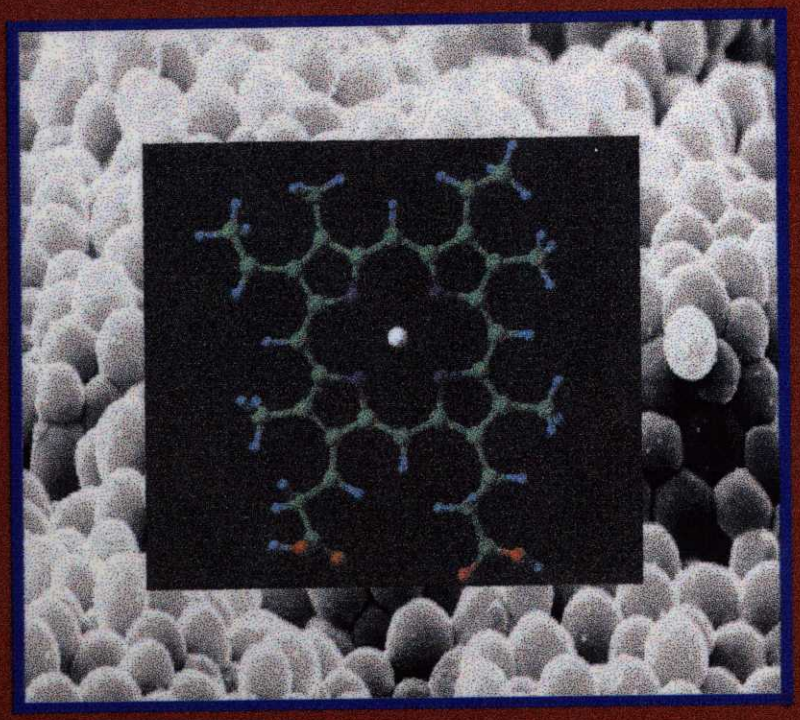

The *HEM1* gene of *Kluyveromyces lactis*

Mónica González Domínguez

COVER

The photo of yeast cells was taken with a Jeol JSM35/C scanning electron microscope (magnification X2000). The heme molecule was retrieved from the NMR structure of the Cytochrome c551. Images are available on the following web sites:
http://www.swam.ac.uk/chemeng/stevep/stevep1.htm (yeast cells)
http://www-nmr.cabm.rutgers.edu/~gardino/heme.html (heme)

FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA CELULAR Y MOLECULAR
UNIVERSIDAD DE LA CORUÑA

EL GEN *HEM1* DE *Kluyveromyces lactis*

Memoria para aspirar al grado de
de Doctor en Biología
presentada por

Mónica González Domínguez

Octubre de 1999

El presente trabajo, **El gen *HEM1* de la levadura *Kluyveromyces lactis* (*The HEM1 gene of Kluyveromyces lactis*)**, presentado por Dña. Mónica González Domínguez para aspirar al grado de Doctor en Biología, ha sido realizado bajo nuestra dirección en el Departamento de Biología Celular y Molecular de la Universidad de A Coruña.

Revisado el texto, estamos conformes con su presentación para ser juzgado.

A Coruña a 22 de Junio de 1999

V°B°
LAS DIRECTORAS DEL TRABAJO
Dra. María Esperanza Cerdán Villanueva
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El viajero acusa la fatiga del tiempo. Pesa hasta la satisfacción que recompensa al caminante sin tregua cuando completa un trecho. Ya no divisa el punto de partida, y ha olvidado los imprevistos de la ruta sorteados con más o menos acierto. Pero su memoria conserva el ejemplo de los que salieron a su paso para allanar el camino.

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El viajero recuerda que hubo caminantes con los que descansó en los cruces del destino.

A Janet, por haber descubierto mis ojos para la vida y la Ciencia (*"Libre, libre, grita la flecha en el aire. Mas no sabe que su destino está signado por la puntería del arquero"*). A Mikel, por abrir la senda que debía seguir (*"El destino es el escenario de nuestra batalla"*). A Silvia, Ainhoa y Raquel, por animar mi paso a fuerza de sentimiento.

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Aún cansado, el viajero se sienta y mira al mar. Sabe que debe reanudar la marcha porque su destino es caminar. Su corazón ya apunta al horizonte, aunque sus pies parecen todavía anclados. Navegará contra viento y marea para encontrar su océano de paz. Y mañana, allá donde se encuentre, nunca olvidará el puerto de donde partió.

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"If I create from the heart,
nearly everything works;
if from the head,
almost nothing".

Marc Chagall

Vincent van Gogh. ***The starry night.*** **1889**

A la memoria de Antonio Domínguez Arcas,
que se fue para quedarse siempre.
Que tu estrella sea mi Norte en las
noches sin luz.

Tu nieta

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ABBREVIATIONS

Acetyl CoA: acetyl Coa
ADP: adenosine 5'-triphosphate
ALA: 5-aminolevulinic acid
Ala: alanine
ALAS: 5-aminolevulinic acid synthase
ATCC: American Type Culture Collection
ATP: adenosine 5'-triphosphate
BCIP: 5-bromo-4-chloro-3-indolyl phosphate
BSA: bovine serum albumin
CM: complete medium
Coprogen: coproporphyrinogen
Cys: cysteine
Da: dalton
Dig: digoxigenin
Dnase: deoxyribonuclease
DTT: dithiothreitol
dNTP: deoxynucleoside triphosphate
EDTA: ethylenediaminetetraacetic acid
EMBL: European Molecular Biology Laboratory
GDP: guanosine 5'-diphospahte
GTP: guanosine 5'-triphosphate
HSP: heat shock protein
IPTG: isopropyl-1-thio-β-D-galactoside
kb: kilobase
MOPS: 3-(N-morpholino)propane sulfonic acid
mRNA: messenger ribonucleic acid
Mw: molecular weight
NAD: nicotinamide adenine dinucleotide
NBT: nitroblue tetrazolium
NCBI: National Center for Biotechnology Information
nm: nanometers
nt: nucleotides
O.D.: optical density
ORF: open reading frame
PAGE: polyacrilamide gel electrophoresis
PBG: porphobilinogen
PMSF: phenylmethylsulfoside
pI: isoelectric point
Pro: proline
Protogen: protoporphyrinogen
rRNA: ribosomal ribonucleic acid
Proto: Protoporphyrin
Pwo: *Pyrococcus woesei*
SDS: sodium dodecyl sulfate
SSC: sodium chloride/sodium citrate (buffer)
Taq: *Thermus aquaticus*

TBS: Tris-buffered saline
TCA: trichloroacetic acid
Tris: tris(hydroximethyl)aminomethane
Urogen: uroporphyrinogen
UV: ultraviolet
YEp: yeast episomal plasmid
YIP: yeast integrative plasmid

INTRODUCTION

1.1. BIOLOGICAL IMPORTANCE OF HEME

1.1.1. Coming back to the origin: heme, a ferroprotoporphyrin

Heme is the generic term to define the ferroprotoporphyrin IX, regardless of the oxidation state of the chelated iron. Porphyrins are compounds of undoubted biological importance. Along evolution, abiotic formation of porphyrins provided the first pigments necessary for the eventual synthesis of chlorophylls, and consequently facilitated the emergence of simple photosynthetic organisms on the primordial earth.

The porphyrin nucleus consists of a macrocycle of four pyrrole rings linked by four methene bridges (Fig. 1.1). Eight side chains can be attached, giving each porphyrin its remarkable physical characteristics. An important feature of this complex ring is its metal-binding capability, and this metal (magnesium in chlorophyll and iron in hemes) confers the biological importance to the porphyrin. Porphyrins are essential for life in the biosphere: i)chlorophylls allow the conversion of solar radiation to chemical energy through photosynthesis; ii)hemes are the prosthetic groups of cytochromes which transport electrons along the respiratory chains for a coupled synthesis of ATP.

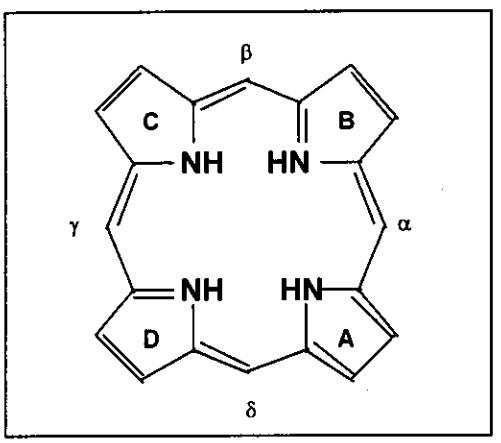

Figure 1.1. The structure of porphyrin macrocycle. The pyrrole rings are designated as A, B, C and D, and the four methene bridges are indicated as α, β, γ and δ.

