Oxygen-Dependent Transcriptional Regulator Hap1p Limits Glucose Uptake by Repressing the Expression of the Major Glucose Transporter Gene RAG1 in Klyuyveromyces lactis

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The HAP1 (CYP1) gene product of Saccharomyces cerevisiae is known to regulate the transcription of many genes in response to oxygen availability. This response varies according to yeast species, probably reflecting the specific nature of their oxidative metabolism. It is suspected that a difference in the interaction of Hap1p with its target genes may explain some of the species-related variation in oxygen responses. As opposed to the fermentative S. cerevisiae, Klyuyveromyces lactis is an aerobic yeast species which shows different oxygen responses. We examined the role of the HAP1-equivalent gene (KIHAP1) in K. lactis. KIHAP1 showed a number of sequence features and some gene targets (such as KICYC1) in common with its S. cerevisiae counterpart, and KIHAP1 was capable of complementing the hap1 mutation. However, the KIHAP1 disruptant showed temperature-sensitive growth on glucose, especially at low glucose concentrations. At normal temperature, 28°C, the mutant grew well, the colony size being even greater than that of the wild type. The most striking observation was that KIHAP1 repressed the expression of the major glucose transporter gene RAG1 and reduced the glucose uptake rate. This suggested an involvement of KIHAP1 in the regulation of glycolytic flux through the glucose transport system. The ΔKhap1p mutant showed an increased ability to produce ethanol during aerobic growth, indicating a possible transformation of its physiological property to Crabtree positivity or partial Crabtree positivity. Dual roles of KIHAP1 in activating respiration and repressing fermentation may be seen as a basis of the Crabtree-negative physiology of K. lactis.

In both prokaryotes and eukaryotes, the transcription of many genes is controlled by oxygen availability. This regulation has been extensively studied in Saccharomyces cerevisiae. However, this species is rather exceptional among yeasts because of its extreme fermentation-oriented physiology. Other species that have comparable sets of genes in their genomes (45) conspicuously differ from S. cerevisiae in their mode of carbon metabolism in general and in their response to oxygen in particular. The difference may reflect evolutionary variation of the regulatory networks that characterize each species. In the present work, an aerobic species, Klyuyveromyces lactis, was chosen for a comparative study of oxygen-linked regulation. The genomic sequence of this yeasts is entirely known (EMBL accession numbers CR3821121 to CR382126), and its carbon metabolism is well documented (7, 8, 51).

In S. cerevisiae, the transcriptional activator Hap1p is involved in cell response to oxygen via heme (25). A number of genes appear to be targets of this regulator, but the range and mode of these interactions are only partially known. In S. cerevisiae, oxygen-regulated genes are of two kinds: the “aerobic genes,” which are activated under aerobic conditions, and the “hypoxic genes,” which are fully expressed only under anoxic or hypoxic conditions and repressed by oxygen. This regulation occurs at the level of transcription. Indeed, several transcription factors responding to oxygen have been identified. They include (i) Rox1p and Mot3p, which repress the transcription of the hypoxic genes under aerobic conditions; (ii) conversely, Mga2p, which activates the transcription of the hypoxic genes under hypoxia; and (iii) Hap1p, which activates aerobic genes (26, 29, 32, 46, 53, 56). The activities of Rox1p and Hap1p are in turn controlled by heme. In most organisms, the role of heme is thought to be central in the oxygen-sensing mechanism. It has been suggested that heme synthesis is regulated by oxygen concentration at three steps of its synthetic pathway (25): coproporphyrinogen III oxidase (Hem13p), protoporphyrin oxidase (Hem14p), and ferrochelatase (Hem15p). Heme control of Hap1p appears to be direct and stringent. In S. cerevisiae, the target genes of Hap1p include CYC1 (encoding iso-1-cytochrome c), CYC7 (encoding iso-2-cytochrome c), CYT1 (encoding cytochrome c1), CTT1 (encoding catalase), and YHB1 (encoding a flavohemoprotein), as well as the tran-
scriptional repressor-encoding genes ROX1 and MOT3 (31). Thus, Hap1p plays a pivotal role in the molecular events underly-dering the oxygen response. Gene microarray-assisted surveying has suggested that Hap1p potentially regulates at least 24 genes in S. cerevisiae (46).

S. cerevisiae and K. lactis show quite different responses to oxygen. S. cerevisiae, known as a Crabtree-positive species, ferment sugars even in the presence of oxygen, while K. lactis, which is Crabtree negative, metabolizes sugars preferentially without oxygen-regulated processes. The mechanisms that link glucose repression to the expression of respiratory genes are, however, unclear in K. lactis. For example the Hap2/3/4/5p complex is known to be involved in this regulation in S. cerevisiae (21), but this connection is often absent or uncertain in K. lactis (1, 6, 7, 9, 37). Such data prompted us to examine in K. lactis the role of the supposed major regulator Hap1p.

