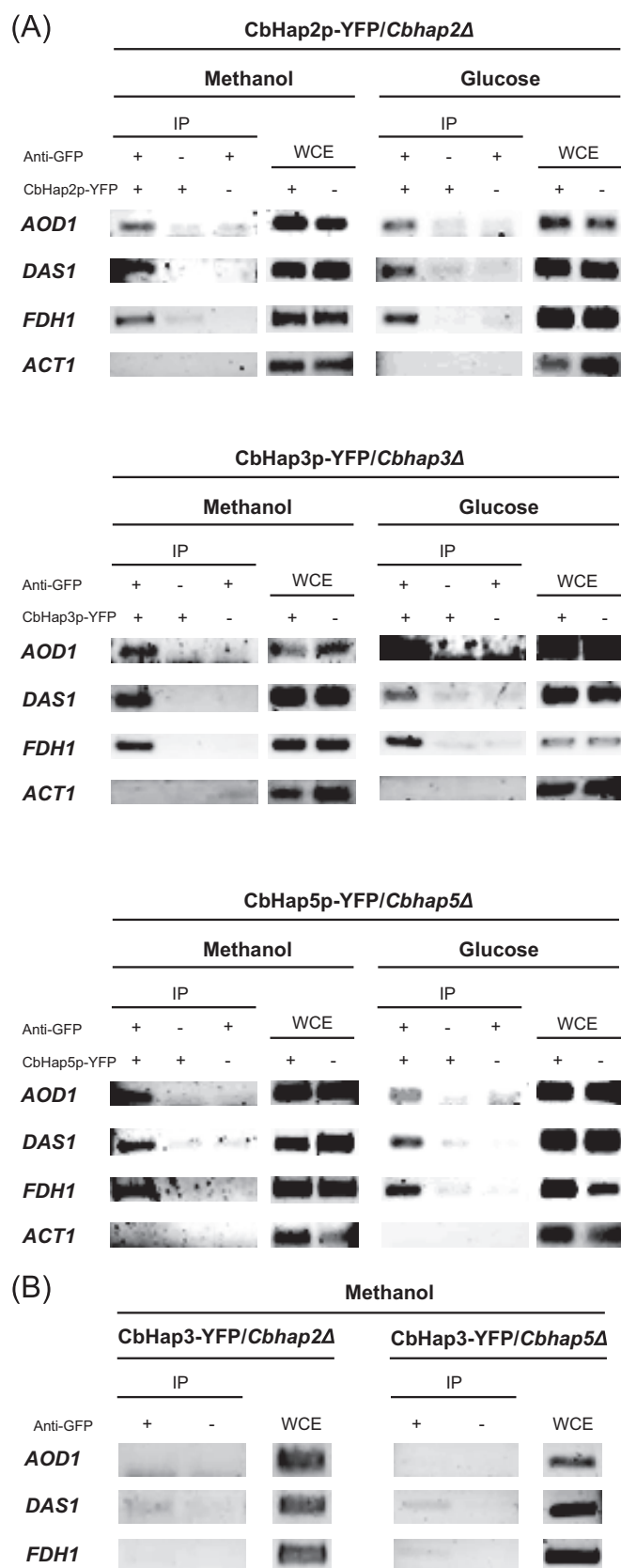


FIG 4 Binding of CbHap2p, CbHap3p, and CbHap5p to methanol-inducible promoters. ChIP assays were performed with cells grown on methanol or glucose. YFP-tagged proteins and native proteins were immunoprecipitated

dependent glucose repression, CbTrm2p-dependent derepression, and CbTrm1p-dependent methanol-specific induction. Glucose repression/derepression is also observed in *S. cerevisiae* when glucose is exhausted and cells are shifted to nonfermentable carbon sources, such as ethanol and glycerol, whose metabolism requires respiration. The Hap complex is well known as a transcriptional activator of genes involved in respiratory metabolism and promotes the transition from fermentation to respiration (27, 29); however, its role in methylotrophic yeasts has not yet been clarified.

In this study, we identified three genes, *CbHAP2*, *CbHAP3*, and *CbHAP5*, as components of the Hap complex in *C. boidinii* and demonstrated that these genes were necessary for methanol-inducible gene expression but not for the metabolism of other nonfermentable carbon sources. Since the methanol utilization pathway requires respiration consuming molecular oxygen, it seemed to be reasonable that the Hap complex plays a role in methanol metabolism. However, unexpectedly, the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains retained normal growth on glucose and nonfermentable carbon sources other than methanol (Fig. 1C). This result was quite different from the demonstrated function of the Hap complex in *S. cerevisiae*, which is required for growth on nonfermentable carbon sources. These results suggested that CbHap2p, CbHap3p, and CbHap5p have specific functions necessary for growth on methanol. *C. boidinii* might have other proteins that have the same function as the Hap complex in *S. cerevisiae*. However, we could not find any other genes homologous to *CbHAP2*, *CbHAP3*, and *CbHAP5* in the *C. boidinii* draft genome sequence.

