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ORIGINAL PAPER

A stress response related to the carbon source and the absence of *KlHAP2* in *Kluyveromyces lactis*

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Abstract The *Kluyveromyces lactis HIS4* gene (*KlHIS4*) is transcriptionally regulated by the carbon source. The promoter region encompassing positions −238 to −139 is responsible for this regulation according to *lac*Z reporter assays. Electrophoretic Mobility Shift Assay (EMSA) experiments on *KlHIS4* promoter (positions −218 to −213, Fragment 6, F6) show a specific gel-shift band, CS1, whose intensity is carbon-source dependent in *K. lactis hap2* (*klhap2*) knock-out strains. The *klhap3* mutation is not able to cause this effect by itself, but the combination of *klhap2* and *klhap3* mutations has an enhanced effect on CS1 band formation. Introducing a heat shock element (HSE) at the sequence in the F6 fragment (mutated F6, F6*) increases the binding activity in the *klhap2* mutant. *KlHIS4* mRNA levels in the *klhap2* or the double *Klhap2/3p* mutant do not correlate with the increase in CS1 binding activity, indicating that the factor causing CS1 is acting and only detectable in vitro. EMSA experiments with *K. lactis* wild-type cells under temperature stress conditions show a band enhancement (Ts1), similar in size to CS1. Cross-competition experiments between F6 and F6* show that F6* competes more efficiently than F6 for both CS1 and Ts1 formation, indicating the involvement of the HSE in the formation of the specific gel-shift bands. Moreover, the similar gel-shift patterns suggest that both bands are caused by the same heat shock-like factor under different stress conditions. Therefore, the enhancement of the CS1 band signal in the *klhap2* (and *klhap2/3*) mutants is due to the increase in heat shock-like factors in the protein extracts from these mutant cells grown in a non-fermentable carbon source. This *Klhap2*-dependent stress effect was not previously described in *K. lactis.*

Keywords Yeast · Hap2/3/4-complex · Transcription · *Kluyveromyces* · Carbon-source

Abbreviation
EMSA Electrophoresis mobility shift assay

Introduction

Carbon-source-dependent transcriptional regulation is an important step in gene expression regulation and has a special relevance for yeast biotechnological applications (from fermentation to biomass production). One of the transcriptional activators best characterized in *S. cerevisiae* is the Hap2p/3p/4p/5p complex, which binds to the CCAAT sequence. This complex is involved in several regulatory functions such as reprogramming gene expression during the shift from fermentation to respiration, ammonium assimilation, mitochondrial development as well as coordination of nuclear and mitochondrial gene expression [3, 5, 18]. Hap2p and Hap3p subunits are required for DNA-binding activity [15, 23]. Hap4p is the activator subunit and its transcription is glucose repressible [2, 6] and only after the Hap2p/3p/5p complex binds to DNA the Hap4p interacts with this complex [12].

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In *Aspergillus nidulans* and humans the three subunits are necessary to form the DNA-binding complex, as described for *S. cerevisiae* [1, 21]. Human cells contain at least three distinct CCAAT-binding proteins, all appearing to be protein complexes composed of heterologous subunits. One of these, CP1, has DNA-binding properties that are virtually identical to those of the yeast Hap2p/Hap3p complex. CP1 consists of at least two subunits, CP1A and CP1B (homologous to Hap3p and Hap2p, respectively), and both are required for DNA binding [1, 4]. Moreover, the proteins of CP1 and Hap2p/Hap3p are functionally interchangeable in vitro and the human/yeast hybrid complexes that can be formed retain the ability to specifically recognize CCAAT elements. It has been demonstrated that *A. nidulans* HapB is also interchangeable with Hap2p and CP1B [21].

In *Kluyveromyces lactis*, *KlHAP2* and *KlHAP3* genes have been isolated by functional complementation of the inability of *hap2* and *hap3 S. cerevisiae* mutants to grow in non-fermentable carbon source. However, *Klhap2* and *Klhap3* mutants have the capacity to grow in non-fermentable media [13, 14], which contrasts with the phenotype described for *S. cerevisiae* mutants [10, 16]. Moreover, analysis of the transcriptional regulation of *KlCYC1* showed that fragments encompassing the CCAAT UAS2 consensus give retarded bands whose intensity is not altered in a *Klhap2* or *Klhap3* mutant background, indicating that they are not related to the KlHap complex [17].

