

## Two Proteins with Different Functions Are Derived from the *KIHEM13* Gene<sup>∇</sup>

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**Two proteins that differ at the N terminus (l-*KICpo* and s-*KICpo*) are derived from *KIHEM13*, a single-copy-number gene in the haploid genome of *Kluyveromyces lactis*. Two transcriptional start site (tss) pools are detectable using primer extension, and their selection is heme dependent. One of these tss pools is located 5' of the first translation initiation codon (TIC) in the open reading frame of *KIHEM13*, while the other is located between the first and second TICs. In terms of functional significance, only s-*KICpo* complements the heme deficiency caused by the  $\Delta$ *hem13* deletion in *K. lactis*. Data obtained from immune detection in subcellular fractions, directed mutagenesis, chromatin immunoprecipitation (ChIP) assays, and the functional relevance of  $\Delta$ *KIHEM13* deletion for *KIHEM13* promoter activity suggest that l-*KICpo* regulates *KIHEM13* transcription. A hypothetical model of the evolutionary origins and coexistence of these two proteins in *K. lactis* is discussed.**

Cell complexity depends on regulatory mechanisms that control gene expression and adapt levels of specific proteins to internal and environmental changes. One gene can produce one protein or several proteins through mechanisms controlling transcription, mRNA processing, translation, and post-translational events. In yeast, changes in gene transcription and the regulation of mRNA translation play critical roles in the response to oxygen availability.

In the yeast *Kluyveromyces lactis*, the *HEM13* gene (*KIHEM13*) encodes coproporphyrinogen oxidase (Cpo), which catalyzes the sixth step of heme biosynthesis. Cpo promotes oxidative decarboxylation of the 2- and 4-propionyl groups of coproporphyrinogen III to form the vinyl groups of protoporphyrinogen IX. Northern blot analyses have revealed that *KIHEM13* is a hypoxic gene and that its transcription is regulated by oxygen and heme (10). In *Saccharomyces cerevisiae*, the gene homologous to *KIHEM13* is essential in the absence of a heme precursor under aerobiosis (normoxia, with approximately 20% O<sub>2</sub>) and hypoxic conditions (less than 0.05% O<sub>2</sub>). *KIHEM13* complements a  $\Delta$ *hem13* mutation in *S. cerevisiae* and restores its capacity to grow in the absence of hemin (protoporphyrin IX) (2). Sequence alignment of the protein derived from the *KIHEM13* open reading frame (ORF) with the *S. cerevisiae* homolog demonstrates the presence of 52 additional amino acids at the N terminus of *KICpo*. This extension includes the amino acids between the first and second in-frame ATG

codons in the *KIHEM13* ORF. Therefore, computer-aided translation from the second in-frame ATG (the second translation initiation codon [TIC-2]) produces a *Saccharomyces*-like Cpo, while translation from the first in-frame ATG (TIC-1) gives rise to an N-terminally extended Cpo (Fig. 1A).

This study demonstrates that in *K. lactis*, *KIHEM13*, a single-copy-number gene, produces two proteins that differ in the N-terminal region (l-*KICpo* and s-*KICpo*). Diverse evidence supports a mechanism in which the use of alternative transcription start sites (tss) allows two different polypeptides to be synthesized. Complementation with s-*KICpo*, but not with l-*KICpo*, restores the growth of *K. lactis*  $\Delta$ *hem13* mutants in media without hemin supplementation. Differential use of tss pools during hypoxia and other functional data indicate that l-*KICpo* plays a role in the transcriptional regulation of *KIHEM13* expression. A hypothetical model of the origin and coexistence of these two proteins that have differing functions is proposed.

### MATERIALS AND METHODS

**Yeast strains and growth conditions.** The *K. lactis* strains used in this study were NRRL-Y1140 (CBS2359), MW190-9B (*MAT $\alpha$  lac4-8 uraA Rag<sup>+</sup>*), and CBS $\Delta$ KU80 (CBS2359  $\Delta$ *Kku80*) (16). The *S. cerevisiae* strain BY4741 (*MAT $\alpha$  his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0*), used for efficient recombination, was obtained from EUROSCARF (<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/yeast.html>). Yeast cells were grown at 30°C in YPD (2% glucose, 2% Bacto peptone, 1% yeast extract) or synthetic complete medium (CM) (29). Hypoxic conditions were generated in anaerobic jars with the AnaeroGen gas pack system (Oxoid Ltd.). For hypoxic growth, media were supplemented with Tween and ergosterol as described previously (2). When required, media were supplemented with hemin at a final concentration of 50  $\mu$ g/ml.

**Nomenclature used for gene regions, primers, and plasmids.** In the numbering of sequences, the A in the first ATG of the *KIHEM13* ORF was considered position +1; negative positions were counted 5'-wards and positive positions 3'-wards. Using this nomenclature for the *KIHEM13* ORF, TIC-1 was at position +1, TIC-2 at position +157, and the TGA stop codon at position +1126. In the primer sequences, capital letters represent the genomic sequences and lowercase letters represent additional sequences added for design convenience. Restriction sites are shown in italics. The letters "w" and "c" at the end of primer names indicate the forward and reverse strands, respectively.

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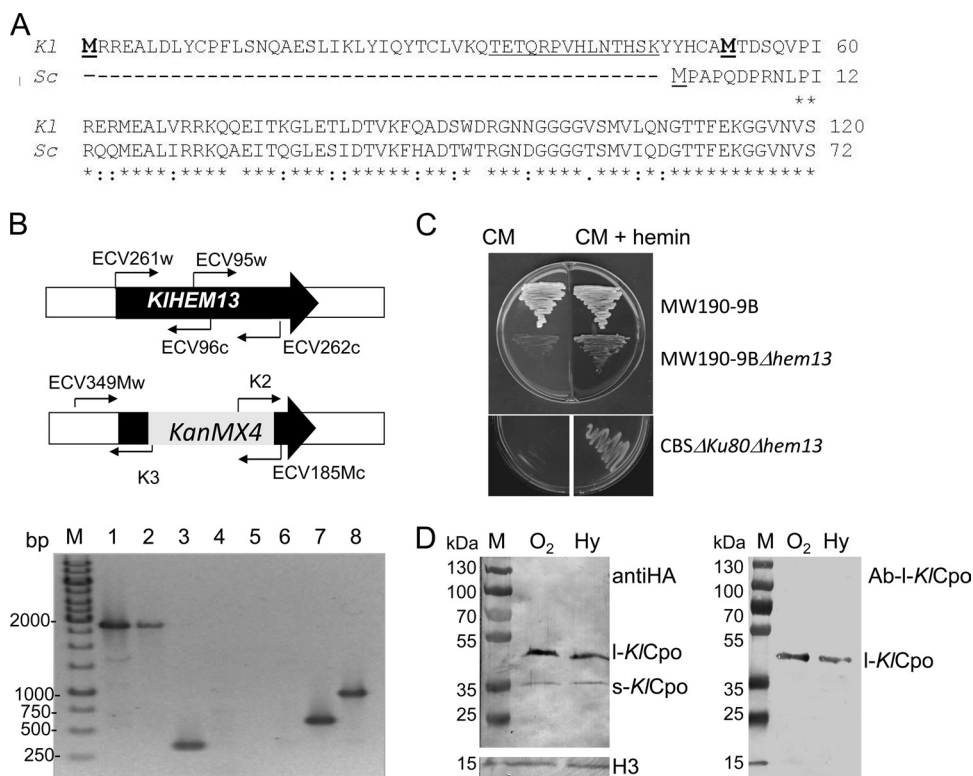