MATERIALS AND METHODS

Strains and culture media. Yeast strains are listed in Table 1. Yeast cells were routinely grown in a complete medium containing 1% Bacto yeast extract, 2% Bacto peptone (YP), and 2% glucose. The minimal medium contained 0.67% yeast nitrogen base (Difco) without amino acids (YNB) and a carbon source as specified. For auxotrophic requirements, relevant amino acids were added at the concentration of 40 μg ml⁻¹ and adenine or uracil was added at 20 μg ml⁻¹. The Δheml and Δheml Δhap1 mutants were grown in media supplemented with 30 μg ml⁻¹ of 5-aminolevulinic acid (6-ALA) (for heme-deficient conditions) or with 30 μg ml⁻¹ of ergosterol and 0.2% Tween 80 (for heme-deficient conditions). Tween 80 (polyoxyethylenesorbitan monoleate) is used as a source of unsaturated fatty acids (3, 4). For hypoxic cultures, the complete medium supplemented with 30 μg ml⁻¹ of ergosterol and Tween 80 was flushed with nitrogen for 1 h unsaturated fatty acids (3, 4). For hypoxic cultures, the complete medium supplemented with 30 μg ml⁻¹ of ergosterol and Tween 80 was flushed with nitrogen for 1 h.

TABLE 1. Yeast strains

| Yeast strain | Genotype | Reference or source |
|--------------|----------|---------------------|
| K. lactis    |          |                     |
| PM6-7A      | MATα    | H. Fukuhara, Institut Curie |
| 2350/152    | MATα    | M. Wosiewski-Louvel, University of Lyon 1 |
| MW270-7B    | MATα    | This work           |
|             | leu2    |                     |
| MW270-7BΔkhapl |        |                     |
|             | Kihapl:1::KanMX | This work |
|             |          |                     |
| S. cerevisiae |          |                     |
| 334-ΔHAP1   | MATα    | This work           |
| W303-1A     | MATα    |                     |
| W303-ΔHEM1  | MATα    |                     |
| W303-ΔHAP1  | MATα    |                     |
| W303-ΔHEM1ΔΔHAP1 | MATα |                     |
| W303-ΔHAP1(K) | MATα |                     |

Cloning of KIHAP1. Hap1p of S. cerevisiae has a typical zinc finger motif, CTICRKKKVKC (15, 40). According to this sequence, oligonucleotides were synthesized that contained mixed degenerate codons (Table 2, oligonucleotide 1). The mixed nucleotides were labeled with γ-32P[ATP and used as a hybridization probe to search the HAP1 ortholog in the K. lactis genome. Genomic DNA (strain PM6-7A) was digested with EcoRI and HindIII and Southern blot hybridization gave an unambiguous signal with an EcoRI-HindIII fragment of 3.6 kb and an EcoRI fragment of 7.1 kb (data not shown). Therefore, the DNA fragments of the 3.6-kb size range were collected and inserted into pUC19 vector to construct a partial library. Colony hybridization detected a positive clone (named pBW-1). Sequencing of its 3.6-kb insert revealed a partial open reading frame orthologous to the N terminus of S. cerevisiae HAP1 (ScHAP1). To clone the sequence downstream of this partial open reading frame, inverse PCR (39) was applied with a self-circularization mixture of EcoRI-digested genomic DNA of K. lactis (Table 2, oligonucleotides 2 and 3). The PCR product was cloned into pCR-Script Amp SK+ vector. The resulting plasmid (named pBW-2) contained a 3.8-kb DNA fragment overlapping the 3.6-kb insert of pBW-1. Colony hybridization and Southern blot hybridization were carried out at 45°C in a solution containing 0.75 M NaCl, 0.075 M trisodium citrate, 5% Denhardt’s mixture, and 0.5% sodium dodecyl sulfate. Denatured herring sperm DNA was included at the concentration of 100 μg ml⁻¹.

Disruption of KIHAP1. To construct the Kihapl null mutant, the "split-marker recombination" procedure was used as described previously (17). The DNA fragments upstream and downstream of the KIHAP1 open reading frame were PCR amplified (Table 2, primers 4 and 5 and primers 6 and 7) and cloned into pKa and pAN vectors, respectively. The resulting plasmids were transformed into K. lactis strain MW270-7B. The expected structure of integration was confirmed by Southern hybridization: the entire open reading frame of KIHAP1, exactly from the initiation codon ATG to the stop codon TAG, has been deleted and replaced by the KanMX selection marker to give the ΔKihapl mutant.

Northern blot analysis. Total RNA was isolated (42), fractionated on an agarose-formaldehyde denaturing gel, and immobilized onto a Hybond-N membrane (Amersham). Hybridization was performed at 65°C in a buffer containing 7% sodium dodecyl sulfate, 0.5 M sodium phosphate buffer, pH 7.2, and 10 mM EDTA. Probes were synthesized by PCR (Table 2, oligonucleotides 8 to 33) and labeled with 32P using the Ready to Go DNA labeling kit (Pharmacia). The density of hybridization bands was quantified with the ImageQuant 5.2 and normalized for RNA loading against signal of KIACT1.