In a previous work, we demonstrated a carbon-source-dependent transcriptional regulation of *KlHIS4* (which as *S. cerevisiae HIS4*, encodes for a multifunctional protein catalyzing three steps of histidine biosynthesis). *KlHIS4* mRNA levels are higher in glycerol than in glucose and its regulation is different from that of *S. cerevisiae* where the *HIS4* transcription level decreases in glycerol [11]. This difference and the presence of a CCAAT sequence on *KlHIS4* promoter were the reasons to study the possible involvement of the *K. lactis* Hap complex carbon-source-dependent transcriptional regulation of this gene. In this report, we describe findings showing a *klhap2*-dependent cellular stress response, not previously shown in *K. lactis*.

Materials and methods

Kluyveromyces lactis strains and growth conditions

The *K. lactis* strains used were NRRL-Y1140 (*MATa)* ATCC8585 as wild-type. In *β*-galactosidase experiments, MW190-9B (*MATa lac4-8 uraA Rag +)* by Wésolowki-Lovel was used. MW270-7B/16 (*MATa leu2 uraA metA1 HAP2::ScURA3*), WMH7302-D1 (*MAT leu2 uraA trp1 metA1 ade2 his2-2 HAP3::ScLEU2)* and WMH2302-3 (*MATa leu2 uraA trp1 metA1 ade2 his2-2 HAP3::ScLEU2 HAP2::ScURA3*) were kindly provided by M. Bolotin-Fukuhara and L. Grivell, and will be referred to in the text as *klhap2*, *klhap3* or *klhap2/3*, respectively. *Saccharomyces cerevisiae* strains BWG1-7a (*Mata ade1-100 his4-519 leu2-3 leu2-112 ura3-52*) by Guarente and Mason [8] and J01-1a (*Mata ade1-100 his4-519 leu2-3 leu2-112 ura3-52 Δhap2*) by Olsen and Guarente [15] were used for EMSA assays (data not shown).

Rich medium was prepared by standard methods [19] with 4% glucose (YPD), or 2% glycerol plus 2% ethanol (YPGE).

For the temperature-shift experiments, cells grown in YPD media with 2% glucose were cultured at 25°C to an OD_{600} of 1 and cultures were split and one half shifted to 37°C for 90 min while the other was maintained at 25°C.

β-galactosidase experiments

Two fragments of *KlHIS4* promoter were cloned into pXW1 plasmid in frame with the *lacZ* gene. The constructions were called pML10 and pML11, where pML10 has a promoter fragment from −236 to −1, positions with respect to ATG, the first of the coding regions, obtained by PCR using the oligos OMLPR5 5′-TAGGATCCGGAC TTCTTCTTCGAG-3′ and OMLPR0 5′-TTTTCTTTCA AAGCTTTGACA-3′. pML11 has a promoter fragment from −139 to −1 also obtained by PCR using the oligos OMLPR0 and OMLPR6 5′-TAGGATCCACTGCAAAAA TCAAGC-3′. In both cases the plasmid pKH4Pst [11] was used as template. *Hind*III and *Bam*HI sites, used to clone the fragments, have been underlined.

After transforming the MW190-9B *K. lactis* strain, two independent isolates from each transformation were grown in YPD and YPGE and the *β*-galactosidase experiments were performed as in Guarente [9]. *β*-galactosidase activity is expressed in arbitrary units.

Electrophoretic mobility shift assays

Crude extracts were prepared from yeast cells incubated in rich media with 4% glucose or 2% glycerol plus 2% ethanol. Extracts and gel retardation assays were performed as in Ramil [17]. EMSA assays were carried out with two *KlHIS4* promoter fragments called Fragment 6 (F6) and mutated Fragment 6 (F6*).