FIG. 1. One gene and two proteins. (A) Alignment of the N-terminal regions of the ORFs translated from the *KIHEM13* (*KI*) and *ScHEM13* (*Sc*) genes. The first and second in-frame Mets from the *K. lactis* protein are boldface and underlined. (B) Verification of the null strains. (Top) Schemes showing the primers used. (Bottom) PCR amplifications from MW190-9B (lanes 1, 3, 5, and 6) or MW190-9B- $\Delta$ *hem13* (lanes 2, 4, 7, and 8) DNA with primers ECV261w and ECV262c (lanes 1 and 2), ECV95w and ECV96c (lanes 3 and 4), ECV349Mw and K3 (lanes 5 and 7), or K2 and ECV185Mc (lanes 6 and 8). Lane M, 1-kbp GeneRuler (Fermentas). (C) Growth of wild-type and  $\Delta$ *Klhem13* strains in complete medium (CM) alone or supplemented with hemin. (D) Western blot of proteins expressed in MW190-9B from the pSK1-*KIHEM13*-3HA construct C-terminally tagged with HA and hybridized with anti-HA (left) or Ab-I-K/Cpo, which is directed against the peptide underlined at the N terminus in panel A (right). Hybridization with ab21054, an HRP-conjugated anti-histone H3 antibody from Abcam (H3), was used as a loading control. I-K/Cpo, protein translated from the first TIC; s-K/Cpo, protein translated from the second TIC. The strains were grown under aerobiosis (O<sub>2</sub>) or hypoxia (Hy). The molecular sizes of the proteins used as molecular markers (PageRuler prestained protein ladder; Fermentas) are given to the left of each panel.

**Construction of the *K. lactis*  $\Delta$ *hem13* strains.** The *KIHEM13* gene was amplified from MW190-9B genomic DNA using PCR with primers ECV184Mw (gggggtacc TTTTTGGCATGGAAGGAAGG, at position -1025) and ECV185Mc (gggggtacc TAGGTGGAGAAGGTGCATGGG, at position +1751) and was cloned into the KpnI site of the YIplac204 vector (9). This construct was used as a template for inverse PCR carried out with the divergent primers ECV250Mc (ggggcgccgcGG CTAGCTAGTTCCACCG, at position -129) and ECV251Mw (ggggcgccgcGG CCGTCGACAATACTTTT, at position +1253). Thus, after religation, deletion of the ORF was obtained. A NotI site included in the primers allowed the *kanMX4* cassette for Geneticin (G418) selection to be introduced (28). This construct, YIplac204-*Klhem13::kanMX4*, was used as a PCR template to obtain a linear fragment containing the marker for G418 resistance and two flanking regions of homology to the *K. lactis* genome using primers ECV261Mw (GGAAGGAACGC GATCTTCAGC, annealing at position -1014) and ECV262Mc (CAGCAGCAC AATGTCCGAACC, annealing at position +1715). *K. lactis* CBSΔKU80 and MW190-9B cells were transformed using this cassette by following the procedure of Ito et al. (13). Two rounds of selection in YPD-G418 (40 μg/ml) were carried out to eliminate false positives. Correct integration into the *K. lactis* genome was verified using PCR as described previously (26). Genomic DNA samples isolated from parental strains and their respective *K. lactis*  $\Delta$ *hem13* mutants were used as templates for amplification with sets of primers designed to test the genotype. Internal primers K2 (CGCCTTAATTAACCGGGGAT) and K3 (GCCTCGACATCAT CTGCCAG) were designed for annealing inside the *kanMX4* cassette. In order to verify correct homologous integration, external primers ECV185Mc (described above) and ECV349Mw (ggggagctcACGACGAAGTTTGTATTATTTTTTTT, at position -1046) were designed flanking *KIHEM13*, but external to the regions of homology used for the recombination event. Amplification with each pair of exter-

nal/internal primers produced PCR products of the expected sizes. The expected size of the fragment obtained with ECV261w and ECV262c (described above) and the absence of amplification of an internal fragment of the ORF were tested with primers ECV95w (GGACGTTGCCTTGATACCCAAA, at position +687) and ECV96c (GACACGGGATCCTGGAGTTC, at position +1008). Further verifications of null phenotypes were accomplished using Cpo enzymatic assays and phenotypic analyses of the growth defect in CM in the absence of hemin.

**Cell fractionation.** *K. lactis* cells ( $\Delta$ *hem13* and wild type) were grown in heme-supplemented medium as described above. Cells were harvested at an optical density at 600 nm (OD<sub>600</sub>) of 2, washed (to remove adsorbed heme), and broken with glass beads (diameter, 0.45 mm) in 0.1 M potassium phosphate buffer, pH 7.2. The cell extract was centrifuged for 5 min at 3,000 × g to remove intact cells. The supernatant is referred to as the homogenate (H). An aliquot of the homogenate was centrifuged for 30 min at 45,000 × g to sediment membranous fractions containing nuclei (MF). The supernatant was referred to as the "soluble fraction" (SF). The pellet (the MF) was resuspended in potassium phosphate buffer and was solubilized by sonication.

**Cpo activity.** Cpo activities were measured as described previously (18) on a Photon Technology International (PTI) spectrofluorometer in 1-ml cuvettes at 30°C. Tween 80, EDTA, dithiothreitol (DTT), and freshly prepared coproporphyrinogen solutions were added as described previously (18). The coupling enzyme was highly purified *S. cerevisiae* protoporphyrinogen oxidase (100 U/assay). The excitation wavelength was 410 nm, and the emission wavelength was 632 nm. One unit of Cpo activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of protoporphyrinogen per hour at 30°C under standard conditions.

**C-terminal tagging of *KIcPo*.** *KIHEM13* was modified using the pFA6a-3HA-*kanMX6* cassette (20). The 3HA-*kanMX6* cassette was amplified using PCR with two primers, ECV436Mw (CAAGTATTGAAGAACCCTGTTGAATGGGTTcggatccccgggtaattaa) and ECV437Mc (ATACATGAGACGTTACATAAATAAAGGCATgaattcgagctgtttaaac). These primers were designed to fuse the *KIHEM13* ORF in frame to a DNA sequence encoding three copies of the hemagglutinin (HA) epitope. They had regions of homology to the pFA6a-3HA-*kanMX6* cassette (lowercase letters in sequences) and to *KIHEM13* (capital letters), near a BsmI restriction site at position +1127 that overlapped with the TGA stop codon of *KIHEM13*. Plasmid pSK1-*KIHEM13* (2) was linearized by partial digestion with BsmI and was used together with the 3HA-*kanMX6* PCR product to cotransform the *S. cerevisiae* strain BY4741. The transformed cells were selected in CM without uracil (CM-Ura) and in CM-Ura plates with 40 µg/ml G418. Growth in CM-Ura selected cells carrying plasmid pSK1-*KIHEM13*, and growth in media with G418 selected recombinant plasmids carrying the tagged protein. The recombinant plasmid was isolated from the transformed BY4741 cells and was amplified in DH10B bacteria by using LB medium supplemented with kanamycin (20 µg/ml) for selection. After verification by sequencing, plasmid pSK1-*KIHEM13*-3HA was used to transform the *K. lactis* strain MW190-9B.