Protein-DNA binding assay. Formation of the Hap1p-DNA complex was assayed by a gel retardation procedure according to the method of Fytlovich et al. (20). Double-stranded synthetic oligonucleotides Sc-UASCYC1 and Kl-UASCYC2 (Table 2, oligonucleotides 34 and 35 and oligonucleotides 36 and 37, respectively) were labeled with 32P and incubated with cell extracts (see below) in 20 μl of incubation buffer (6% glycerol, 20 mM HEPES buffer, pH 7.5, 50 mM KCl, 1.5 mM MgCl2, 10 μM ZnCl2, and 0.5 to 5 μg of denatured salmon sperm DNA as a nonspecific competitor). Binding reactions were carried out at 4°C for 15 min, and the reaction mixtures were loaded onto a 4% polyacrylamide gel in 0.5X TBE buffer, pH 8.3 (45 mM Tris base, 45 mM H3BO3, 1.2 mM EDTA, 2% glycerol). After electrophoresis at 4°C, the gel was transferred to the surface of Whatman 3MM paper, dried, and autoradiographed. The cell extracts were prepared as follows. S. cerevisiae strain 334-ΔHAP1 was transformed with the plasmid pDP-KI-HAP1 (carrying KIHAP1 fused to the inducible promoter GAL10-CYC1). To prepare control extracts, the empty vector pDP8p-10 was also used for transformation. The transformants were grown in liquid minimal medium containing 2% glucose as carbon source. At the cell density of A600 = 1.5, KIHAP1 expression was induced by the addition of galactose (final concentration, 2%). After 8 h, cells were harvested by centrifugation and washed with extraction buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The cells suspended in the extraction buffer (1 ml for 1 g of cells) were disrupted in a Carver press. The broken cells were centrifuged for 30 min at 27,000 × g, and the supernatant fraction was collected and stored at −80°C until use.
Measurement of glucose uptake rate. The cells were grown in YP medium supplemented with 2% glucose and harvested at an optical density at 600 nm (OD_{600}) of 1 to 1.5. The cells were washed twice with ice-cold 100 mM potassium phosphate buffer, pH 6.5, and suspended in the same buffer, at a concentration of about 80 mg (dry weight) ml^{-1}. To start the uptake reaction, 100 l of cell suspension was added with 100 l of a buffered (pH 6.5) aqueous solution of 200 mM [14C]glucose (603 dpm/nmol). Aliquots of 48 l were taken at different time intervals (7, 15, 30, and 60 s), uptake was stopped by dilution with 10 ml of ice-cold water, and the aliquots were filtered through a glass microfiber disc and washed twice with 5 ml ice-cold water. The blank in each experiment was determined by adding labeled substrate simultaneously with the cold water. The filters were placed in scintillation vials, and 5 ml of Ultima Gold (Packard) was added; the retained radioactivity was determined in a liquid scintillation counter.

Glucose concentration and ethanol concentration determination. Yeast cells were harvested by centrifugation after a 36-h precultivation at 28°C in YP medium containing 2% glucose, reinoculated into 50 ml YP medium containing 5% glucose in a flask to an OD_{600} of 3.0, and then incubated at 28°C on a shaker (220 rpm). Aliquots (0.5 to 1.0 ml) were taken from the cultures at appropriate time intervals for analysis of cell OD 600, glucose consumption, and ethanol production. Glucose levels in medium were determined by use of dinitrosalicylic acid reagent as described previously (38). Ethanol levels in culture supernatant were assayed enzymatically using a procedure with NAD-dependent alcohol dehydrogenase (Sigma) based on the work of Kagi and Vallee (27).

Nucleotide sequence accession number. The nucleotide sequence of KlHAP1 has been assigned GenBank accession number AY648979.