Specific oligonucleotides were used after annealing as Fragment 6: oligo oMLPR18: 5′-TTACTTCTTCTTCGAG TTTTCATTGGCTTTCCTGTGTTTTTTTAT-3′ (Watson strand, encompassing positions −236 to −200) and oMLPR19: 5′-TTTATGAAAAAAACACAGGAAAGCC AATGAAAACTCGAAGAAGAAGT-3′ (Crick strand, encompassing positions −236 to −200). The CCAAT

mutated sequence was included in mutated Fragment 6 (F6*) obtained by annealing oligos: oMLPR20: 5′-TTTA CTTCTTCTTCGAGTTTTCA*CCTT*CTTTTCCTGTGTTTT TTTCAT-3′ (encompassing positions −236 to −200 Watson strand) and oMLPR21: 5′-TTTATGAAAAAAAC ACAGGAAAG*AAGGT*GAAAATCTGAAGAAGAAGT-3′ (encompassing the same positions at the opposite strand). The Hap2p/3p/4p/5p consensus sequences are underlined and mutated bases are in italics. After annealing, the generated fragments contain at the 5′ end protruding TTT to label by Klenow and α − 32P-ATP. The binding reactions were carried out at 30°C for 20 min in buffer A [17] with labeled DNA probe, corresponding to 10,000 cpm, 2 μg of calf thymus DNA as carrier and 15 μg (for the *klhap* mutants) or 30 μg (for the temperature shift) of protein extracts.

Northern analyses

Northern experiments were performed as previously described by Zitomer and Hall [24]. The 1-kb fragment obtained by PCR, with OMLPR6 5′-TAGGATCCACTG CAAATCAAGC-3′ and OML15 5′-CATGCCCATAAAG GG-3′ and pM756 [7] as template, was used as the *KlHIS4* probe. The *KlLEU2* probe was also obtained by PCR, with OMLKL1 5′-CCTTAATAGCTTCGTCAG-3′ and OMLKL2 5′-TTTAACGCATCCTACATTTG-3′ using NRRL-Y1140 genomic DNA as template.

A U3 probe was used for loading correction. The U3 probe was obtained by PCR with U3F 5′-CGACGTA CTTCAGTATGTAA-3′ and U3R 5′-ATTTGTACCCAC CCATAGAG-3′ and hybridized with the *S. cerevisiae snR17A* and also cross-hybridized with the *K. lactis* homologous gene. Hybridized bands were quantified using the program ImageQuant (Molecular DynamicsTM). All hybridizations were carried out at least in duplicate.

Computer searches: Yutaka Akiyama′s "TFSEARCH: Searching Transcription Factor Binding Sites" program was used to analyze the putative binding sites on the *KlHIS4* promoter sequences (http://www.rwcp.or.jp/papia/).

Results

The region encompassing positions −236 to −139 is responsible for *KlHIS4* carbon source regulation.

To analyze if the region encompassing the CCAAT box was responsible for *KlHIS4* carbon source regulation, β-galactosidase experiments were performed using two reporter plasmids, pML10 that includes a 236 bp promoter with the CCAAT box, and pML11 with a 139-bp promoter without this sequence. As shown in Fig. 1 in cells expressing pML10, we detected a small but reproducible

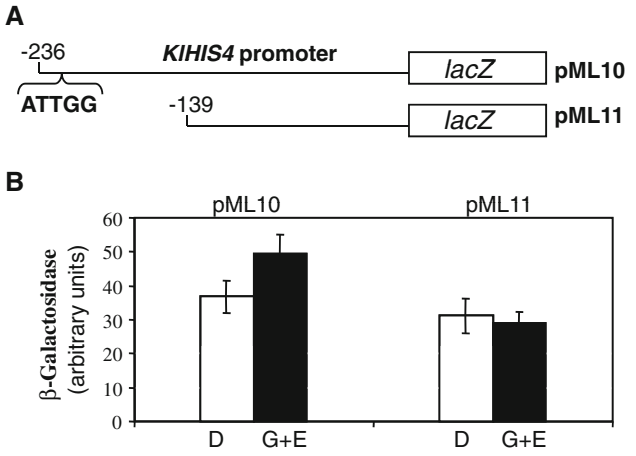

Fig. 1 **a** Diagram of *KlHIS4* promoter fragments fused to *lacZ* gene, constructions pML10 and pML11. **b** β-galactosidase activity graph of *K. lactis* wild-type strain MW190-9B transformed with the reporter plasmids grown in glucose (D, *white bar*) or glycerol plus ethanol (G + E, *black bar*). β-galactosidase activity is expressed in arbitrary units. The *numbers* represent the average of four independent experiments performed in duplicate

1.5-fold increase in β-galactosidase activity in glycerol plus ethanol (G + E), with respect to cells grown in glucose (G). In cells expressing the pML11 deletion, this increase is lost (Fig. 1); therefore, the promoter region from −236 to −139 is responsible for *KlHIS4* carbon source regulation.