1.1.2. Heme dynamics in the cell

Apart from serving as a prosthetic group in globins, catalases and cytochromes, heme fulfils different roles as an effector molecule in systems that utilise oxygen. There is an important number of biological processes influenced by heme, and therefore its synthesis, distribution and degradation are closely controlled.

Heme biosynthesis is regulated by feed-back mechanisms that prevents the highly deleterious over-accumulation of this metabolite. Once synthesised in the mitochondria, heme must be distributed to other cellular compartments including microsomes, peroxisomes, cytosol and nucleus. Taking into account the high affinity of heme for proteins and lipids, it has been suggested that a carrier may mediate this sorting. No clear picture on the intracellular trafficking of heme has been drawn so far, and the existence of a free-heme pool is under debate. The affinity and concentration of heme-binding molecules *in vivo* would preclude the existence of such a pool (Smith, 1990). Supposing that heme might be free, at least transiently in the course of incorporation into proteins, its contribution to regulation remains doubtful (Brown *et al.*, 1990). Further research is needed to elucidate the pathways followed by the newly synthesised heme until its final locations inside cells.

Heme degradation has also been widely studied (Brown *et al.*, 1990). Although the macrocycle ring is very stable, oxidative breakdown of this structure occurs in both non-biological and biological systems. In higher eukaryotes, the enzyme heme oxygenase catalyses heme breakdown yielding the bile pigments, biliverdin and bilirrubin. In plants, the bile pigments form part of phycobiliproteins such as the phytochrome and the phycocianin. In yeast, devoid of any heme-oxygenase activity, the decay of heme levels would stem from dilution, as cell mass increases during growth in anaerobiosis.

1.1.3. Heme, a multifunctional regulator

Heme not only determines the structure and activity of hemoproteins, but also controls the biosynthesis of many of them intervening at different levels (Fig. 1.2). The functions of heme are largely conserved from yeast to humans, and recent evidence indicates that heme might regulate diverse processes through similar mechanisms (Lathrop and Timko, 1993; Zhang and Guarente, 1995).

Section 1.3 will deal with the effects of heme on transcription in *Saccharomyces cerevisiae*. Other actions related to protein translation and translocation will be presented herein.

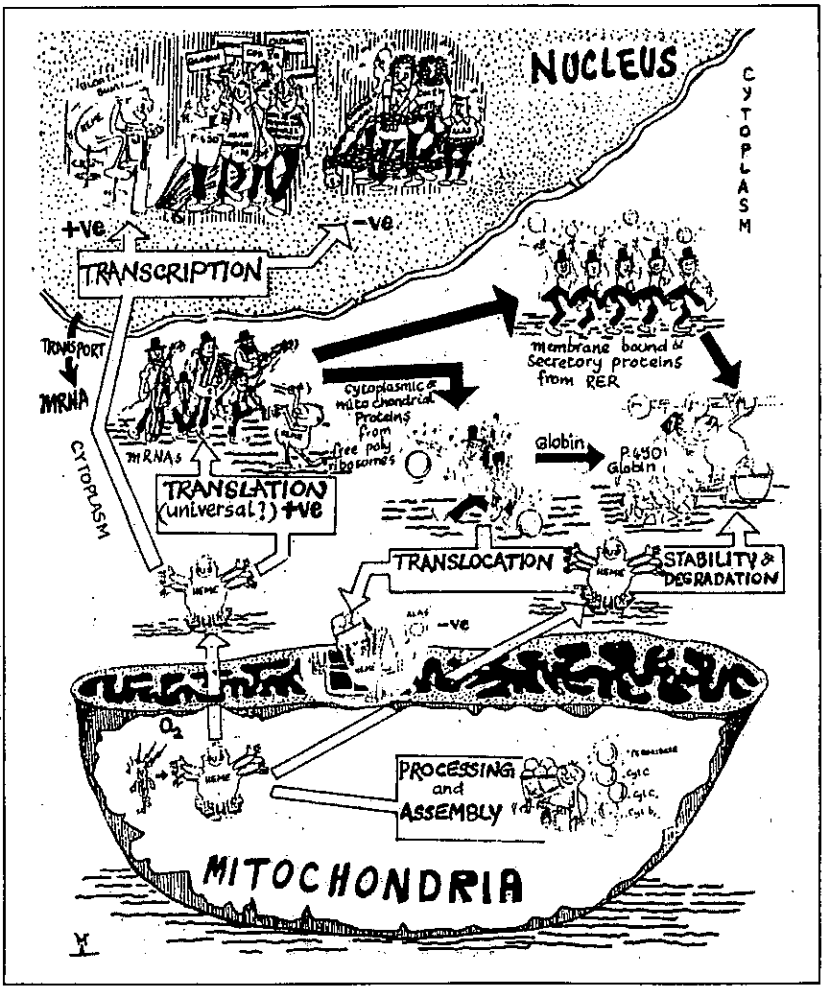

Figure 1.2. Functional versatility of heme. Once synthesised in the mitochondria, heme reaches different cellular compartments where it regulates diverse processes related to oxygen consumption (adapted from Padmanaban *et al.*, 1989).

Phosphorylation of the eukaryotic factor eIF-2 represents an important mechanism for the general regulation of the initiation of translation. Among the eIF-2 kinases characterised so far is HRI (Heme-Regulated Inhibitor), which prevents protein synthesis in heme-deficient reticulocytes (Chen *et al.*, 1991). This mechanism couples the heme availability with the synthesis of globin, the major product of erythroid cells. Figure 1.3 illustrates schematically how heme exerts its effect on translation. After formation of the initiation complex, eIF-2 bound to GDP is released from it, and then interacts with GEF (Guanidyl Exchange

Factor) which promotes the exchange of GDP for GTP, regenerating eIF-2-GTP for a new translation initiation event. When eIF-2 is phosphorylated, it has a great affinity for GEF, and consequently it does not dissociate and cannot take part in a new initiation complex. Under heme-deprivation conditions HRI is active and phosphorylates the eIF-2 factor, leading to the inhibition of translation. When heme is available, HRI is inactive and protein synthesis continues.

The molecular bases of HRI regulation by heme are not yet understood, though the presence of HRM (heme regulatory motifs) in its sequence would suggest a direct interaction between heme and the kinase through these motifs. Yang *et al.* (1992) reported a correlation between the formation of inter-subunit disulphide bridges in the HRI homodimer and the regulation of the kinase by porphyrin compounds *in vitro*. In a hypothetical model, heme would abolish the kinase activity of HRI by promoting the formation of inter-subunit disulphide bridges (Werk, 1994). More recently, Chefalo *et al.* (1998) have proposed that HRI has a regulatory heme-binding site.

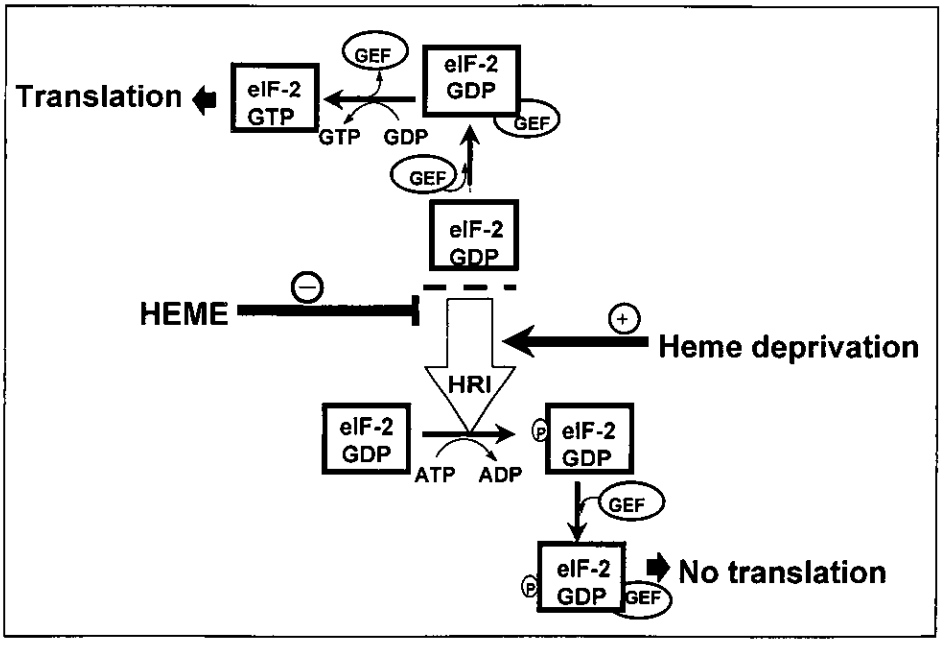

Figure 1.3. Effect of heme on protein synthesis. Heme regulates translation through the kinase activity of the HRI. In the presence of heme HRI is inactive and translation can proceed; whereas in the absence of heme, HRI is active and translation is inhibited.