**Protein extracts.** Protein extracts for Western blotting and chromatin immunoprecipitation (ChIP) assays were obtained in phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.144% Na<sub>2</sub>HPO<sub>4</sub>, 0.024% KH<sub>2</sub>PO<sub>4</sub> [pH 7.4]), and those for the measurement of β-galactosidase activity were obtained in Z-Buffer as described previously (2). Protein concentrations were determined using the method of Bradford (3).

**Western blotting.** Western blotting and control of loading were carried out as described previously (1). The HA probe (F-7) (sc-7392; Santa Cruz Biotechnology, Inc.), directed against the HA-tagged proteins (l-*KIcPo* and s-*KIcPo*) at their C termini, was used according to the manufacturer's instructions. A polyclonal probe specific for l-*KIcPo*, directed to its N terminus (Ab-l-*KIcPo*), was obtained by chemical synthesis of the TETQRPVHLNTHSK peptide (positions T34 to K47) and rabbit immunization (at the facilities of GenScript USA Inc.). Membranes were incubated with a 1:325 dilution of Ab-l-*KIcPo* in PBS with 0.25% nonfat dry milk and 0.1% Tween 20 at room temperature for 25 min. The secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology, Inc.), was diluted 1:200 in PBS with 0.1% Tween 20, and membranes were incubated at room temperature for 25 min. An HRP-conjugated antibody against human histone H3 (ab21054; Abcam) was used as a loading control, since it cross-reacts with the homologous protein from *K. lactis*. The immune complexes on the membrane were visualized using diaminobenzidine (Sigma-Aldrich Co.) and H<sub>2</sub>O<sub>2</sub>.

**Protein identification by mass spectrometry.** Samples were analyzed using matrix-assisted laser desorption–tandem time of flight (MALDI-TOF/TOF) mass spectrometry by following procedures described previously (24). Peptides were identified using ProteinPilot software, version 4.0 (AB Sciex Pte. Ltd.).

**RT-PCR.** RNA was isolated from MW190-9B cells grown in CM to an OD<sub>600</sub> of 0.8 with the Aurum Total RNA Mini kit (Bio-Rad). To ensure that the RNA preparation was DNA free, it was treated with RQ1 RNase-free DNase (Promega) according to the manufacturer's instructions. After this treatment, the absence of DNA was verified by the failure to amplify a control region without the addition of reverse transcriptase (RT). The cDNA was obtained by using 1 ng of RNA, 2 pmol of primer oligo(dT)<sub>18</sub>, and the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. After cDNA synthesis, primer ECV550Mc (ATTTACTCCTGTTGCATCTG, annealing at position +475) and forward primers ECV551Mw (ACTGAAATGAGAAGAG AAGCA, annealing at position –6) and ECV548Mw (TGGTAAAGCAAACA GAAACT, at position +108) were used for PCR amplification of the corresponding fragments, including TIC-1.

**Determination of tss using primer extension.** Total RNA (60 µg) was incubated at 70°C for 10 min with 0.5 µg of primer ECV300Mc (ATGTAAAGTTT TATCAATGATTCTGC, at position +74) or primer ECV405Mc (AGTATCC AAAGTTTCCAGAC, at position +252) in two independent reactions. The mixtures were cooled briefly on ice and were reverse transcribed with the SuperScript II reverse transcriptase kit according to the manufacturer's protocol (Invitrogen) by using [α-<sup>32</sup>P]dATP for labeling. After cDNA synthesis, RNA was hydrolyzed by alkali treatment. The cDNA was precipitated with an equal volume of isopropanol at –80°C for 2 h and was pelleted by centrifugation at 13,000 rpm for 30 min. The purified cDNA samples were dissolved in 10 µl water. Two sequencing reactions were carried out using the Sanger method on template DNA from the pSK1-*KIHEM13* clone, and the same primer was used for the primer extension reaction. Reaction mixtures were loaded onto a 7% polyacryl-

amide gel and were exposed to screens for visualization in the PhosphorImager system (Pharmacia Biotech) after electrophoresis and vacuum drying.

**Fusion of *KIHEM13* promoter regions to the *lacZ* reporter gene and measurement of β-galactosidase activity.** The upstream region of *KIHEM13* from –1025 to +14 was amplified with ECV163Mw (gggggatccTTTTTTGGCATGGAAGG AACG) and ECV154c (gtcgacGCTTCTCTTCATTTTCAGTT), and after digestion with BamHI and SalI, the product was cloned into the pXW1 vector (5) upstream of, and in frame with, the *lacZ* reporter gene. The upstream *KIHEM13* region including the sequence between TIC-1 and TIC-2 (to position +190) was cloned into the pXW1 polylinker in a similar manner, but using the reverse primer ECV394Mc (gggggaagcTTCTCTCTAATTGGCACTTGTG), which contains a HindIII site for cloning. The upstream region of *KIHEM13* from –494 to +190 was amplified with ECV396Mw (gggggtcgaccattcatctgtaactgcaacc) and ECV394Mc. These constructs, named pTIC-1-*lacZ*, pTIC-2-*lacZ*, and pTIC-2<sub>494</sub>-*lacZ*, respectively, were verified by sequencing and were used to transform *K. lactis* strain MW190-9B and its *Δhem13* derivative (this work) as required. β-Galactosidase activity was measured at 30°C using protein extracts (as described above) in four replicates. Average values ± standard deviations are presented. Values are presented as nmol of *ortho*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg protein.

***KIHEM13* constructs in pCXJ18.** Constructs of *KIHEM13* expressed from its own promoter and terminator regions, or containing only the desired ORF (starting from TIC-1 or TIC-2) but under the control of heterologous promoter and terminator regions, were prepared in the *K. lactis* centromeric (*KICEN2*) vector pCXJ18 (4). The pCXJ18-*KIHEM13* construct was obtained from an available *KIHEM13* clone; *KIHEM13*, including its own promoter and terminator regions, was digested with HindIII and BamHI and was inserted into the pCXJ18 polylinker. The pCXJ18-l-*KIcPo* and pCXJ18-s-*KIcPo* constructs were obtained as follows. Two DNA sequences, including the ORF from the first (from +1 to +1126) or second (from +157 to +1126) in-frame ATG start codon, respectively, were amplified from genomic DNA obtained from the *K. lactis* strain MW190-9B using PCR. In both cases, the amplified DNA sequences contained BsmFI and NotI restriction sites (included in the design of the primers) at the 5' and 3' ends, respectively, and were used to insert the clones into the pKLAC1 vector (New England Biolabs Inc.). These constructs were amplified using PCR with primers ECV694AVc (gggggtcgacGAAGTCCATATTTGACACCCGG) and pKLAC17391w (gggggatcaATTAAGTTGGGTAACGCCAGG), containing HindIII and BamHI sites, respectively. The PCR products were digested with HindIII and BamHI and were cloned into the same sites of the pCXJ18 vector. In these constructs, transcription was under the control of the *LAC4* promoter and the *LAC4* terminator from the pKLAC1 vector. All the constructs were verified by sequencing.