A specific carbon-source-dependent retarded band is enhanced in *klhap2* mutants

To test if the CCAAT sequence was responsible for the carbon-source regulation, the labeled Fragment 6 (F6) encompassing the region −236 to −200 was incubated with protein extracts from wild-type, *klhap2*, *klhap3* and *klhap2/3* double mutant strains grown either in glucose or in glycerol plus ethanol. The EMSA assay is shown in Fig. 2. In the four strains, a retarded band, CS1, was found; its intensity was stronger in non-fermentable carbon sources than in glucose (compare F-nF in all strains, Fig. 2a). The band intensity increased in the *klhap2* mutant with respect to the wild-type under the same conditions (compare lanes 4 and 5, corresponding to *klhap2*, with lanes 2 and 3 of the wild-type, in Fig. 2a). In the case of the *klhap3* mutant, the band intensity was similar to that of the wild-type (compare lanes 6 and 7, corresponding to *klhap3*, with lanes 2 and 3 of the wild-type, in Fig. 2a), therefore, the absence of KlHap3p is not relevant in CS1 formation. However, the combination of *klhap2/3* mutations produced a stronger signal than the *klhap2* mutation alone, indicating that Klhap3p is also involved, in the formation of CS1, perhaps for their combined role in the *K. lactis* Hap complex.

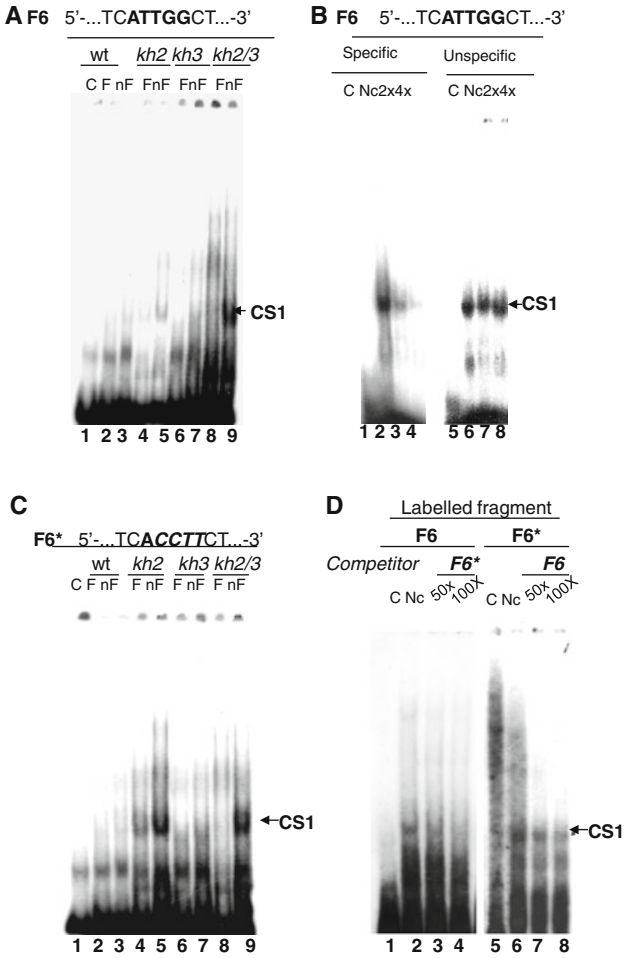

Fig. 2 **a** EMSAs using *KlHIS4* promoter fragment from −236 to −200 Fragment 6 (F6). **b** Competition assays with F6 fragment with *klhap2/3* mutant growing in YPGE, to test the CS1 band specificity**.** *Lanes 3–4* non-labeled/F6 fragment. *Lanes 7–8* non-labeled *KlHIS4* promoter fragment from positions −264 to −237. Reactions with X referring to the number of excess fold for the competitor. **c** Experiment with mutated CCAAT sequence F6*. The assays were performed with wild-type (wt), *klhap2* (*klh2*), *klhap3* (*klh3*) and double mutant *klh2/3*) protein extracts obtained from cultures grown in YPD (F) or YPGE (nF). **d** Cross-competition experiment between mutated and non-mutated fragment 6 using the protein extracts from *klhap2/3* cells grown in YPGE. *Nc* no competitor, *c* reaction control without protein extract. The CCAAT consensus is indicated in *bold letters* and the mutated base pair in *italics*