At the postranslational level, heme affects the import of the cytochrome *c* and the 5-aminolevulinic acid synthase (ALAS) into mitochondria. In the biogenesis of cytochrome *c*, a precursor is firstly

synthesized in the cytosol without heme (apocytochrome *c*). Subsequently, the apocytochrome *c* enters the mitochondria where the enzyme cytochrome *c* lyase (CCHL) incorporates the prosthetic group, heme. Numerous works have been addressed to the study of the mitochondrial import of cytochrome *c* in yeast (Nicholson and Neupert, 1989; Dumont *et al.*, 1991; Wang *et al.*, 1996). Figure 1.4 represents a recent model proposed by Wang *et al.* (1996) for the mitochondrial import of cytochrome *c* in *S. cerevisiae*. Once synthesized in the cytosol, apocytochrome *c* binds to a putative factor and is led to mitochondria without a typical targeting signal. The protein diffuses reversibly across the mitochondrial outer membrane (OM). As the protein passes through the OM, it gains access to the CCHL which is exposed at the outer side of the mitochondrial inner membrane (IM). The CCHL catalyses the covalent attachment of heme to the apoprotein, which triggers the folding of the resulting holocytochrome *c* into its native globular structure. The initial translocation of the apocytochrome *c* across the outer membrane is a freely reversible process. However, after the folding which follows the attachment of heme, the holocytochrome *c* gets trapped in the intermembrane space (IMS).

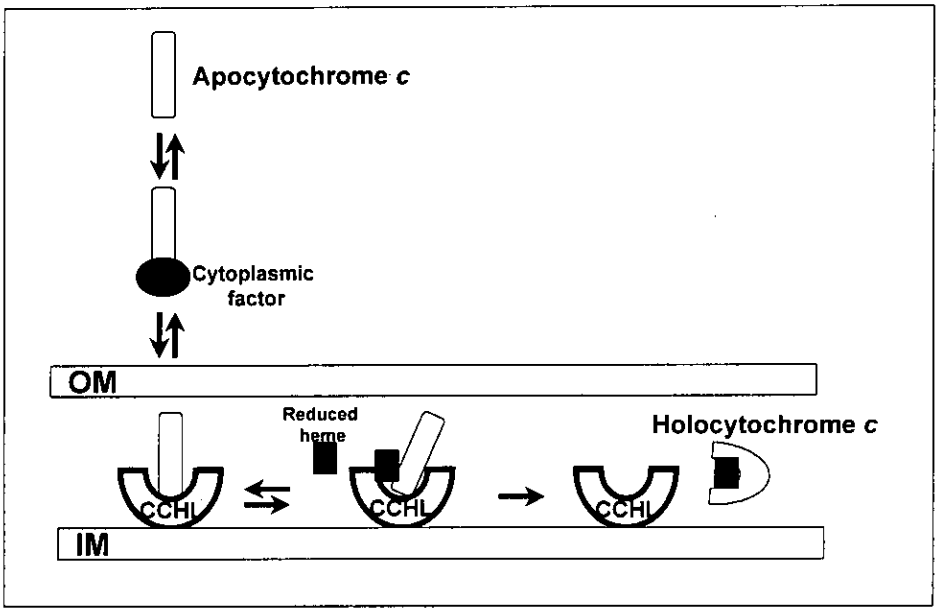

Figure 1.4. Model for the biogenesis of cytochrome *c*. The attachment of heme to the apocytochrome *c* triggers a conformational change to give a mature holocytochrome *c*. The conversion to the holoform, catalysed by the CCHL, drives unidirectionally the cytochrome *c* into the mitochondria against a reversible translocation

The enzyme ALAS catalyses the first reaction in the heme biosynthetic pathway. This protein is translated by cytosolic ribosomes and then targeted to the mitochondria. In the last decades, different authors reported that heme inhibits the transport of ALAS into mitochondria both *in vivo* and *in vitro* (Yamauchi *et al.*, 1980; Yamamoto *et al.*, 1983; Srivastava *et al.*, 1983; Hayashi *et al.*, 1983). More recently, Lathrop and Timko (1993) have identified the specific regulatory motifs involved in this inhibition by heme.

Heme is also necessary for the proper assembly of the ten subunits of the cytochrome oxidase complex in *S. cerevisiae*. In heme-sufficient cells, antibodies directed against subunit VI co-immunoprecipitate subunits I, II and III; whereas, only subunit VI is precipitated in heme-deficient cells. Therefore, heme is required for the association of the subunits in the assembly of the holoenzyme (Saltzgaber-Müller and Schatz, 1978).

1.1.4. Mechanism of heme actions

Although the mechanisms underlying the regulatory function of heme are not well established, some insights are emerging. Recent works suggest that heme should be considered not only as a ligand of hemoproteins but also as a redox-sensitive cofactor (Poyton and Burke, 1992; Burke *et al.*, 1997; Kwast *et al.*, 1998). Following examples illustrate the mechanisms proposed to explain the action of heme in different systems.

1.1.4.1. *Heme-based* sensors

The hemoprotein FixL forms part of the signal transduction system by which oxygen regulates the nitrogen fixation in *Rhizobium meliloti* (Gilles-González *et al.*, 1994). FixL belongs to a heterogeneous family of environmental sensors combining a non-conserved N-terminal sensor and a kinase activity. In FixL, heme is the sensor which detects and transmits the oxygen signal acting as a redox-sensitive effector. In the presence of oxygen the spin state of the heme iron is altered, and the metal moves out of the porphyrin plane as a result of transition from low to high spin; this displacement results in a long-range conformational change that activates the kinase activity (Gilles-González *et al.*, 1995).

FixL defines a new class of hemoproteins well-adapted to sense and to transduce the signal of oxygen: heme-based sensors (Bunn and Poyton, 1996). Other proteins to be considered as heme-based sensors are flavohemoglobins from bacteria (Poole *et al.*, 1994) and yeast (Zhao *et al.*, 1996), together with the NADPH oxidase from mammals (Kwast *et al.*, 1998).

1.1.4.2. HRM: heme regulatory motifs

The presence of short sequences termed *heme regulatory motifs* (HRM) has been reported in many proteins functionally related to heme. HRM consists of a Cys-Pro dipeptide flanked by a hydrophobic residue downstream, and by a positively charged amino acid upstream. Spectroscopic and chromatographic analyses reveal that the HRM represents a new heme-binding module, different from the characteristic histidine/methionine or bis-histidine pairs of cytochromes and globins (Zhang and Guarente, 1995; McCoubrey *et al.*, 1997). HRM seems to bind heme in a reversible and transient manner, rendering the proteins with such elements specially suitable to sense variations in the intracellular levels of heme.

Table 1.1. Occurrence of the heme regulatory motifs. The conserved core Cys-Pro is highlighted in bold. Heme binds to the Cys residue of the motif.

PROTEIN	ORGANISM	MOTIF	POSITION	REFERENCE
Hap1p	*S. cerevisiae*	KCPI	280	Pfeifer *et al.*, 1989
		KCPV	299	
		RCPV	323	
		RCPV	348	
		KCPV	389	
		RCPI	415	
		KCPV	1192	
CCHL	*S. cerevisiae*	GCPV	26	Dumont *et al.*, 1987
		ECPV	42	
	N. crassa	SCPM	35	Drygas *et al.*, 1989
		KCPV	67	
	C. albicans	ACPI	26	Cervera *et al.*, 1996*
	C. elegans	QCPL	36	Palmer, 1996*
		ACPV	57	
	Human	GCPM	25	Schaefer *et al.*, 1996
		GCPV	35	
		ECPI	66	
	Mouse	GCPM	29	Schaefer *et al.*, 1996
		GCPV	39	
CC1HL	*S. cerevisiae*	KCPV	10	Zollner *et al.*, 1992
Heme oxygenase	Rat	KCPF	264	Rotenberg *et al.*, 1990
		NCPF	281	McCoubrey *et al.*, 1997
HRI	Rabbit	ACPY	347	Chen *et al.*, 1991
		RCPA	548	

(*)These sequences have not been published yet, and the reference corresponds to the NCBI GenBank citations. HRM of the ALAS will be shown in Chapter III.