**Directed mutagenesis.** Plasmid pSK1-*KIHEM13*-3HA was subjected to directed mutagenesis in order to eliminate the codon for the second in-frame Met present in *KIcPo*. For directed mutagenesis, the Megaprimer PCR protocol was used (27). The mutagenic oligonucleotide AVV019w (ACATTCTAAATATTA TCATTGTGCGcGACAGATTCACAAGTGCCAAATTAGA), for changing Met to Ala, and ECV120Mc (AGGCGAAGAAATTTACTCCTG) were used to obtain the megaprimer, and PCR mutagenesis was carried out by our laboratory protocol using Dream-*Taq* (Fermentas). After the PCRs, products were first treated with DpnI and then transformed into *Escherichia coli* ECOS Blue (Yeastern Biotech Co.), and the DNA was amplified with the GeneJET Plasmid Miniprep kit (Fermentas). After the transformation of MW190-9B yeast cells, the absence of s-*KIcPo* production from this construct was verified by Western blotting.

**ChIP assays.** Protein extracts were prepared from cells of strain MW190-9B transformed with plasmid pSK1-*KIHEM13*-3HA or with its derivative pSK1-*KIHEM13*-3HA-2TIC mutant, obtained by mutagenesis, and were grown in the selective medium CM-Ura under aerobic or hypoxic conditions. ChIP assays were carried as described previously (19) using magnetic Dynabeads (Invitrogen S.A.) according to the manufacturer's instructions and anti-HA (sc-7392; Santa Cruz Biotechnology, Inc.) for precipitation of the HA-tagged protein. Each assay was carried out in duplicate from two independent cultures.

The promoter region of *KIHEM13*, from –494 to –200 and from –434 to –200, was amplified with primers ECV396Mw (gggggtcgacCATTTCATCGTA ACTGCAACC) and ECV555Mc (gggggtCGACAGTCCGAGACCACAAA GATGAATA) or primers ECV554Mw (ggggggatccCGATAGCCTTTTCTTG ACCCAATT) and ECV555Mc, respectively. Negative controls for amplification from the HA immunoprecipitates were included. The primers used, AVV026 (GCCTGTACCACAGGTTTCG-3') and AVV027 (AGAGTTTCACACAAT TTCCGC), were designed to amplify a region of the promoter of the unrelated KLLA0E17623g ORF.

## RESULTS

**Only one gene encodes Cpo in *K. lactis*.** A null allele of *KIHEM13* was constructed in the *K. lactis* haploid strains CBS $\Delta$ KU80 and MW190-9B. Correct integration of the disruption cassette was verified and is demonstrated for strain MW190-9B (Fig. 1B). Similar results were obtained with CBS $\Delta$ KU80 (data not shown). Both  $\Delta$ *KIhem13* null strains were unable to grow in the absence of hemin (Fig. 1C). Cpo enzymatic activity was determined in CBS $\Delta$ KU80 and its CBS $\Delta$ KU80- $\Delta$ *KIhem13* derivative using fluorometric assays (18). Cpo enzymatic activity was absent in the CBS $\Delta$ KU80- $\Delta$ *KIhem13* strain. Cpo activity in the strain with wild-type *HEM13*, CBS $\Delta$ KU80, was detected only in the soluble fraction ( $40 \pm 4$  U/ $\mu$ g protein), not in the membranous fraction. This result is in agreement with the subcellular location of Cpo activity in the soluble cytosolic fraction of *K. lactis* cells, as reported for *S. cerevisiae* (17). Analysis of the complete sequence of the *K. lactis* genome with BLAST tools at Génolevures (<http://www.genolevures.org>) yielded no other *K. lactis* ORFs with homology to *HEM13* genes. These data confirm that only one gene, *KIHEM13* (KLLA0F18546g), encodes a functional Cpo enzyme in *K. lactis*.

**Two *KICpo* proteins are translated from *KIHEM13*.** In order to ascertain which of the two in-frame TICs present in *KIHEM13* was used for translation, a recombinant *K. lactis* strain expressing *KICpo* tagged at the C terminus with three copies of HA was constructed. After growth of this strain under aerobic or hypoxic conditions, protein extracts were obtained; 30  $\mu$ g of total protein was fractionated using electrophoresis in 12% polyacrylamide gel electrophoresis (PAGE) gels, and Western blotting was performed with an HA-specific primary antibody. Two proteins with different  $M_r$ s were obtained (Fig. 1D). The  $M_r$  calculated for the slowly migrating band had the expected size for the *KICPO*-3HA construct translated from TIC-1 (*l-KICpo*). The  $M_r$  calculated for the rapidly migrating band was coincident with that expected for the protein translated from TIC-2 (*s-KICpo*). In order to verify the nature of these two proteins, the corresponding bands were fractionated by PAGE, excised from the gel, and analyzed using MALDI-TOF/TOF. The sequences of the internal peptides obtained confirmed that the two proteins were *KICpo*, but peptides from the N terminus and C terminus were not present in the pools detected, and therefore it was not possible to verify by these methods whether the proteins differed in their N-terminal regions. In order to test whether *l-KICpo* contained the N-terminal region corresponding to translation from TIC-1 and whether *s-KICpo* lacked this region, Western blotting was performed using a specific antibody (Ab-*l-KICpo*) directed against a peptide encoded by the region between the first and second TICs (underlined in Fig. 1A). The results (Fig. 1D) revealed that this antibody interacts with *l-KICpo* but not with *s-KICpo*. Considering that (i) both proteins contained the C-terminal tag, (ii) only *l-KICpo* interacted with Ab-*l-KICpo*, (iii) experimentally calculated  $M_r$ s were as expected from TIC-1 and TIC-2 translation, and (iv) no introns could be identified when *KIHEM13* was analyzed using the yeast intron program EXPLORA (14), it was concluded that the two *KICpo* proteins detected were derived from translation initiation from TIC-1 (*l-KICpo*) and TIC-2 (*s-KICpo*). The production of different proteins by translation from differ-

ent TICs has been described previously in eukaryotic systems, including yeasts (21, 22).