### TABLE 2. List of oligonucleotides

| Purpose and oligonucleotide no. | Target | Sequence (5'→3') |
|---------------------------------|--------|-----------------|
| KlHAP1 cloning probe (containing degenerate codons) | | |
| 1 | TG(C/T)ACCAT(C/T)TGTCG(A/C/G/T)AA(AG)AG(A/G)AA(A/G)GT(C/T)AA(A/G)JGT | |
| Inverse PCR for KlHAP1 | | |
| 2 | TGGATTGGCTATAATTG | ACCACACAAAGTTTCC |
| 3 | CCGGATCCGAGAGAATATCGCTAATGCC | |
| KlHAP1 disruption | | |
| 4 | ACGAAGCATCATCACATGGTACATAG | |
| 5 | TCATTCTTGTGCTGTTA | |
| 6 | CTACACAGATCCTGGGTACAT | |
| 7 | CAAATCATGATG | |
| Northern hybridization probes | | |
| 8 | KlHAP1 | ATGAGCTCATATTACCTCACTCTGGAACAG |
| 9 | KICYC1 | GGTGTTGAGTTCCTCTGGCAGGACATGGACAA |
| 10 | KICYT1 | TCCACAGCTCATACAAAGAAG |
| 11 | KIHMG1 | AAGAAGCATATCCCAGTCCAG |
| 12 | KIERG1 | CAAAATCATGATG | |
| 13 | KHEM13 | GTTCATCTTACATGAGTCG |
| 14 | RAG1 | TTCAATTTGGCAGCAGAC |
| 15 | HGT1 | TCCATCTGATTTTGAATG |
| 16 | KIYB1 | AATGGGAGTTCCTCATTTG |
| 17 | RAG1 | TCAAAATCATGAGTCG |
| 18 | KIPOX1 | TTAGTCAGATGCTCAGTGG |
| 19 | KLEEB1 | CGGCATCAATATGGGCAAC |
| 20 | KIACT1 | TGGTCGCTGGCTTATGTT |
| 21 | Sc-UAS_{CYC1} (Hap1p binding site in CYC1 promoter) | ScCYC1(UAS1B-342) | CTCACTTGGCCGGGTTTACGAGATGGACAG |
| 22 | ScCYC1(UAS1B-313) | CGGTACCTCCTCCGTAACCCGCCGCAAG |
| 23 | KL-UAS_{CYC1} (Hap1p binding site in KICYC1 promoter) | KICYC1(UAS1B-276) | CTATCATTTGCTGGAACATCGG |
| 24 | KICYC1(UAS1B-305) | GTGTCTTGGATCGTACG |
| 25 | ScCYC1(UAS1B-342) | CTCTTTGGCCGGGTTTACGAGATGGACAG |
| 26 | ScCYC1(UAS1B-313) | CGGTACCTCCTCCGTAACCCGCCGCAAG |

**RESULTS**

**KIHAP1 complements the hap1 mutation of *S. cerevisiae***. Compared with Hap1p, the distinct functional domains, including the Zn(II)₂Cys₆ binuclear cluster (box Zn) followed by a coiled-coil dimerization domain (box CC), three repression modules (RPM; indicated with solid bars), several heme-responsive modules (HRM; indicated with stippled bars; seven in *S. cerevisiae* and eight in *K. lactis*), and an activation domain (box at C terminus). The stretch of 12 glutamine residues (box Q) present in the C-terminal part of the dimerization domain in Hap1p is absent in KlHap1p. Numbers indicate amino acid residue positions in the proteins. The distances are not in proportion.

![Diagram](https://example.com/diagram.png)

**FIG. 1.** Comparison of functional modules between *S. cerevisiae* Hap1p and *K. lactis* KlHap1p. Both proteins are schematically represented and composed of a typical Zn(II)₂Cys₆ binuclear cluster (box Zn) followed by a coiled-coil dimerization domain (box CC), three repression modules (RPM; indicated with solid bars), several heme-responsive modules (HRM; indicated with stippled bars; seven in *S. cerevisiae* and eight in *K. lactis*), and an activation domain (box at C terminus). The stretch of 12 glutamine residues (box Q) present in the C-terminal part of the dimerization domain in Hap1p is absent in KlHap1p. Numbers indicate amino acid residue positions in the proteins. The distances are not in proportion.

**KIHAP1 was as efficient as that by the *S. cerevisiae* HAP1 gene** (Fig. 2B, line 5). These experiments showed that KlHap1p, similarly to the *S. cerevisiae* protein, has a role essential for growth under hypoxic or heme-deficient conditions when being introduced into *S. cerevisiae* (20, 49).

**KIHap1p binds to the consensus cis element CGGN₆CGG of the CYC1 and KICCY1 promoters.** In *S. cerevisiae*, Hap1p binds to the cis element CGGN₆CGG of the target gene promoters (23, 34), although this may not be the only mode of interaction (24). Both Sc-UAS₇CYC1 and Kl-UAS₇CYC1 contain the CGGN₆CGG motif. The physical interaction of KlHap1p with this sequence was shown by a gel retardation experiment (Fig. 3). To do this experiment, we overproduced KlHap1p by introducing a multicopy KIHAP1-carrying plasmid into the *S. cerevisiae* Δhap1 strain (334-Δhap1) under the control of a galactose-inducible promoter. Labeled oligonucleotides Sc-UAS₇CYC1 and Kl-UAS₇CYC1 were used for binding assays. We found that (i) in the presence of hemin, the formation of a KlHap1p-DNA complex was observed (Fig. 3, lanes 4 and 6), and (ii) in the absence of hemin, a diffuse weak band with slower migration was formed (Fig. 3, lanes 3 and 5). Clearly hemin was important for in vitro binding of KlHap1p to the cis element of the cytochrome gene. At the same time, a high-molecular-weight complex appeared on the top of the gel (Fig. 3, lanes 3 to 6), as previously reported (20, 54). All these data confirmed that KlHap1p could bind DNA by the same mechanism as that used by Hap1p.

**Expression of KIHAP1 in *K. lactis***. Figure 4 shows the transcript level of KIHAP1 under several growth conditions. The cells grown aerobically on glucose, galactose, or glycerol did not show any significant difference in the transcript level. By contrast, the transcription was markedly increased (2.4-fold) under hypoxia, at both exponential and stationary phases of growth. This response to hypoxia is similar to that known for ScHAP1 (20, 49).