1.2. 5-AMINOLEVULINIC ACID SYNTHASE AND THE FIRST STEP OF HEME BIOSYNTHESIS

1.2.1. Heme biosynthesis: overview on the enzymatic pathway

The metabolic pathway for the biosynthesis of tetrapyrroles is remarkably similar in all organisms, and starts with the formation of a highly reactive aminoketone: the 5-aminolevulinic acid (ALA). ALA is produced from different substrates depending on the organism: in animals, yeast and α-proteobacteria, the ALA synthase (EC 2.3.1.37) catalyses the condensation of glycine and succinyl-CoA to yield ALA, carbon dioxide and Coenzyme A (Fig. 1.5 B); in plants and most eubacteria, ALA derives from glutamate through three enzymatic steps (Fig. 1.5 A).

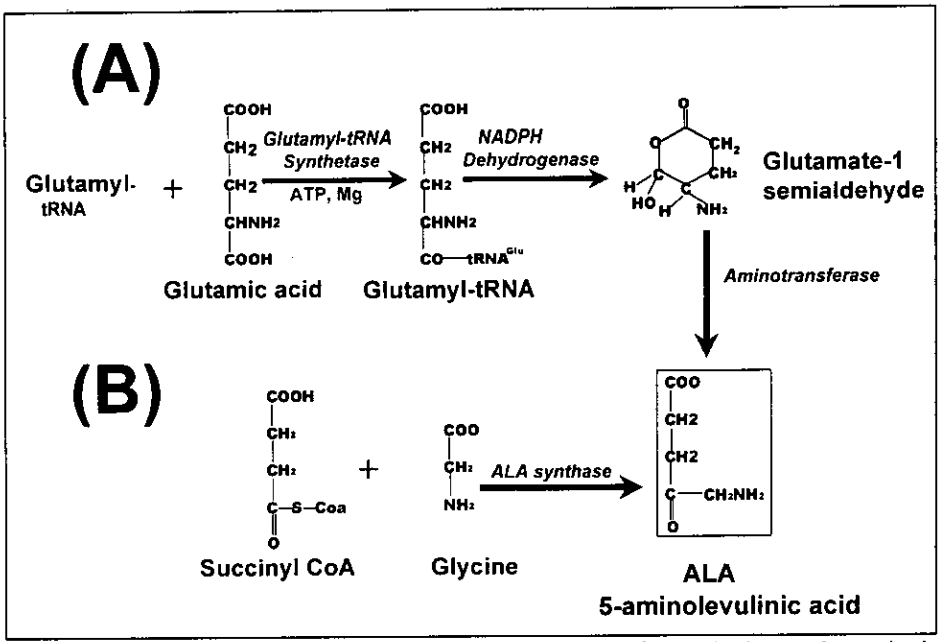

Figure 1.5. Synthesis of the 5-aminolevulinic acid (**A**)from glycine and succinyl-CoA; (**B**)from glutamate.

The ALA is converted to a macrocycle structure along three steps. First, two molecules of ALA condense to generate the porphobilinogen. Second, four molecules of porphobilinogen polymerise to give an unstable compound called preuroporphyrinogen. Last, preuroporphyrinogen is cycled yielding the intermediate uroporphyrinogen. The uroporphyrinogen is at a branch point of the pathway, and it may go through the route to produce heme or chlorophylls, or, alternatively, corrins (Fig. 1.6).

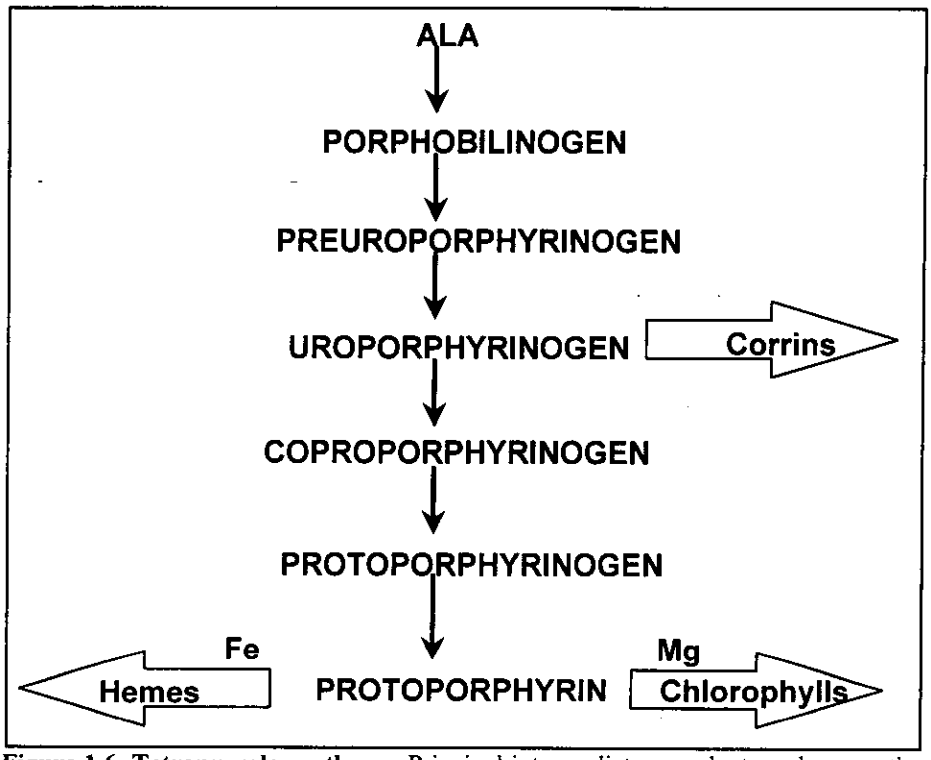

Figure 1.6. Tetrapyrroles pathway. Principal intermediates, products and connections are shown in a schematic form.

In the heme biosynthetic pathway, the four acetyl side chains of the uroporphyrinogen are decarboxylated to methyl groups, producing the coproporphyrinogen. The coproporphyrinogen undergoes an oxidative decarboxylation of the two propionyl groups on the A and B rings to generate the vinyl groups of the subsequent intermediate, the protoporphyrinogen. The protoporphyrinogen is then oxidised to protoporphyrin. Finally, a ferrous iron is inserted into the macrocycle of the protoporphyrin to give heme. The last reactions present interesting features in their mechanisms: i)in eukaryotes, both oxidations require molecular oxygen as electron acceptor; ii)in all cells, there is also a specific requirement for ferrous iron in the last reaction. Thus, the biosynthesis of heme depends absolutely on the availability of oxygen and the supply of reduced iron (Dailey, 1990). Figure 1.7 shows the sequence of reactions and the enzymes involved in the heme biosynthetic pathway.