To analyze CS1 specificity, EMSA competition assays were performed under the same conditions (Fig. 2b). As a specific competitor we used a non-labeled F6 fragment and an unspecific competitor, non-labeled *KlHIS4* promoter fragment from positions −264 to −237. CS1 band intensity decreases clearly in the presence of two to four-fold excess of specific competitor (Fig. 2b, lanes 3–4) while it remains with similar intensity in the presence of two to four-fold excess of unspecific competitor (Fig. 2b, lanes 7–8). This result proves that CS1 is a specific band.

Involvement of the CCAAT sequence in CS1 binding activity

To determine if the CS1 band was dependent on the ATTGG (CCAAT at the opposite strand) element, the sequence was changed to A*CCTT* Fragment 6* (F6*) and the EMSA result is shown in Fig. 2C. In this mutated fragment, the CS1 band was highly enhanced in the *klhap2* mutant with respect to the wild-type both in glucose and in glycerol plus ethanol (compare lanes 2 and 4 or 3 and 5 in Fig. 2c). The pattern in *klhap3* was not changed with respect to the result in Fig. 2a and the double *klhap2/3* mutant shows a similar pattern to that for *klhap2* alone (compare lanes 4 and 5 with 8 and 9 in Fig. 2c). The latter result initially suggested the involvement of the CCAAT element and the relevance of *klhap2* mutation for CS1 formation.

We also performed a cross-competition experiment between mutated and non-mutated F6 fragments using the *klhap2/3* extracts for an enhanced signal. As shown in Fig. 2d, the F6* competes with labeled F6 showing a decrease in CS1 signal (Fig. 2d, lanes 2–4), while the non-mutated F6 is not as efficient in competing with F6* since it is not able to cause a drastic decrease in CS1, even with 100-fold excess (Fig. 2d, lanes 6–8).

We tested if the F6* mutation could introduce, or extend, a binding site causing the CS1 band enhancement. After searching for new binding sites introduced, we found that the mutation included in F6* adds an extra HSE (F6 has already three sites) as putative binding site for a heat shock factor (also shown in Fig. 4a).

Therefore, a cross-competition experiment, using the *klhap2/3* mutant extracts, shows that the enhancement of the CS1 signal in the F6* is specific, and due to the HSE existing on F6, and the extra HSE (instead of the CCAAT) is responsible for that band enhancement.

KlHIS4 mRNA levels in *Klhap* mutants do not correlate with the CS1 enhancement

To test if the CS1 band enhancement in *Klhap* mutants correlates to changes in *KlHIS4* mRNA levels we performed Northern experiments using wild-type and *Klhap* mutant strains grown under the same conditions as those used for the binding experiments. In the *klhap2* mutant, the *KlHIS4* carbon-source regulation is lost (Fig. 3a, compare lanes 1–2 with 3–4) and in both *klhap3* and *klhap2/3* mutants there is a lower signal (Fig. 3a, lanes 5 and 6 or 7 and 8). *KlLEU2* is not regulated by carbon source in a wild-type strain (Fig. 3a, lanes 1 and 2) and in the *hap* mutants there is a diminished signal as in *KlHIS4* (Fig. 3a, lanes 5–8). Although the result on *KlHIS4* indicates that the KlHap complex has some regulatory role in the expression

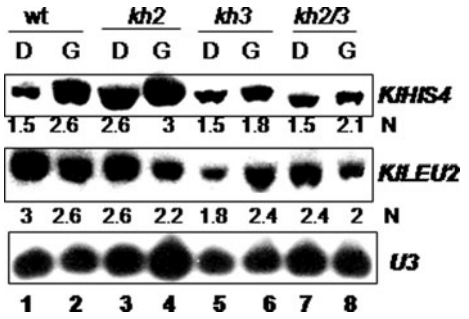

Fig. 3 Northern experiments showing the transcriptional effect of *klhap2/3* mutations. Wild-type (wt), *klhap2 klh2*), *klhap3* (*klh3*) and double mutant (*klh2/3*) RNA obtained from cultures in YPD (D) or YPGE (G). The hybridizations with *KlHIS4*, *KlLEU2* or *U3* (used for normalizing values) were performed on the same blot. *N* normalized values