***KIHEM13* is transcribed from two different tss pools, and selection of a specific tss depends on the availability of oxygen and heme.** To better characterize the promoter regions in the 5' upstream region of *KIHEM13* and their relative positions to the TICs, the precise positions of tss on the *KIHEM13* promoter were determined using primer extension. The data (Fig. 2) confirmed the presence of two tss pools. One pool (tss-1) was located 5' of TIC-1 and included three weak starts at G positions -87, -80, and -69. A second tss pool (tss-2), between TIC-1 and TIC-2, included two strong starts at C positions +46 and +56 (positions -111 and -101 if the relative positions from TIC-2, which would be the only possible starting AUG codon for an mRNA transcribed from this tss-2 pool in this case, are considered). The positions of the determined tss relative to the TICs are represented on the *KIHEM13* sequence (Fig. 2B). The tss pools were detected during aerobiosis or hypoxia. However, the tss at position -69 in the tss-1 pool is specific for hypoxia and disappeared when hemin was added (Fig. 2A). The long transcript could be produced to a minor extent from tss-1 under both aerobic and hypoxic conditions. The short transcript would be more abundant than the larger transcript under both conditions, but production of the long transcript would increase during hypoxia owing to heme-regulated use of the tss at the -69 position (Fig. 2C).

In order to confirm the functionality of these tss pools, total RNA was isolated from *K. lactis* cells grown under aerobiosis or hypoxia, and cDNA was obtained. This cDNA was used as a template in PCR amplifications using two pairs of primers, ECV551Mw/ECV550Mc and ECV548Mw/ECV550Mc. The long transcript, starting 5' of TIC-1, is present in the total RNA isolated from *K. lactis* cells under aerobic and hypoxic conditions (Fig. 3A).

The upstream regions of *KIHEM13* from -1025 to TIC-1 (pTIC-1-*lacZ*) and to TIC-2 (pTIC-2-*lacZ*) were fused with the reporter gene *lacZ*, and  $\beta$ -galactosidase activity in protein extracts obtained from transformed *K. lactis* cells after aerobic and hypoxic growth was measured. The results presented in Fig. 3B indicate that the sequence located upstream of TIC-1 had poor activity in terms of promoting the expression of the reporter gene. When this sequence was extended 3' of TIC-2, its promoter activity increased by 3 orders of magnitude. From these data it was concluded that the importance of the region between TIC-1 and TIC-2 in the control of *KIHEM13* transcription, under aerobic and hypoxic conditions, is due to the presence of two major initiation sites in the tss-2 pool. However, minimal transcription is possible through the tss-1 pool in the region upstream of TIC-1. These results are compatible with the use of alternative tss to generate two *KIHEM13* transcripts differing at their 5' termini.

***s-KICpo* but not *l-KICpo* expression complements the heme deficiency of the  $\Delta$ *KIhem13* mutant strain.** The results indicated that the two proteins, *l-KICpo* and *s-KICpo*, were obtained from the single gene *KIHEM13*. Therefore, the question of whether the two proteins were functionally equivalent was investigated. Constructs that expressed only one of the two proteins were obtained from a heterologous *LAC4* promoter, avoiding the co-expression caused by the use of the native *KIHEM13* promoter. The pCXJ18-*l-KICpo* (expressing *l-KICpo*) and pCXJ18-*s-*

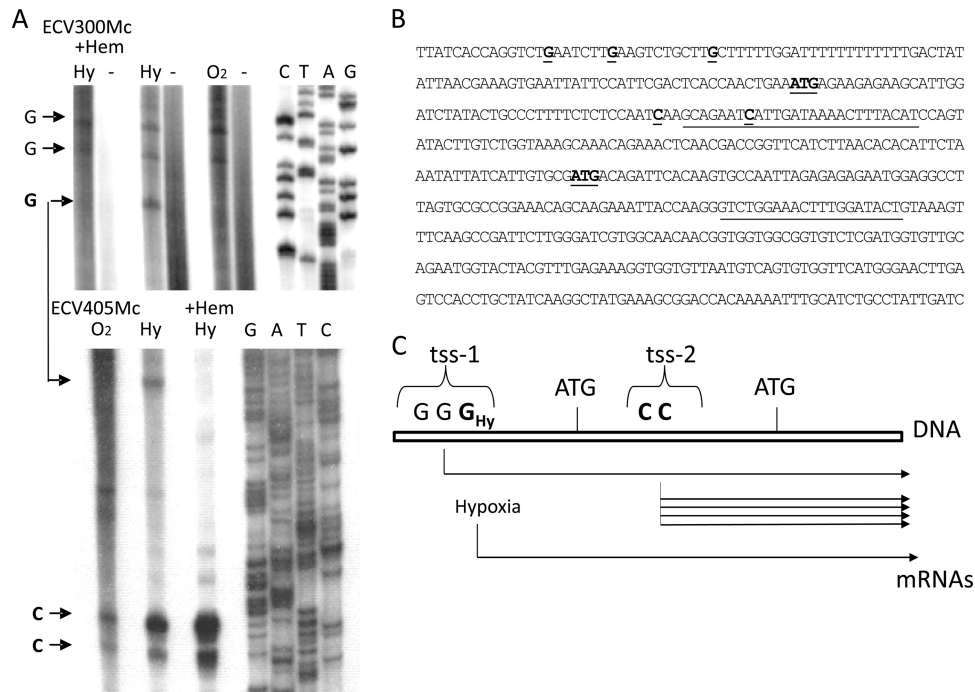


FIG. 2. Determination of transcriptional start sites in the *KIHEM13* upstream region. (A) Primer extension analysis. Aerobic or hypoxic conditions are indicated as in Fig. 1. +Hem, medium supplemented with hemin, -, negative control. (B) Sequence of the upstream region of *KIHEM13* and the ORF (partial). The positions of primers ECV300Mc and ECV405Mc are underlined. The ATGs of the first and second TICs and the experimentally determined tss are underlined. (C) Scheme showing the relative positions and use of tss pools and TICs.

*KICpo* (expressing *s-KICpo*) constructs and the control pCXJ18-*KIHEM13* construct (expressing both *l-KICpo* and *s-KICpo*) are described in Materials and Methods. These plasmids were used to transform the MW190-9B and MW190-9B- $\Delta hem13$  strains, and the selected transformed cells were plated on the selective synthetic medium CM-Ura with galactose as a carbon source in the absence or presence of hemin. Complementation of the heme deficiency of the  $\Delta hem13$  mutant strain implies that the protein expressed from the plasmid construct has Cpo activity. This was evident with the pCXJ18-*s-KICpo* construct and the control pCXJ18-*KIHEM13* construct but not with pCXJ18-*l-KICpo*. Therefore, only *s-KICpo* complements the mutant phenotype (Fig. 4A). However, despite the lack of complementation, expression of *l-KICpo* from the pCXJ18-*KIHEM13* and pCXJ18-*l-KICpo* constructs in the  $\Delta hem13$  strain was confirmed using Western blotting with the *l-KICpo*-specific antibody (Fig. 4B). In conclusion, *l-KICpo* is expressed from these constructs but lacks Cpo activity.