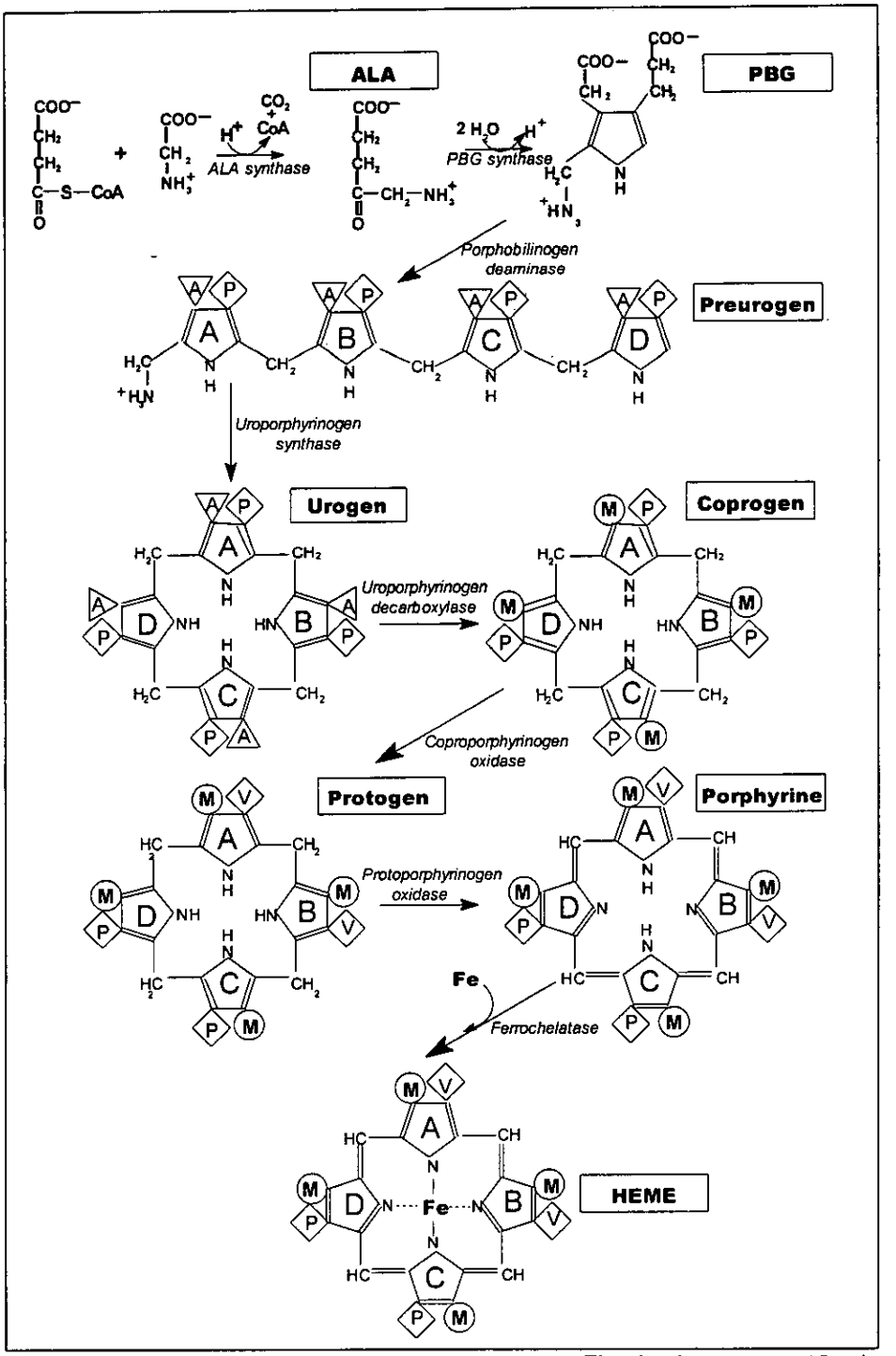

Figure 1.7. Heme biosynthesis, reactions and enzymes. The simple precursor ALA is converted into the complex macrocycle of heme through eight enzymatic reactions. Names of the intermediates are abbreviated.

1.2.2. 5-aminolevulinic acid synthase: occurrence and properties

1.2.2.1. Isolation and purification of the 5-aminolevulinic acid synthase

The ALAS was described in 1958 simultaneously by Shemin and his co-workers in bacterial extracts (Kikuchi *et al.*, 1958), and by Neuberg and his group in avian preparations (Gibson *et al.*, 1958). Since that time, the enzyme has been purified from many sources. Early attempts to isolate and purify ALAS were hindered by the low concentration of this enzyme in mitochondria, its susceptibility to proteolytic degradation and its capacity to form aggregates. Purified ALASs are homodimers, with subunit Mw ranging from 40,000 to 70,000 da.

1.2.2.2. The reaction

The enzyme ALAS catalyses the condensation between glycine and succinyl-CoA to yield ALA (Fig. 1.5 B). In eukaryotes the enzyme is located in the mitochondria, reflecting the requirement for succinyl-CoA, one of the tricarboxilic acid cycle intermediates. The enzyme ALAS also needs pyridoxal-5′-phosphate (PLP) as an essential cofactor.

Alignment of all reported ALAS sequences reveals the presence of a conserved glycine-rich sequence (GAGAGG) which might constitute part of the cofactor binding site, similar of that found in other PLP-dependent enzymes. Gong *et al.* (1996) performed an analysis by mutagenesis on the functional significance of each amino acid residue present in the glycine-rich motif. The replacement of either one of the first two glycines or the arginine between them reduces drastically the enzymatic activity, altering both the Km and Vmax. Thus, the glycine loop is critical for catalysis and for substrate binding. Another well-conserved amino acid is the catalytic lysine to form a Schiff base linkage to PLP (Hunter and Ferreira, 1999).

1.2.2.3. Genetics of the 5-aminolevulinic acid synthase

Cloning and sequencing of cDNAS and genes encoding ALAS from different species allowed to deduce and analyse the primary structure of the respective proteins. Table 1.2 compiles a list of the ALASs ORFs cloned to date. Comparison of the amino acid sequences of ALASs from bacteria to mammals shows extensive similarities (Cox *et al.*, 1991; Ferreira and Gong, 1995). The highest homology is centred in the C-terminal region of the mature proteins corresponding to the catalytic domain of the enzyme.

The expression of genes encoding ALAS have been analysed in several prokaryotes and in many eukaryotes:

i)Prokaryotes

The facultative photosynthetic bacterium *Rhodobacter sphaeroides* synthesises bacteriochlorophylls, hemes and corrinoids. Depending on the physiological conditions for growth, the relative amounts of the tetrapyrroles vary greatly. Bacteriochlorophyll predominates during photosynthetic growth, and its production can be repressed without interrupting heme or corrinoid production or causing accumulation of intermediates. This complex regulation of tetrapyrrole biosynthesis led to hypothesise the existence of a bacteriochlorophyll-specific ALAS isozyme, what was supported by the finding of two ALAS genes, HemA and HemT (Neidle and Kaplan, 1993). Transcription of HemT was observed in a mutant strain devoid of the gene HemA, but not in a wild type background (Neidle and Kaplan, 1993).

ii)Eukaryotes

S. cerevisiae has only one ALAS coded by the *HEM1* gene (Labbe and Labbe-Bois, 1990), but tissue-specific isozymes, coded by independent genes, appear in higher eukaryotes: the housekeeping or non-specific form (ALAS-H or ALAS-N), which is expressed in all tissues and mostly in the liver; and the erythroid-specific isozyme (ALAS-E), which is exclusively expressed in erythroid cells.

In non-erythroid cells, heme exerts a negative feedback control on ALAS production at the transcriptional and translational level (Andrew *et al.*, 1990). In erythroid cells, the transcription of the ALAS-E gene is positively regulated by heme (Sassa and Nagai, 1996), although a heme-dependent inhibition at the translational level has been reported (Smith and Cox, 1997). Moreover, the 5′-untranslated region of the human ALAS-E mRNA carries an iron-responsive element which confers modulation by iron (Bhasker *et al.*, 1993).

In eukaryotes, ALAS is synthesised in the cytosol as a larger precursor which undergoes a proteolytic cleavage to give the mature form of the enzyme during its transport into mitochondria. The import of ALAS into mitochondria is prevented by heme both in erythroid and non-erythroid cells (Yamauchi *et al.*, 1980; Srivastava *et al.* 1983; Hayashi *et al.*, 1983; Yamamoto *et al.*, 1983). In 1993, Lathrop and Timko identified the elements responsible for the inhibition of the import by heme in the presequence of the ALAS-E from rat: the heme regulatory motifs, HRM. These HRM appear in all eukaryotic ALASs, excepting the *S. cerevisiae* enzyme.

Table 1.2. 5-aminolevulinic acid synthases from different organisms.

ORGANISM	REFERENCE
Rhodopseudomonas palustris	Inui and Yamagata, 1998 *
Escherichia coli	Ikemi *et al.*, 1992
Agrobacterium radiobacter	Drolet and Sasarman, 1991
Bacillus subtilis	Petricek *et al.*, 1990
Rhodobacter sphaeroides (HemT)	Neidle and Kaplan, 1993
Rhodobacter sphaeroides (HemA)	Neidle and Kaplan, 1993
Rhodobacter capsulatus(HemA)	Hornberger *et al.*, 1990
Bradyrhizobium japonicum	McClung *et al.*, 1987
Rhizobium meliloti	Leong *et al.*, 1985
Paracoccus denitrificans	Page and Ferguson, 1994
Saccharomyces cerevisiae	Urban-Grimal *et al.*, 1986
Kluyveromyces lactis	González-Domínguez *et al.*, 1997
Aspergillus nidulans	Bradshaw *et al.*, 1993
Drosophila melanogaster	Ruíz de Mena *et al.*, 1997 *
Plasmodium falciparum	Wilson *et al.*, 1996
Rat (housekeeping)	Munakata *et al.*, 1993
Rat (erythroid)	Yamamoto *et al.*, 1988
Mouse (erythroid)	Schoenhaut *et al.*, 1986
Chicken (housekeeping)	Snoswell *et al.*, 1985
Chicken (erythroid)	Riddle *et al.*, 1989
Opsanus (housekeeping)	Cornell, 1994*
Opsanus (erythroid)	Hellmich and Cornell, 1992*
Human (housekeeping	Bawden *et al.*, 1987
Human (erythroid)	Bishop, 1990

(*)These sequences have not been published yet and the reference corresponds to the NCBI GenBank citations.