**Genes under the control of KIHap1p.** In order to identify the target genes of KlHap1p, we constructed a strain with a disrupted *klh1* gene (see Materials and Methods). The
FIG. 2. Complementation of S. cerevisiae Δhap1 mutants by KIHAP1. (A) Increase of cytochrome c synthesis by introduction of KIHAP1 into the Δhap1 mutant of S. cerevisiae. KIHAP1 (plasmid under the control of the S. cerevisiae HAP1 promoter) was inserted into the multicopy vector YEp352, resulting in the plasmid pKl-HAP1. The plasmid pSc-HAP1 containing the S. cerevisiae HAP1 gene was used as a positive control. The Δhap1 mutant W303-ΔHAP1 was transformed with pSc-HAP1 (curve 1), pKl-HAP1 (curve 2), or the empty vector YEp352 (curve 3). All strains were grown at 28°C for 2 days on plates of complete medium containing 2% glucose. Cytochrome spectra were determined at liquid nitrogen temperature in a Cary 400 spectrophotometer according to the protocol of Claisse et al. (13), and three resulting curves were placed at different heights in panel A so that they could be compared with each other. The absorption peak at 550 nm corresponds to cytochrome c. (B) KIHap1p complements the defective phenotype of the S. cerevisiae Δhap1 Δhem1 strain in the presence of ergosterol and Tween 80. Line 1, W303-1A (HEM1 HAP1); line 2, W303-ΔHEM1 (hem1 HAP1); line 3, W303-ΔHEM1-ΔHAP1 (hem1 hap1) transformed with the empty vector YEp352; line 4, W303-ΔHEM1-ΔHAP1 with pKI-HAP1; line 5, W303-ΔHEM1-ΔHAP1 with pSc-HAP1. A serial dilution of cultures was deposited on YP-2% glucose medium supplemented with 30 μg ml⁻¹ ergosterol and 0.2% Tween 80 (right panel, YP-glucose + ET, heme-deficient condition), as well as on the medium supplemented with 30 μg ml⁻¹ β-ALA as control (left panel, YP-glucose + β-ALA, heme-sufficient condition). Cells were grown at 28°C for 3 days.

FIG. 3. Binding of KIHap1p to the upstream activation sequence element CCGN₆CCG in the promoters of CYC1 and KICYC1. Cell extracts were prepared from (i) S. cerevisiae strain 334-ΔHAP1 transformed with the plasmid pDP-Kl-HAP1, which contained the KIHAP1 gene fused to the GAL10-CYC1 promoter, and (ii) the same strain transformed with the empty vector pDP8-10 as a control (lanes 1 and 2). The cell extracts were incubated with ³²P-labeled probes (Sc-UAS_CYC1, lanes 1, 3, 4, and 7; Kl-UAS_CYC1, lanes 2, 5, 6, and 8), either in the absence (lanes 3 and 5) or in the presence (lanes 1, 2, 4, 6, 7, and 8) of 25 μM hemin. One hundred nanograms of unlabeled Kl-UAS_CYC1 or Sc-UAS_CYC1 was added as a competitor (lanes 7 and 8, respectively). Other details are specified in Materials and Methods.

transcript levels of several K. lactis genes, which are orthologous to the known target genes of Hap1p in S. cerevisiae (46), were compared between the wild-type and ΔKlhap1 strains under different growth conditions. Results are presented in Fig. 5.

KICYC1, KICT1, and KIQCR2 genes encode the unique cytochrome c, the cytochrome c₁, and subunit II of ubiquinol cytochrome c reductase of K. lactis, respectively (9, 11, 22). As with their S. cerevisiae counterparts, which are regulated by Hap1p (16, 43, 46), the expression of these three K. lactis genes was markedly reduced (>2.5-fold) by the KIHap1p mutation when oxygen was limited.

KIHMG1 is an ortholog of S. cerevisiae HMG1 encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase. The S. cerevisiae gene is known to be under the control of Hap1p (46, 47). The K. lactis ortholog was also activated (2.5-fold) by KIHap1p under hypoxic conditions.

ERG11 of S. cerevisiae encoding lanosterol 14α-demethylase (cytochrome P450) is controlled by Hap1p (48). By contrast, the expression of its K. lactis ortholog was not affected by KIHap1p mutation under any of the conditions tested.

HEM13 of S. cerevisiae encodes coproporphyrinogen III oxidase. Its counterpart KIHEM13 has previously been reported to be subject to heme- and oxygen-dependent negative regulation (23), as is the case for the S. cerevisiae gene (2, 49). Our experiment indicated that KIHap1p had such a repressive ef-
FIG. 4. Expression of KlHAP1 under various growth conditions. The K. lactis reference strain 2539/152 was grown under aerobic conditions ($O_2$) in YP medium containing 2% glucose (Glu), 2% galactose (Gal), or 2% glycerol (Gly) or under hypoxic conditions ($N_2$) in the Gal medium (galactose was used to avoid possible glucose repression). Total RNA was extracted at exponential and stationary phases of growth. The KlHAP1 probe was prepared by PCR amplification (primers are shown in Table 2). KlACT1 was used as a loading control. Signal quantitation and normalization were carried out as described in Materials and Methods, and the value 1.0 in each panel indicates the reference of normalized intensity with which other signals were compared.