**Autoregulation of the *KIHEM13* promoter.** The *l-KICpo* protein does not complement the heme deficiency of a  $\Delta Kihem13$  mutant strain (Fig. 4A). In addition, Cpo activity in the *K. lactis* wild-type strain was present only in the cytosolic soluble fraction (SF). These data excluded the possibility that *s-KICpo* could be present in the membranous fraction that contains the nuclei (MF) but did not exclude the possibility that *l-KICpo* could be present in this fraction, since it has no catalytic activity. Western blotting was carried out with protein extracts from both fractions, using anti-HA and the specific antibody Ab-*l-KICpo*. The data presented in Fig. 5A demonstrate that *l-KICpo* is found in both the soluble and membranous frac-

tions, while *s-KICpo* is present only in the soluble fraction. As expected, histone H3 was detected in the total extract (H) and the MF but not in the SF.

In order to explore whether *l-KICpo* could have a nuclear function and regulate *KIHEM13* transcription, the interaction between the protein and the promoter was investigated *in vivo* using ChIP. The *K. lactis* strain MW190-9B was transformed with pSK1-*KIHEM13*-HA; protein extracts were obtained after aerobic or hypoxic growth; and ChIP assays were performed. The results revealed that under hypoxic conditions, an HA-tagged immune-precipitated protein was complexed with DNA in a region that extended from -494 to -200 in the *KIHEM13* promoter and with a smaller subregion from -434 to -200 in the same promoter (Fig. 5B, lanes 8). To prove unambiguously that *l-KICpo*, and not *s-KICpo*, was binding to the *KIHEM13* promoter, a mutant *KIHEM13* gene without the ATG codon for the second in-frame Met was obtained (pSK1-*KIHEM13*-3HA-2TIC mutant). The *K. lactis* strain MW190-9B was transformed with this mutant construct, and ChIP assays were performed after growth during hypoxic conditions. The results obtained confirm that *l-KICpo* binds to the *KIHEM13* promoter (Fig. 5C).

A functional role for this region of the *KIHEM13* promoter, which is coimmunoprecipitated with the protein in ChIP assays, was evident in terms of autorepression during hypoxia. The promoter region of *KIHEM13* from -494 to +190 was fused in frame to the *lacZ* reporter in the pXW1 plasmid to make the construct pTIC-2<sub>494</sub>-*lacZ*. Cells from the *K. lactis* strain MW190-9B and its  $\Delta Kihem13$  derivative were transformed with this construct, and  $\beta$ -galactosidase activity was

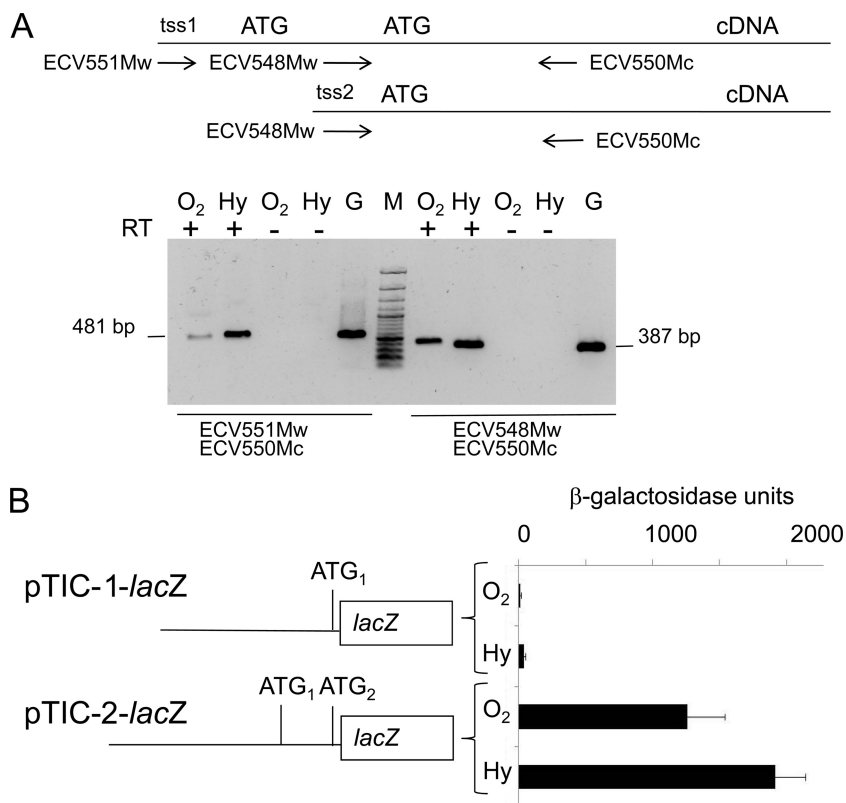


FIG. 3. Analyses of the 5' region of *KIHEM13*. (A) Amplification from cDNAs obtained with oligo(dT) from total mRNA isolated from MW190-9B using the primer pairs shown. Aerobic and hypoxic conditions are indicated as in Fig. 1. G, positive control for amplification, carried out with genomic DNA; RT+ and RT-, reactions with and without the addition of reverse transcriptase, respectively; M, molecular size marker (100-bp GeneRuler; Fermentas). The sizes of the PCR products are indicated. (B) Expression of the *lacZ* reporter gene driven by two *KIHEM13* regions, described in the text and schematized on the left.

determined in protein extracts obtained from cells grown under hypoxic conditions. Comparative analysis of reporter gene activity demonstrated that the expression directed by the *KIHEM13* promoter was lower in strain MW190-9B than in its  $\Delta Kihem13$  derivative (Fig. 5D). These data are compatible with a regulatory feedback control on *KIHEM13* expression by I-KICpo.

## DISCUSSION

Sequence alignment of the protein derived from *KIHEM13* with *S. cerevisiae* Cpo shows the presence of 52 additional amino acids at the N terminus of KICpo. This extension includes the amino acids between two in-frame TICs in the *KIHEM13* ORF. Other proteins from ascomycetes with homology to KICpo accessed through Génolevures (<http://www.genolevures.org/>) do not have similar N-terminal extensions. This N-terminal extension does not contain a known sorting signal for either nuclear localization or other cellular localization as assayed with PSORT (<http://psort.hgc.jp/>).