1.3. HEME IN *Saccharomyces cerevisiae*: BIOSYNTHETIC PATHWAY AND REGULATORY FUNCTIONS

1.3.1. Hemoproteins in *Saccharomyces cerevisiae*: presence and distribution

Although a vast majority of hemoproteins are located inside the mitochondria, they are also present in other subcellular compartments. The mitochondrial repertoire comprise components of the respiratory chain complexes, such as the cytochromes *c* and *a*-a_3 from the cytochrome *c* oxidase (complex IV); or the cytochromes c_1 and *b*, associated to the ubiquinol-cytochrome *c* reductase (complex III) (Beckmann *et al.*, 1987); and the cytochrome b_2 (L-lactate dehydrogenase) (Daum *et al.*, 1982).

Hemoproteins found outside mitochondria include catalases and some biosynthetic enzymes. *S. cerevisiae* possesses two catalases, the cytoplasmic catalase T (*CTT1*) (Spevak *et al.*, 1983) and the peroxisomal catalase A (*CTA1*) (Cohen *et al.*, 1985). The microsomal fraction also contains hemoproteins: the cytochrome b_5, required for oxidative desaturation of fatty acids; and the cytochrome P450 (*ERG11*), which catalyses the first step in ergosterol biosynthesis (Aoyama *et al.*, 1987). Another protein related to heme, although not a hemeprotein *senso stricto*, is the transcriptional activator Hap1p (heme activated protein), with a wide-ranging function in gene regulation by oxygen in *S. cerevisiae* (Pfeifer *et al.*, 1989; Creusot *et al.*, 1988; Verdière *et al.*, 1988).

Given the variety of the processes in which the hemoproteins intervene, the expression of their respective encoding genes is specifically regulated, though most of them respond to oxygen and heme. The cytochromes and other hemoproteins taking part in cellular respiratory functions are poorly required in hypoxia, and the expression of the corresponding genes is kept at low levels under these oxygen-deficient conditions. In contrast, hemoproteins involved in biosynthesis of essential metabolites, such as methionine, unsaturated fatty acids or ergosterol, are needed constitutively regardless of the environmental conditions.

1.3.2. Overview of the heme biosynthetic pathway in *Saccharomyces cerevisiae*

Initial evidence about the regulation of heme and hemoproteins synthesis stemmed from the study of porphyrias, a group of illnesses where porphyrin and heme metabolism is altered by inborn or acquired enzymatic defects. The studies on the biosynthesis of heme had their beginnings in animal cells. Nevertheless, the research in *S. cerevisiae*, a simple and well-known eukaryotic cell, contributed to a better understanding of this metabolic pathway and its regulation. Since *S. cerevisiae* is a facultative aerobe, respiratory mutants defective in heme synthesis were easily isolated (Gollub *et al.*, 1977; Urban-Grimal and Labbe-Bois, 1981; Kurlandzka and Rytka, 1985; Carvajal *et al.*, 1990). These mutants have provided valuable information about the regulation of the heme biosynthetic pathway and also about the effect of heme on the synthesis of cytochromes and other hemoproteins.

1.3.2.1. Cellular location

The heme biosynthetic pathway in *S. cerevisiae* consists of eight enzymatic steps partitioned between the cytoplasmic and mitochondrial compartments (Fig. 1.8). In spite of their different intracellular locations, all the enzymes are encoded by nuclear genes. The route is identical to that described in other eukaryotic cells, except for the enzyme coproporphyrinogen oxidase, which is cytoplasmic in *S. cerevisiae* but mitochondrial in other eukaryotic systems (Camadro *et al.*, 1986).

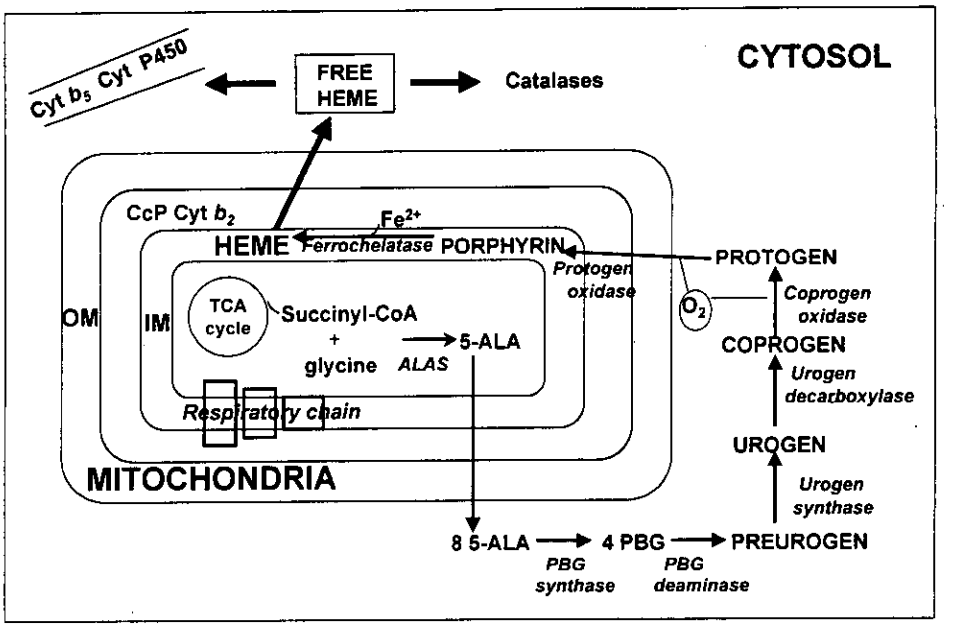

Figure 1.8. Heme biosynthesis in *S. cerevisiae* and subcellular distribution of the hemoproteins. The pathway is partitioned between the mitochondria and the cytosol. After the synthesis, heme is incorporated into different hemoproteins inside and outside the mitochondria.

1.3.2.2. Isolation of *HEM* genes

Many of the isolated heme-deficient mutants of *S. cerevisiae* carried mutations in the nuclear genes encoding enzymes of the heme biosynthetic pathway. In these heme-deficient mutants, devoid of mitochondrial cytochromes, the respiratory metabolism is abolished and, therefore, they do not grow on non-fermentable carbon sources, such as lactate or glycerol. They also require unsaturated fatty acids and ergosterol for growth owing to the absence of cytochromes b_5 and P450. In addition, mutants defective in the early steps of the pathway show methionine-auxotrophy. These phenotypic properties were all used to describe the *S. cerevisae* heme-deficient mutants.

The availability of *hem* mutants allowed to clone the corresponding *HEM* genes by functional complementation. All the structural genes of the heme biosynthetic pathway in *S. cerevisae* have been already isolated and analysed to a certain extent (Table 1.3).

Table 1.3. *S. cerevisiae* genes coding for heme biosynthetic enzymes

GENE	ENZYME	REFERENCE
HEM1	5-aminolevulinic acid synthase	Arrese *et al.*, 1983; Urban-Grimal *et al.*, 1986; Bard and Ingolia, 1984
HEM2	Porphobilinogen synthase	Myers *et al.*, 1987
HEM3	Porphobilinogen deaminase	Keng *et al.*, 1992
HEM4	Uroporphyrinogen synthase	Amillet and Labbe-Bois, 1995
HEM6	Uroporphyrinogen decarboxylase	Diflumeri *et al.*, 1993
HEM13	Coproporphyrinogen oxidase	Zagorec *et al.*, 1988
HEM14	Protoporphyrinogen oxidase	Glerum *et al.*, 1996; Camadro and Labbe, 1996
HEM15	Ferrochelatase	Labbe-Bois, 1990

1.3.2.3. Regulation

After the isolation of *HEM* genes, the regulation of the heme biosynthetic pathway could be examined at transcriptional level. Although the first studies on enzymatic activities revealed variations in response to changes in the oxygen tension, the intracellular level of heme or the carbon source, transcriptional analyses of the corresponding genes did not confirm these previous data (Pinkham and Keng, 1994).

The genes *HEM1* and *HEM3* may be co-regulated since they exhibit a similar pattern of constitutive expression, resulting from a complex combination of positive and negative regulatory elements in the promoters (Keng and Guarente, 1987; Keng *et al.*, 1992). *HEM13*, coding the coproporphyrinogen oxidase (CPO), is the only gene of the pathway subjected to a clear transcriptional regulation. The level of *HEM13* mRNA raises 40-50 fold in response to oxygen/heme-deficiency (Zagorec *et al.*, 1988). This induction would reflect the adaptation of cells to oxygen deprivation. The CPO encoded by *HEM13* which needs oxygen for the decarboxylation of coproporphyrinogen. CPO becomes rate-limiting for heme production under hypoxia because of the oxygen-deficiency. Cells try to compensate this situation by increasing the amount of CPO, in order to maximise the low levels of oxygen for a residual synthesis of heme (15-25% of the aerobic levels) in these restrictive conditions (Labbe-Bois and Labbe, 1990).