FIG. 5. Regulation by KlHap1p of the transcription of target genes in K. lactis. The wild-type strain MW270-7B and its $\Delta$Klhap1 isogenic mutant were grown in YP medium containing 2% glucose (Glu; lanes 1 and 5), 0.02% glucose (loGlu; lanes 2 and 6), or 2% ethanol (EtOH; lanes 3 and 7) under aerobic conditions ($O_2$). Under hypoxic conditions ($N_2$; lanes 4 and 8), the YP-2% glucose medium was supplemented with ergosterol and Tween 80 and flushed with nitrogen gas. Total RNA was extracted at the exponential phase of growth. Gene probes were obtained by PCR amplification (primers are listed in Table 2). KlACT1 was used as a loading control. Signal quantitation and normalization were carried out as described in Materials and Methods, and the value 1.0 in each panel indicates the reference of normalized intensity with which other signals were compared.

dect on the KlHEM13 expression, but to a limited degree (1.4-fold) under oxygen-limited conditions.

YHB1 encodes a flavohemoglobin that could play a role against the effects of oxidative stress (55). Previous reports showed that the expression of YHB1 was high under oxygen-replete conditions and low under hypoxia (14, 55). In contrast to that, KlHAP1 expression was increased under hypoxic conditions. Furthermore, it appeared that KlHap1p had little effect on the expression of KlYHB1.

Interestingly, the overall amino acid sequence of KlHap1p has some similarity with those of Oaf1p and Pip2p of S. cerevisiae. Oaf1p and Pip2p are key transcription factors when the sequences orthologous to these genes have been detected in the K. lactis genome. We found that the K. lactis ortholog of EEB1/EHT1 (but not that of POX1) was indeed regulated by KlHap1p (Fig. 5). EEB1 and EHT1 encode acyl coenzymeA: ethanol O-acetyltransferase in S. cerevisiae (41). Thus, KlHap1p probably has a role in medium-chain fatty acid ethyl ester biosynthesis in K. lactis, the culture of which smells fruity and is easily distinguishable from that of S. cerevisiae.

The phenotype of the $\Delta$Klhap1 mutant suggests important roles of KlHAP1 in glucose metabolism of K. lactis. To further investigate the KlHAP1 function in K. lactis, we examined the phenotype of the $\Delta$Klhap1 mutant grown under different conditions. The S. cerevisiae hap1 mutant was also examined for comparison.

The S. cerevisiae hap1 mutant did not present any modified phenotype in the culture conditions shown in Fig. 6. In contrast to the S. cerevisiae mutant, the $\Delta$Klhap1 mutant showed an increased sensitivity to the temperature 37°C in glucose media, in particular at lower concentrations of glucose (upper panels in Fig. 6). At 28°C in media with higher levels of glucose, a careful observation of individual colonies led to a finding that the colonies formed by the $\Delta$Klhap1 mutant were markedly larger than those of the wild type (lower panels in Fig. 6). Such differences between Klhap1 and hap1 mutants in glucose media might suggest that KlHAP1 could have a particular role in K. lactis, one so far unknown for HAP1 of S. cerevisiae.

KlHAP1 negatively controls the transcription of RAG1. When yeast grows on sugars under hypoxia or under conditions limiting respiration, the fermentation requires a much higher flow of substrate than does the respiratory mode of growth. Sugar uptake often becomes the limiting step of fermentation. In this sense, oxygen availability may possibly modify the gene activities involved in sugar uptake. K. lactis has two glucose permease genes, RAG1 (for low-affinity transport; $K_m$ of about 20 to 50 mM) (12, 52) and HGT1 (for high-affinity transport; $K_m$ of about 1 mM) (5). It has been reported that the colonies of the strain having the rag1 deletion were smaller than those
Therefore, we examined whether KlHap1p could influence the expression of the two glucose transporter genes. The results of Northern hybridization clearly revealed that at a high glucose concentration, \( \text{RAG1} \) transcript level was significantly higher in the \( \Delta \text{Klhap1} \) mutant than in the wild type while full expression of \( \text{HGT1} \), in contrast, seemed to be dependent on KlHap1p (Fig. 7A). These effects were also confirmed in minimal medium (data not shown). It appeared therefore that KlHap1p negatively regulated \( \text{RAG1} \) transcription specifically at a high glucose concentration.