*KIHEM13* is a single-copy-number gene in *K. lactis*, and Cpo activity depends on its expression (Fig. 1B and C). Two tss pools (tss-1 and tss-2) have been mapped to the 5' region of *KIHEM13* (Fig. 2). The relative positions of the tss pools with reference to the TICs indicate that initiation of transcription from tss-1 would produce mRNA molecules containing TIC-1

and TIC-2, while initiation from tss-2 would produce mRNA molecules containing only TIC-2. Two proteins with different functions, I-KICpo and s-KICpo, are produced from this single gene. The s-KICpo protein has Cpo activity and complements the  $\Delta hem13$  deletion, unlike I-KICpo (Fig. 4). Some evidence suggests that I-KICpo (the only form present in the membranous fraction containing the nuclei) has a regulatory role in terms of transcriptional regulation of *KIHEM13* during hypoxia (Fig. 5). The utilization of multiple tss, frequently very close to one another and forming tss pools, is a frequent event in yeasts, but its functional significance is not fully understood (11). The alternative use of tss has been related to the production of protein isoforms with the same function but different cellular locations. Genes containing more than one in-frame TIC in their 5' regions may give rise to isoforms with different subcellular localizations due to heterogeneous N-terminal ends, depending on which of the AUG codons is used to initiate translation. Often, such genes have alternative transcription start sites, leading to the synthesis of two different mRNAs. For example, the genes coding for the yeast aminoacyl-tRNA synthetases (21) or glutathione reductase (22) use this mechanism. Generally, however, this has not been related to the utilization of alternative promoters or to the production of proteins with various functions, as reported here for *KIHEM13*. To our knowledge, this is the first report of the use of alter-

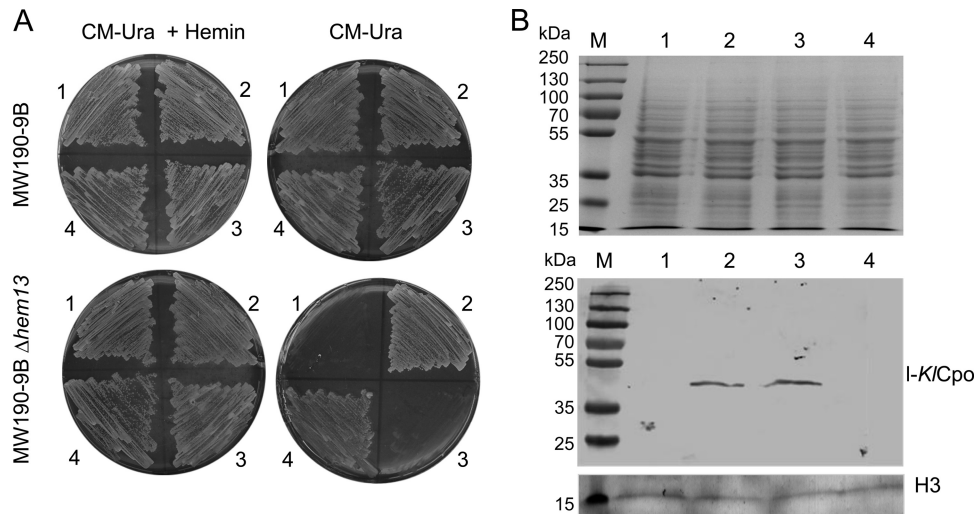


FIG. 4. Test of complementation of the heme deficiency of the MW190-9B- $\Delta$ hem13 strain by l-KICpo and s-KICpo expression. (A) Growth of cells transformed with pCXJ18 (sector 1), pCXJ18-*KIHEM13* (sector 2), pCXJ18-l-KICpo (sector 3), or pCXJ18-s-KICpo (sector 4) in selective medium. The addition of hemin to the medium is indicated (+ Hemin). (B) (Top) Protein-stained gel before blotting. (Bottom) Western blot demonstrating the expression of l-KICpo from the pCXJ18-*KIHEM13* and pCXJ18-l-KICpo constructs. Hybridization with ab21054, an HRP-conjugated anti-histone H3 antibody from Abcam (H3), was used as a loading control. Lane numbers correspond to sector numbers in panel A.

native tss as a mechanism for producing functionally different proteins in budding yeasts. However, this phenomenon has been described in the fission yeast *Schizosaccharomyces pombe*, although in that system one of the two transcripts is poorly translated, and its function remains to be elucidated (25).

Alternative selection of tss as a mechanism for producing different proteins has been described in plants and mammals (6, 15). Examples of regulatory mechanisms based on tss selection have been reported in higher eukaryotes. Start site selection within many murine core promoters differs among tissues, and dynamic usage of tss in these promoters is associated with CpG islands, promoter structures, and imprinting (15). In the *KIHEM13* promoter, transcription from the tss-2 pool results in s-KICpo production under aerobic and hypoxic conditions (Fig. 2A and 3A). This is in accordance with the finding that only s-KICpo is able to complement the heme deficiency caused by *hem13* deletion in *K. lactis* (Fig. 4A). Cpo activity is necessary for cell viability during aerobiosis and hypoxia, and transcription from tss-2 would ensure cell survival under these conditions. The tss at position -69 in the tss-1 pool is specific for hypoxia and disappears in the presence of hemin (Fig. 2A). This feature argues in favor of l-KICpo having a function that is more relevant during hypoxia. This hypothesis is supported by the finding that during hypoxia, a specific complex is formed that contains the tagged protein and a promoter region of *KIHEM13* (delimited from -434 to -200 in our experiments [Fig. 5B and C]), which is also implied in the control of *KIHEM13* expression under this condition (Fig. 5D). However, analysis of the l-KICpo sequence does not reveal any known DNA-binding domains. It is possible that *in vivo*, the interaction of l-KICpo with DNA may be anchored by another, as yet unknown regulatory factor that binds directly to the *KIHEM13* promoter during hypoxia. However, the data presented here do not preclude a direct interaction.

A functional explanation for the repression exerted by l-KICpo on the *KIHEM13* promoter could be that this regulatory

mechanism is directed to avoiding toxicity caused by an excess of Cpo activity that could eventually cause accumulation of the product, protoporphyrinogen IX, and consequently a bottleneck in subsequent steps of the heme biosynthetic pathway. Indeed, this compound accumulates when protoporphyrinogen oxidase, the next enzyme in the biosynthesis of heme, has decreased activity, and this metabolic failure produces variegate porphyria in humans (7).

An intriguing question concerns why the l-KICpo form lacks catalytic activity. There are precedents, proteins in which a terminal extension blocks the catalytic site. For instance, proteases lack enzymatic activity until they are processed. Of course, processing is not the only mechanism suitable for opening the catalytic site, and conformational changes produced by interaction with other protein partners or regulatory molecules have to be considered as well. The crystal structure of *S. cerevisiae* Cpo has been resolved (23), and on the basis of sequence homology, a similar structure is predicted for s-KICpo (data not shown). However, homology modeling of the N-terminal extension present in l-KICpo is not possible, because there are no homologous models for this region. Currently, research is under way to purify and crystallize the two KICpo forms, l-KICpo and s-KICpo, in order to determine their structures and investigate correlations between structure and function.