1.3.3. Regulatory functions of heme in *S. cerevisiae*

1.3.3.1. Oxygen and/or heme responsive genes

In the yeast *S. cerevisiae* the expression of a large number of genes is regulated in response to oxygen availability (Table 1.4). Many of these genes are expressed optimally in the presence of oxygen (aerobic genes), and encode apocytochromes and enzymes implicated in respiration or in processes related to oxygen toxicity. Some, however, are highly expressed when oxygen levels are restricted (hypoxic genes). Among the hypoxic genes, many of them code for isoforms of proteins synthesised also in aerobiosis. Gene pairs (aerobic/hypoxic) encoding homologous proteins but with different expression patterns in response to oxygen levels are: *TIF51B/ANB1; COX5a/COX5b; CYC1/CYC7* and *ACC2/ACC3*. Hypoxic isoforms might be more suitable to function in low oxygen tension environments (Zitomer and Lowry, 1992). Table 1.4 shows that the effects of oxygen and heme act mostly in parallel, i.e., those genes positively regulated by oxygen are also positively regulated by heme and *vice versa*, though there are few exceptions as well.

Table I.4.Oxygen/heme-regulated genes in *S. cerevisae* (modified from Kwast *et al.*, 1998).

FUNCTION	GENE	PROTEIN	HEME EFFECT	OXYGEN EFFECT
I.Mitochondrial respiration/ oxidative phosphorilation		**Cytochrome *c* oxidase**		
	COX4	Subunit IV	Induced	Induced
	COX5a	Subunit Va	Induced	Induced
	*COX5b**	Subunit Vb	Repressed	Repressed
	COX6	Subunit VI	Induced	Induced
	COX7	Subunit VII	Induced	Induced
	COX8	Subunit VIIa	Induced	Induced
	COX9	Subunit VIII		Induced
		Cytochrome *c*		
	CYC1	Iso-1-cytochrome *c*	Induced	Induced
	*CYC7**	Iso-2-cytochrome *c*	Repressed	Repressed
		Ubiquinol cytochrome *c* reductase		
	COR1	Subunit I	Induced	Induced
	COR2	Subunit II	Induced	Induced
	QCR8	Subunit XIII		Induced
	CYT1	Cytochrome c_1	Induced	Induced
		L-Lactate cytochrome *c* oxidorreductase		
	CYB2	Cytochrome b_2	Induced	Induced
		Mitochondrial adenine translocase		
	ACC1	ADP/ATP translocator	None	Induced
	ACC2	ADP/ATP translocator	Induced	Induced
	*ACC3**	ADP/ATP translocator	Repressed	Repressed
II. Heme sterol and unsaturated fatty acids synthesis				
	*HEM13**	Coproporphyrinogen III oxidase	Repressed	Repressed
	HMG1	3-hydroxy-3-metylglutaryl CoA reductase	Induced	Induced
	*HMG2**	3-hydroxy-3-metylglutaryl CoA reductase	Repressed	Repressed
	*ERG11**	Cytochrome P450 lanosterol 14 α–demetylase	Repressed	Repressed
	CPR1	NADPH Cytochrome P450 reductase	Repressed	Repressed
	OLE1	D-9 fatty acid desaturase	Repressed	Repressed
III. Oxidative stress				
	CTT1	Catalase T (cytoslic)	Induced	Induced
	CTA1	Catalase A (peroxisomal)	Induced	Induced
	SOD1	Cu,Zn-superoxide dismutase	Induced	Induced
	SOD2	Mn-superoxide dismutase	Induced	Induced
	YHB1	Flavohemoglobin	Induced	Induced
IV.Translation and transcription factors				
	TIF51A	e-IF-5a	Induced	Induced
	*ANB1**	e-IF-5b	Repressed	Repressed
	ROX1	Heme-dependent repressor	Induced	Induced

(*) Hypoxic genes are marked with an asterisk.

It is widely accepted that heme mediates the effect of the oxygen signal by binding as a ligand to specific transcriptional factors (Zitomer and Lowry, 1992; Pinkham and Keng, 1994; Kwast *et al.*,1998). However, recent evidence suggests that heme might additionally transmit the oxygen signal by an alternative mechanism (Poyton and Burke, 1992; Burke *et al.*, 1997). The current overview of oxygen-sensing pathways in *S. cerevisiae* has been drawn in Figure 1.9. While the research so far has characterised the factors and the *cis*-sites at transcriptional level, the prior events related to the transduction of the signal remain obscure (Bunn and Poyton, 1996).

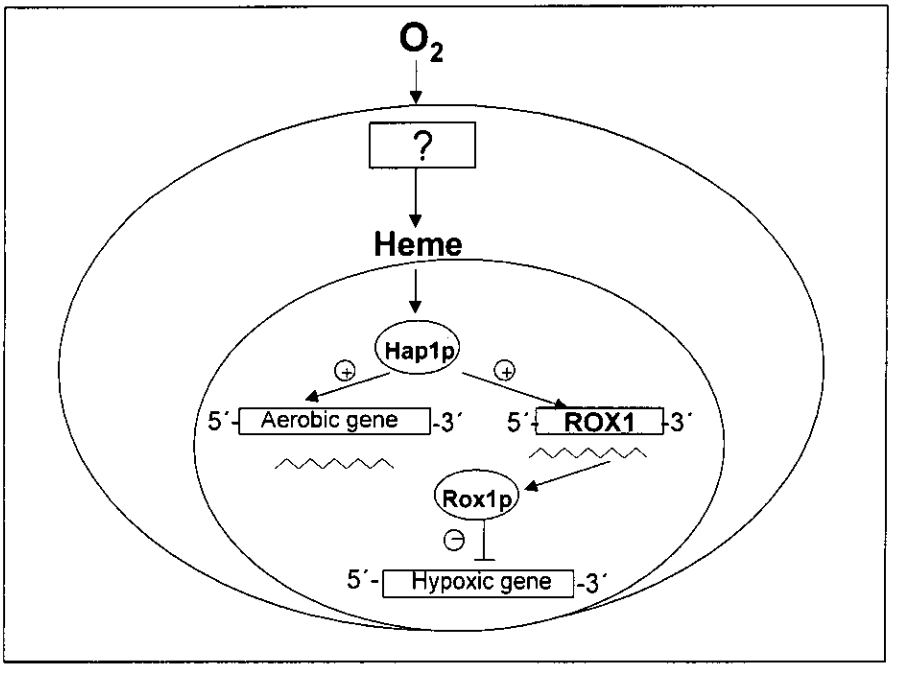

Figure 1.9. A current model of the oxygen-sensing pathways in *S. cerevisiae*. The cellular receptor for the oxygen signal is unknown. Descriptions of Hap1p and Rox1p is in the text (Sec. 1.3.3.2).

1.3.3.2.Heme-regulated transcriptional factors

In *S. cerevisiae* the transcriptional regulation mediated by heme involves at least three factors.

i)Hap1p (heme activated protein)

Hap1p plays a pivotal role in the oxygen regulation mediated by heme. This factor belongs to the Zn-cluster regulatory proteins and its structure consists of: 1)a dimerization and binding domain (residues 1-148); 2)an activation domain (residues 1309-1483); and 3)two regulatory regions, one between residues 235 and 444 (the heme domain), and the other between positions 1048 and 1052 (HRM7)(Pfeiffer *et al.*, 1989; Creusot *et al.*, 1988; Verdière *et al.*, 1988). Seven HRM are dispersed along both regulatory domains, six in the heme domain, together with the mentioned HRM7 (Zhang and Guarente, 1995).

A considerable effort has been devoted to understand the regulation of the Hap1p activity by heme. Fytlovitch *et al.* (1993) proposed the following model: in the absence of heme, Hap1p is weakly bound to its target DNA, but remains transcriptionally inactive because a repressor protein occupies one of the regulatory domains; when heme levels increase, heme displaces the repressor and promotes the transcriptional activity of the factor. Zhang and Guarente (1995) confirmed the interaction of Hap1p with heme, demonstrating that a synthetic peptide containing the HRM binds heme *in vitro*. Further research have provided deep insight into the mechanism by which Hap1 is repressed in the absence of heme, describing the nature of the inactive complex (HMC, high-molecular-weight complex) which is formed, and the involvement of the different domains of the factor (Zhang *et al.*, 1998).