**KlHAP1 represses glucose uptake in \( K. \text{lactis} \).** The finding that KlHAP1 controls the expression of glucose transporter genes in \( K. \text{lactis} \) raised the question of whether such regulation might have a physiological significance in glucose transport. We then determined the rate of glucose uptake in vivo. When a low concentration of glucose (10 mM) was used in the uptake assay system, no radical changes were observed in the uptake rate of the \( \Delta \text{Klhap1} \) mutant compared to the wild type in 2% glucose medium (52). Therefore, we examined whether KlHap1p could influence the expression of the two glucose transporter genes. The results of Northern hybridization clearly revealed that at a high glucose concentration, \( \text{RAG1} \) transcript level was significantly higher in the \( \Delta \text{Klhap1} \) mutant than in the wild type while full expression of \( \text{HGT1} \), in contrast, seemed to be dependent on KlHap1p (Fig. 7A). These effects were also confirmed in minimal medium (data not shown). It appeared therefore that KlHap1p negatively regulated \( \text{RAG1} \) transcription specifically at a high glucose concentration.

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**FIG. 6.** Phenotype of the \( \Delta \text{Klhap1} \) mutant. The strains, including the \( S. \text{cerevisiae} \) wild-type strain W303-1A and its hapl isogenic mutant and the \( K. \text{lactis} \) wild-type strain MW270-7B and its \( \Delta \text{Klhap1} \) isogenic mutant, were grown aerobically to stationary phase at 28°C in YP-2% glucose medium. The cells were serially diluted in 0.15 M NaCl and dropped onto complete medium (YP) or minimal medium (YNB) containing different concentrations of glucose as specified and then grown at 28°C (lower panels) or 37°C (upper panels) for 2 to 4 days. The cells grown at 28°C for 2 days in YP-2% glucose medium (the lower leftmost panel) were used as a reference for cell amount loaded.

**FIG. 7.** KlHap1p controls the transcription of the glucose transporter genes (A) and glucose uptake rate (B) in \( K. \text{lactis} \). (A) The wild-type strain MW270-7B and its \( \Delta \text{Klhap1} \) isogenic mutant were grown aerobically at 28°C in YP medium containing different concentrations of glucose (2% [\( \sim 110 \text{ mM} \), 0.2% [\( \sim 11 \text{ mM} \), and 0.02% [\( \sim 1.1 \text{ mM} \)]) as specified. Total RNA was extracted at the exponential phase of growth. Gene probes were obtained by PCR amplification (primers are listed in Table 2). \( \text{KIACT1} \) was used as a loading control. Signal quantitation and normalization were carried out as described in Materials and Methods, and the value 1.0 in each panel indicates the reference of normalized intensity with which other signals were compared. Note that \( \text{HGT1} \) has two transcripts of different sizes whose precise nature is not known (5). (B) The \( K. \text{lactis} \) wild-type strain MW270-7B and its \( \Delta \text{Klhap1} \) isogenic mutant were grown at 28°C in YP-2% glucose. Cells were suspended in phosphate buffer, and the rate of uptake of \( [\text{14C}] \text{glucose} \) (100 mM) was measured as described in Materials and Methods. The values are means of two independent experiments. In no case was the variation higher than 15% of the mean.
Fermentation in 500 ml flask

A

B

C

D

E

F

FIG. 8. Kinetics of ethanol production of the K. lactis wild-type strain MW270-7B (△) and its ∆Khap1 isogenic mutant (□). Cells were grown at 28°C in 50 ml YP-5% glucose contained in 500-ml flasks (upper panels) or 100-ml flasks (lower panels). Aliquots were taken for analysis of cell OD_{600} (A and D), glucose consumption (B and E), and ethanol production (C and F). Other details are described in Materials and Methods. The values are means of analysis for two flask cultures. Bars indicate the up and down variations.

ΔKhap1 mutant with respect to the wild type (data not shown). However, when a high concentration of glucose (100 mM) was used, we found a 47% increase in the overall rate of glucose uptake compared to that of the wild type (Fig. 7B). It should be noted that the observed glucose uptake was due to both RAG1 and HGT1 products. Since HGT1 transcription was lowered in the mutant under high-glucose conditions (Fig. 7A), the increase of glucose uptake due to the RAG1 product was thought to be even higher than 47%.

The ΔKhap1 mutant has an increased rate of ethanol production. The effect of the Khap1 mutation on RAG1 expression and glucose transport led us to compare the time course of ethanol accumulation between the wild-type MW270-7B and the ΔKhap1 mutant. Two S. cerevisiae strains, the wild-type W303-1A and an isogenic hap1 mutant (disrupted by KanMX to avoid influence on fermentation resulting from auxotrophy), were examined in parallel for each experiment.

Oxygen limitation has been regarded as the primary environmental factor in triggering alcoholic fermentation in K. lactis (30). We therefore carried out the fermentation experiments in smaller flasks (50 ml medium in a 100-ml flask) simulating an oxygen-limiting condition (lower panels in Fig. 8). In conditions where the two strains grew at similar rates (Fig. 8D), the ΔKhap1 mutant consumed glucose much faster than did the wild type (a difference of about 8 to 10 h [Fig. 8E]), in agreement with the results of glucose uptake assays. Consequently, the ΔKhap1 mutant produced ethanol more rapidly than did the wild type (Fig. 8F).