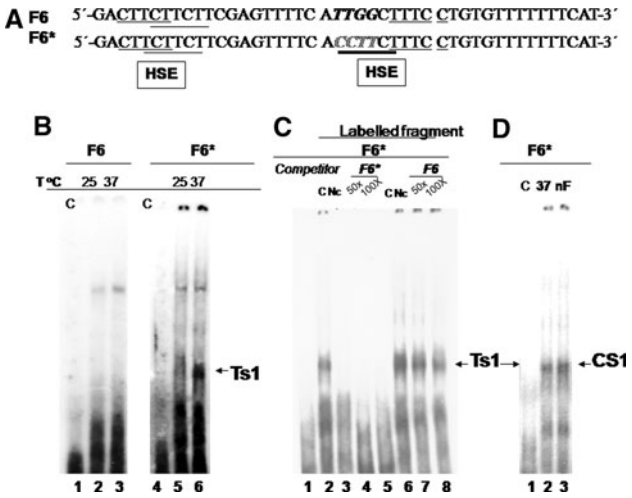

Fig. 4 Effects of a temperature shift on the F6* EMSA pattern. **a** Location of the HSE putative binding sites (*underlined*) found in F6 and F6* sequences. **b** Analysis of the temperature shift (25–37°C) in *K. lactis* Y1140 strain. **c** Cross-competition experiment between mutated and non-mutated fragment. **d** Comparison of gel-shift patterns in temperature-shift or carbon-source stress using wild-type extracts (*lane 2*) or extracts from *klhap2/3* cells grown in YPGE (nF)(*lane 3*). *Nc* no competitor, *c* reaction control without protein extract. Reactions with X referring to the number of excess fold for the competitor

of genes involved in amino acid biosynthesis in vivo, this effect does not correlate with the effects shown with protein extracts from the *klhap* mutants in the EMSA experiments with F6 (Fig. 2a).

In summary, the mRNA levels of *KlHIS4* expressed in the *klhap2* or *klhap2/3* mutants do not correlate with the carbon-source-dependent CS1 band formation using protein extracts from the same mutants under the same experimental conditions. The differences found may be related with the involvement of HSE in the fragments used for binding, and the fact that the small fragments used for binding are not in a promoter context.

K. lactis protein extracts cause a specific gel-retarded band with F6* after a temperature shift

To test our hypothesis, that the mutation introduced in F6* involves a HSF-like binding to the HSE in response to a stress condition causing a CS1 retarded band, we studied the effects of a temperature shift on cell stress. As shown in Fig. 4b (lanes 4–6), after 90 min at 37°C there is a strong enhancement of a retarded band Ts1 similar to CS1 obtained with the *klhap2/3* mutants grown in glycerol plus ethanol. When the same extracts were incubated with F6 this effect was less pronounced (Fig. 4b, lanes 1–3). These results indicate the dependence on the extra HSE (in F6*) for Ts1 formation.

The specificity of this band was confirmed by cross-competition experiments between F6 and F6*, using protein extracts of a wild-type strain after the temperature shift (Fig. 4d). In the experiment performed with labeled F6 the Ts1 band signal is drastically reduced when adding unlabeled F6* as competitor (Fig. 4c, compare lane 2 with lanes 3 and 4), while adding 100-fold excess of F6 the Ts1 band remains. This result shows Ts1 specificity and indicates the need for the new HSE in F6*.

To verify that the carbon-source stress in the *klhap2/3* mutants and the temperature-shift stress in the wild-type cause the same gel-shift pattern, we ran the samples under the two conditions on the same gel. As shown in Fig. 4d (lanes 2 and 3), the gel-shift pattern is identical, and more importantly, the Ts1 and CS1 bands have identical migration. This new information together with its common dependence on the HSE, we can talk in both cases of two different cellular stress conditions allowing the same binding response.