With regard to the evolutionary origin and selective pressure for conservation of the various functions identified in the l-KICpo and s-KICpo proteins, it can be speculated that the "first" function positively selected in *K. lactis* could be the high catalytic Cpo activity, essential for life and carried by s-KICpo. During hypoxia, *KIHEM13* transcription is significantly increased, and accumulation of protoporphyrinogen IX could become toxic. In this context, a new compatible function that decreased the expression of the catalytically active form would be favorable for selection, and a new function for l-KICpo could be established (Fig. 6). It is interesting to speculate about the mechanism by which this new function could arise. It is

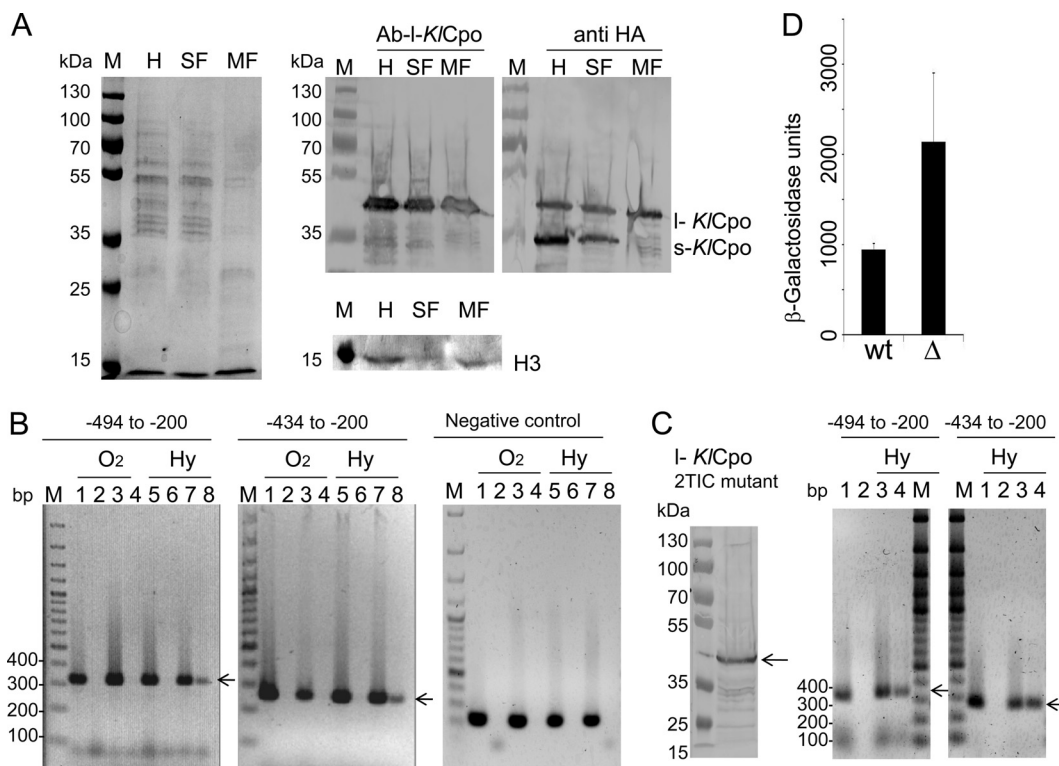


FIG. 5. Nuclear regulatory function of l-KiCpo. (A) (Left) Protein-stained gel before blotting. (Center and right) Western blots showing the expression of l-KiCpo, s-KiCpo, and histone H3 in homogenates from whole-protein extracts (H), from the soluble fraction (SF), and from the membranous fraction containing nuclei (MF), obtained as described in Materials and Methods. The molecular sizes of the proteins used as molecular markers (lanes M) are given to the left. (B) ChIP assays demonstrating *in vivo* binding of the KiCpo protein to different regions of the *KIHEM13* promoter (–494 to –200 and –434 to –200). The negative control shows the absence of binding to the *KIHIS3* promoter. Lanes M, 100-bp GeneRuler (Fermentas). MW190-9B cells that were either left untransformed (lanes 1, 2, 5, and 6) or transformed with the pKS1-HEM13-3HA construct (lanes 3, 4, 7, and 8) were grown under aerobic (O<sub>2</sub>) or hypoxic (Hy) conditions. Odd-numbered lanes, DNA amplification from total input DNA; even-numbered lanes, DNA amplification from specific immunoprecipitates obtained with anti-HA. The primers used for amplification of the two promoter regions are described in the text. (C) (Left) Western blot showing the expression of the l-KiCpo protein in the 2TIC mutant. (Center and right) ChIP assays demonstrating *in vivo* binding of the l-KiCpo protein to regions of the *KIHEM13* promoter during hypoxia. MW190-9B cells were either left untransformed (lanes 1 and 2) or transformed with the pKS1-HEM13-3HA-2TIC mutant construct (lanes 3 and 4). Odd- and even-numbered lanes show DNA amplification as described for panel B. (D) Expression of the *lacZ* reporter gene in MW190-9B (wt) and MW190-9B- $\Delta$ *hem13* ( $\Delta$ ) cells transformed with the pTIC-2<sub>494</sub>-*lacZ* construct described in the text.

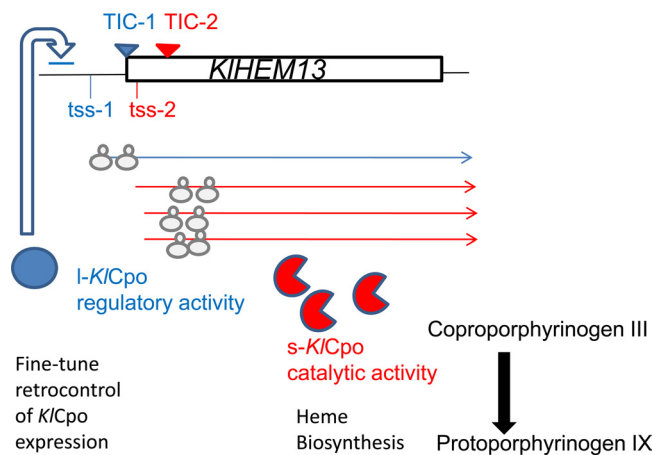


FIG. 6. Hypothetical model of the origins and functions of l-KiCpo and s-KiCpo.

likely that initially the gene was transcribed predominantly from the *tss-2* pool, where the major transcription initiation sites still remain. Therefore, the short transcript that was translated to the catalytically active s-KiCpo would be actively produced. The new heme-regulated use of the transcriptional start site at position –69 in the *tss-1* pool could increase the expression of the long transcript during hypoxia. To understand evolutionary positive selection for the coexistence of these two functions, l-KiCpo expression can be considered to have three advantages. First, it does not exclude s-KiCpo translation. Second, it lacks catalytic activity. Third, it is able to fine-tune hypoxic *KIHEM13* gene expression, preventing the protoporphyrinogen IX excess that could be associated with overproduction of s-KiCpo.

Also of interest is the relationship between these two protein variants in *K. lactis*, l-KiCpo and s-KiCpo, and the phenomenon of moonlighting. Applying the moonlighting criteria (8, 12) to the coexistence of l-KiCpo and s-KiCpo in *K. lactis*, we find two coincidences: (i) they derive from a unique gene (without gene



fusion or alternative splicing) but perform two different functions, and (ii) the two functions are independent in the sense that inactivation of one of the functions, as happens with the catalytic activity in I-KICpo, does not affect the regulatory function. Still, we cannot strictly apply the term “moonlighting” to I-KICpo and s-KICpo, because we are able to fractionate them by PAGE, but we may consider them “moonlighting-like.”

In conclusion, two proteins with different N termini and different functions are derived from the single-copy-number *KIHEM13* gene by a mechanism based on tss selection and the use of two TICs.

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