As described above, the expression of KJHAPI responded to oxygen availability. We therefore performed the fermentation experiments under normoxia (50 ml medium in a 500-ml flask [upper panels in Fig. 8]), which is usually used as an aerobic condition in routine manipulation. Compared to growth under the oxygen-limiting condition, both the wild type and the ΔKhap1 mutant grew to a higher final optical density (OD_{600} of ≈40 [Fig. 8A] versus OD_{600} of ≈25 under the oxygen-limiting condition) and consumed glucose much more slowly (Fig. 8B), showing a strong Pasteur effect (33, 44). Even in this
case, the difference in glucose consumption still existed between the wild type and the ΔKlhap1 mutant (a difference of about 6 h [Fig. 8B]). A similar increase in ethanol production was found in the ΔKlhap1 mutant, but at a lower ethanol concentration (Fig. 8C). Although cultivation in a shake-flask is not regarded as a strictly aerobic condition, we noticed that the K. lactis wild type did not produce ethanol until after 8 h of incubation. Meanwhile, 10 mM ethanol was already detected in the 6-h culture of the K. lactis mutant (a difference of 8 h compared with the wild type did not produce ethanol until after 8 h of incubation. Meanwhile, 10 mM ethanol was already detected in the 6-h culture of the K. lactis mutant). KlHap1p may be involved in the Crabtree effect.

**DISCUSSION**

The spectrum of target genes regulated by Kihap1p and its mode of regulation in K. lactis could deviate from those of Hap1p in S. cerevisiae. The sequence of Kihap1p contains the equivalents of all the functional domains described for the S. cerevisiae counterpart. Therefore, the functional similarity of Kihap1p to ScHap1p can be anticipated from the sequence features. Complementation of the ScHap1p mutation by the Kihap1p gene showed that Kihap1p was an activator in aerobiosis and could also restore growth in heme-depleted conditions. The capacity of heterospecific binding to common cis elements indicated that the Hap1p proteins of the two species share a common specific interaction with these target sites. Our results, together with previous reports (18, 23), show that the basic regulatory scheme “O2 → heme → Hap1p → some respiratory genes” is conserved in both species.

**TABLE 3. Target genes of Hap1p: comparison between S. cerevisiae and K. lactis***

| Target gene | S. cerevisiae | K. lactis |
|-------------|---------------|-----------|
|             | CGG/NCGG sites in the promoter | Regulation by ScHap1p | CGG/NCGG sites in the promoter | Regulation by Kihap1p |
| CYC1        | −349 CGGAAGATCGG −360 | + | −285 CGGGRACATCGG −296 | + |
| CYC7        | −337 CGGGRTPCGG −326 | + | −377 CGGGRATCGG −362 | + |
| CYT1        | −527 CGGATTTCTCGG −516 | + | Not detected | + |
| QCR2        | −494 CGGAAATACCGG −505 | + | −917 CGGGRATCGG −906 | + |
| HMG1        | −472 CGGGRATCAGG −461 | + | −509 CGGGRATCGG −520 | + |
| ERG11       | −620 CGGGRATCGG −631 | + | Not detected | + |
|             | −789 CGGGRATCGG −800 | + | Not detected | n |
|             | −731 CGGGRATCGG −720 | + | −782 CGGGRATCGG −771 | − |
|             | −642 CGGGRATCGG −653 | + | −259 CGGGRATCGG −270 | n |
|             | −778 CGGGRATCGG −767 | + | −595 CGGGRATCGG −606 | n |
|             | −358 CGGGRATCGG −347 | + | −531 CGGGRATCGG −542 | − |
|             | −469 CGGGRATCGG −458 | + | −240 CGGGRATCGG −229 | + |

* The phenotypes of the Δhap1 mutants for each strain were as follows: S. cerevisiae, no obvious change in growth phenotype; K. lactis, growth defect in low-glucose medium at 37°C and increased colony size in high-glucose medium at 28°C.

* A search for putative Hap1p binding site CGG/NCGG (in two orientations) was carried out over a 1-kb region upstream of each gene by using the program at http://rulai.cshl.edu/. One mismatch allowed (C→A, G, T). The numbers refer to the distance from the motif to the ATG initiation codon. Boldface characters indicate nucleotides which match the CGG triplets of the consensus sequence.

* Symbols: +, activated; −, repressed; n, little affected; ?, unknown or unclear.

* The RAG1 gene has two CGG/NCGG sequences (one nucleotide shorter than the consensus sequence) at −629 and −952.
trations in K. lactis. Since the rate of sugar uptake is a major determinant of the glycolytic flux for fermentation (19) and the regulation of glucose uptake is crucial for sensitivity to glucose repression in K. lactis (50), KIHaP1 might have a role in negatively controlling the glycolytic flux by reducing sugar uptake, while it positively regulates the expression of some respiratory genes in K. lactis. The logic of this dual regulation may be that it would help maximize the respiratory pathway and minimize fermentation in this Crabtree-negative species. A possible role of HaP1p in the transcription of sugar transporter genes has not been reported so far for any organism. It is still unclear how KIHaP1 regulates the transcription of the glucose transporter gene.

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