Disruption of *CbHAP2*, *CbHAP3*, and *CbHAP5* caused drastic decreases in  $P_{AOD1}$ ,  $P_{DAS1}$ , and  $P_{FDH1}$  activities (Table 1). Based on these results, components of the Hap complex in *C. boidinii* appear to act as transcription factors that positively regulate methanol induction. While the transcriptional activities of  $P_{DAS1}$  and  $P_{FDH1}$  were completely abolished in the *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* strains,  $P_{AOD1}$  activity was retained at ~20% of the wild-type level (Table 1). These results suggest that not only the Hap complex but also other activators regulate  $P_{AOD1}$  transcriptional activity. The regulation of  $P_{AOD1}$  is distinct from that of other methanol-inducible promoters in *C. boidinii*. *AOD1* gene expression was derepressed in glycerol-containing medium, to ~10% of full induction in methanol-containing medium. Disruption of the *CbTRM1* gene reduced  $P_{AOD1}$  transcriptional activity to the derepressed level, whereas disruption of the *CbTRM2* gene reduced  $P_{AOD1}$  transcriptional activity to the basal level (11). These results suggest that CbHap2p, CbHap3p, and CbHap5p are responsible for methanol-specific induction rather than glucose derepression.

As in other eukaryotic organisms, each component of the Hap complex in *C. boidinii* has a core domain; CbHap3p and CbHap5p have segments similar to the histone fold motif of histones H2B and H2A, which show significant homology to regions of ScHap3p and ScHap5p, respectively (26, 30). CbHap2p also has a

with (+) or without (–) anti-GFP antibody. IP, immunoprecipitation; WCE, whole-cell extract. (A) Binding of CbHap2p, CbHap3p, and CbHap5p to the promoter regions of methanol-inducible genes in the corresponding gene disruptants. (B) Binding of CbHap3p to the promoter regions of methanol-inducible genes in the *Cbhap2Δ* and *Cbhap5Δ* strains. All images are shown as negative images.

unique domain homologous to ScHap2p. Therefore, their basic functions, e.g., nuclear localization and DNA binding, are thought to correspond to those of *S. cerevisiae*. In the present study, we investigated the nuclear localization and DNA-binding activity of CbHap2p, CbHap3p, and CbHap5p. We revealed that all of these proteins were constitutively localized to the nucleus regardless of the carbon source (Fig. 2A) and that the nuclear localization of CbHap3p, which had no NLS in its deduced amino acid sequence, depended on CbHap5p but not on CbHap2p (Fig. 2B). As for the DNA-binding activity, we confirmed that all three proteins specifically bound to methanol-inducible promoters (Fig. 4A) and revealed that the DNA-binding activity of CbHap3p depended on both CbHap2p and CbHap5p (Fig. 4B).

In *S. cerevisiae*, the Hap complex requires the presence of all three components for stable assembly, and each component cannot bind to DNA without forming a heterotrimer (14, 17). In the case of the mammalian counterparts, termed CBF-A (Hap3p), CBF-B (Hap2p), and CBF-C (Hap5p) in rats or NF-YA (Hap2p), NF-YB (Hap3p), and NF-YC (Hap5p) in mice and humans, three proteins assemble in a two-step pathway; CBF-A and CBF-C initially form a stable heterodimer, and CBF-B then binds to form a heterotrimer (31). Also, the structure of the NF-YB/NF-YC heterodimer has been revealed (32). Judging from our present results, it is possible that CbHap3p first binds to CbHap5p to form a heterodimer and is localized to the nucleus by the NLS in CbHap5p and that all three proteins CbHap2p, CbHap3p, and CbHap5p then form a heterotrimer to bind to DNA. As for the direct interaction among components of the Hap complex in *C. boidinii*, we confirmed direct interactions between CbHap3p and CbHap5p and between CbHap2p and CbHap3p by coimmunoprecipitation analysis (Fig. 3). Together with the CbHap2p and CbHap5p dependency of the DNA-binding activity of CbHap3p, there is no doubt that CbHap2p, CbHap3p, and CbHap5p form a complex.

In many organisms, including *S. cerevisiae*, it is known that the Hap complex binds to CCAAT consensus sequences in promoters (29, 33, 34). To investigate whether this motif functions in the transcriptional activation of methanol-inducible promoters in *C. boidinii*, we constructed reporter strains expressing the *ScPHO5* gene under the control of CCAAT-deleted *P<sub>AOD1</sub>* and *P<sub>DASI</sub>* promoters. However, these resulted in little decrease of their transcriptional activities (see Table S2 in the supplemental material). Therefore, it is unlikely that Hap components bind directly to CCAAT sequences on promoters in *C. boidinii*; we still need to determine the binding site on methanol-inducible promoters. Because CbHap2p, CbHap3p, and CbHap5p bound to methanol-inducible promoters regardless of the carbon source, the Hap2p/3p/5p heterotrimer is not sufficient to activate the transcription of methanol-inducible genes. Besides ScHap4p, there are some reports showing that an additional activator is required for transcriptional activation via the interaction with the Hap2p/3p/5p heterotrimer, including the demonstration that Hap4p in the methylotrophic yeast *H. polymorpha* is functional in *S. cerevisiae* (35–39). However, we could not find a gene showing a high level of homology to *SchHAP4* in the *C. boidinii* draft genome sequence. It is possible that some transcriptional activators interact with the Hap2p/3p/5p heterotrimer in *C. boidinii*. We are now revealing the relationship between the CbHap2p/3p/5p heterotrimer and other previously identified activators such as CbTrm1p (8).

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