In summary, the results shown in Fig. 4a–c confirm that the HSE in F6* is the target of a heat shock-like factor acting after a temperature shift and cross-competition experiments indicate that Ts1 is a specific retarded band whose presence, as CS1, is highly dependent on the HSE. The EMSA result in Fig. 4 d confirms that the absence of KlHap2p for *K. lactis* cells growing in a non-fermentable carbon source is a cellular stress condition. The response is equivalent to a temperature shift in a wild-type strain.

Discussion

The results presented show the characterization of a carbon-source-related binding activity (causing the CS1 band) in the absence of KlHap2p. This effect is shown only in extracts from cells grown in non-fermentable carbon

sources, revealing that the *K. lactis* Hap complex is related to regulation under these conditions.

In contrast to that described in *S. cerevisiae*, it has been shown that the *K. lactis klhap2* and *klhap3* mutants still have the capacity to grow in non-fermentable media [14]. Moreover, genes such as *KlCYC1* [17] differ from their *S. cerevisiae* counterparts in their dependence on the Hap complex to activate their transcription in non-fermentable carbon sources. These previous data argued against the *K. lactis* Hap complex being involved in carbon-source-dependent transcriptional activation. Therefore, our results in Fig. 2 are the first evidence showing that KlHap2p is involved in carbon-source regulation.

Adding an extra HSE in F6 (F6*) increases the binding affinity for a heat shock-like factor in the *klhap2* mutant (also enhanced in the *klhap2/3* double mutant), as shown in Fig. 2c (lanes 4 and 5).

If the protein factor causing CS1 band formation is a stress-related factor this would help us explain the results obtained in Fig. 2 and suggests that, in *K. lactis,* the absence of the Hap complex causes a stress response mediated by a heat shock-like factor.

Other works have shown by in vitro data that heat-shock factors can bind to an isolated HSE, but only with low affinity. Increasing the presence of HSE increases the affinity, as reviewed [20]. We consider that this effect is equivalent to the enhancement in CS1 formation after adding an extra HSE in F6*, in both *klhap2* mutant and under temperature-shift stress conditions. We also think that the F6 and F6* fragments have shown to be a useful tool to identify carbon-source-dependent stress conditions in *K. lactis*.

Ucetelli and coworkers [22] have shown that a mutation in *KlPMR1* controls the response of *KlHSP60* under oxidative stress, therefore, the *K. lactis* heat-shock factors can also be involved in other stress responses apart from thermal shock. Therefore, the data presented would show that the absence of *khap2* as a stress condition inducing binding of a heat shock-like factor to HSE in F6 would be more evident by adding an extra HSE (F6*) as summarized in Fig. 5.

The differences found in vitro (by EMSA) and in vivo (by Northern) would reflect that these heat shock-like factors are present in the protein extract probably to act on other target genes, but since the F6 fragment is a "naked" DNA (not in chromatin context), all the consensus sequences are accessible for these factors.

We have shown that the stress response in the *klhap2 or klhap2/3* mutants causing CS1 band (Fig. 2) is equivalent to the Ts1 band after being subjected to heat stress for a wild-type *K. lactis* strain (as shown in Fig. 4). This result reveals a new effect of the *klhap2*, *klhap2/3* mutations in *K. lactis* causing a stress response, not previously

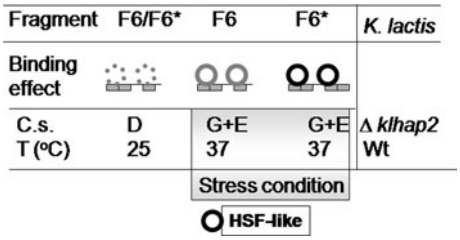

Fig. 5 Model summarizing the different stress responses detected on *KlHIS4* F6/F6* in *K. lactis*. *Small boxes* represent HSE. *Open grey* to *closed black circles* indicate low to high binding signal in these fragments for a stress-related heat shock-like factor, respectively. *C.s.* Carbon source. *Wt K. lactis* wild-type strain for the Hap complex. D Glucose. G + E: cells grown in YPGE

described, which is specially relevant taking into account the importance of the factors belonging to this complex in yeasts and its connection with the transcriptional regulation by the carbon source.

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