Hap1p induces the transcription of a number of aerobic genes, such as *CYC1*, *CTT1* and *CYB2* (Table 1.4), in the presence of oxygen or heme. In the absence of heme, however, the factor may have an opposite effect upon transcription, as it actually switches off the transcription of *SOD2* coding the manganese superoxide dismutase (Pinkham *et al.*, 1997). It is not known yet how the large inactive complex that Hap1p forms in the absence of heme represses transcription.

ii)Hap2/3/4/5 p

This heteromeric complex mainly activates the transcription of genes in response to heme/oxygen and/or growth on non-fermentable carbon sources, i.e., lactate. The Hap2/3/5p trimer acts in DNA-binding, whereas Hap4p is a regulatory subunit required for the activation of the complex. The regulatory effect of heme on the complex is unclear, and the DNA-binding activity appears to be heme-independent (Kwast *et al.*, 1998). Forsburg and Guarente (1989) suggested that heme could affect Hap4p postranscriptionally, but the mechanism involved in the modulation of the

heteromeric complex activity has not been explained yet. Moreover, heme and oxygen are not the only effectors regulating the Hap2/3/4/5p complex. In fact, this heteromer should be regarded as a modular complex responsive to diverse environmental and physiological signals (Pinkham and Keng, 1994).

iii)Rox1p

Rox1p is a repressor which shuts down the transcription of nearly all the hypoxic genes in aerobic conditions (Lowry and Zitomer, 1988). Heme does not participate directly in the mechanism of repression by Rox1p but it is required for the transcription of the *ROX1* gene. In the presence of heme or aerobiosis, Hap1p and other factors activate transcription of the *ROX1* gene and the repressor is produced. After its synthesis, Rox1p enters the nucleus and binds to a specific *cis*-element present in the promoters of the genes under its control. The mere binding of Rox1p is not sufficient for functional repression, and once bound to the DNA, Rox1p recruits a general repression complex Tup1/Ssn6 which turns transcription off (Zitomer *et al.*, 1997).

1.3.3.3. Heme as a redox-sensitive effector in *S. cerevisiae*

It is not clear how the oxygen signal is sensed either in yeast or higher eukaryotes (Bunn and Poyton, 1996). Other obscure feature is how an oxygen-sensor might transmit its signal to a transduction pathway and whether there is more than one oxygen-responsive pathways. Although the molecular approaches have identified many elements involved in the oxygen-sensitive transcriptional circuitries, they have not provided conclusive evidence on how cells actually sense different oxygen levels. In one of the current models, the signal, oxygen, would promote the synthesis of the effector, heme, which would bind as a ligand to different targets; the final effect would depend on the concentration of heme which correlates with the level of oxygen. However, recent works question this scheme, taking into account the following findings (Burke *et al.*, 1997):

i)Transcript levels of many aerobic genes decrease with declining oxygen concentration over a range in which the heme concentration does not vary appreciably in the cell.

ii)After cells are shifted from aerobic to anaerobic conditions, the time course for induction and transcript profiles differ markedly among the hypoxic genes. These data are difficult to reconcile with a loss of a common repressor or effector.

iii)Clamping the redox state of hemoproteins under different conditions of oxygen availability modulate several hypoxic genes.

From these evidences, Poyton and Burke (1992) remark that it should be considered the existence of multiple mechanisms by which heme mediates the signal of oxygen as depicted in Fig. 1.10.

i)Heme acts as a ligand

The presence of oxygen in aerobiosis would induce the synthesis of heme which would serve as a ligand for a transcriptional factor, according to the model proposed for the heme-dependent activation of Hap1p. The concentration of heme, but not the redox state of the iron, is important in this mechanism. This sensing pathway would be efficient during hypoxia, when the intracellular concentration of heme is proportional to the level of oxygen.

ii)Heme acts as a redox-sensitive effector

The action of heme would depend on the changes in the redox state of the iron triggered by variations in the level of oxygen. In this way, heme might modulate the transcriptional factor either directly, being a redox-sensitive ligand for a transcriptional factor, or indirectly, being a redox-sensitive ligand of a kinase (or another regulatory enzyme) which, in turn, would regulate the activity of the mentioned factor. This mechanism would operate at a range of oxygen concentrations which do not significantly alter the intracellular concentration of heme.

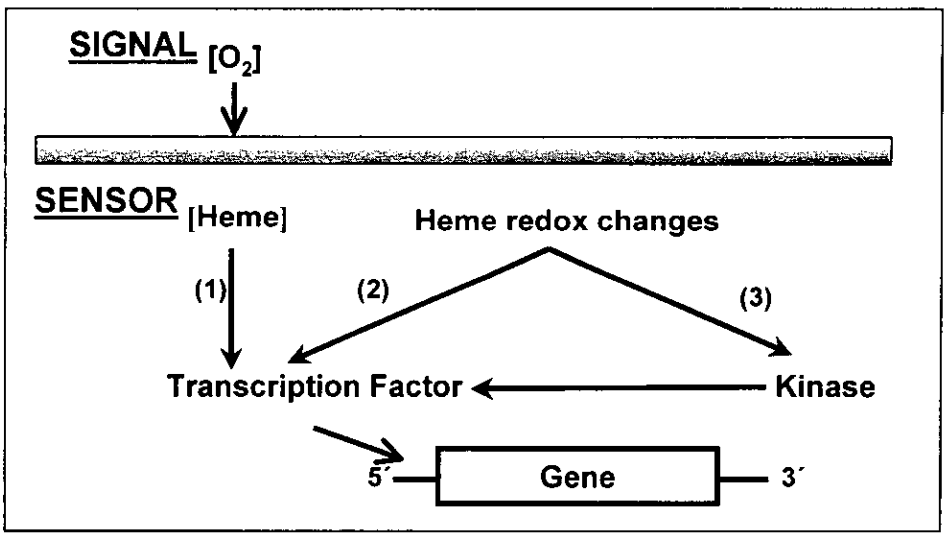

Figure 1.10. Possible roles of heme in the transduction of the oxygen signal for transcriptional regulation in *S. cerevisiae* (adapted from Poyton and Burke, 1992). Heme senses and transmits the signal of oxygen by either **(1)**increasing its concentration and binding to a transcriptional factor as a ligand; or changing its redox state which affects **(2)**directly or **(3)**indirectly (through a kinase) the heme-dependent transcriptional factor.

MATERIALS AND METHODS

2.1. BIOLOGICAL MATERIAL

2.1.1. Bacterial strains, media, growth conditions and transformation

Escherichia coli DH5 or JM109 were used routinely for cloning, maintenance and propagation of plasmids. Recombinant bacteria were grown in LB medium with 80 µg/ml of ampicillin. Cells were rendered competent according to Hanahan (1985), and transformation was accomplished by heat shock.

2.1.2. Yeast strains, media, growth conditions and transformation

Table 2.1 presents the yeast strains used in the course of this study.

Table 2.1. Yeast strains

Strain	Genotype	Reference or source
S. cerevisiae		
S150-2B	*MATa, ura3, leu2, trp1, his3*	Dr. Labbe-Bois, Paris
S150-2B *hem1*	*MATa, ura3, leu2, trp1, his3, hem1Δ*	Dr. Labbe-Bois, Paris
D273-10B	*MATα*	ATCC 24657
K. lactis		
NRRL Y1140	*MATa*	ATCC 8585
MW190-9B	*MATa, lac4-8, uraA, Rag+*	Dr. Wesolowski, Lyon
MW190-9B *hem1*	*MATa, lac4-8, uraA, Rag+, Klhem1::URA3*	This work

Yeast were cultivated at 30°C in rich media YP (2% bactopeptone, 1% yeast extract) with a carbon source (0.5% glucose, 2% glucose); or in CM synthetic complete media (Zitomer and Hall, 1976) containing either 2% glucose or 2% lactate. The *hem1* mutants were grown in media supplemented with ALA; or with Tween 80 (1%), ergosterol (0.002%) and methionine (0.004%). For expression analyses, cultures were grown aerobically or under hypoxia. Hypoxia was generated by bubbling nitrogen at constant pressure through the flasks. Hemin was added to medium when indicated. For mitochondria isolation, cultures were made in lactate medium according to Hermann *et al.* (1994).

Cells were transformed by the procedure of Ito *et al.* (1983) and transformants were plated onto selective plates.

2.2. NUCLEIC ACID PROCEDURES

2.2.1. Vectors and constructs

Table 2.2 lists the vectors used, according to purpose.

Table 2.2. Vectors used in this work.

Vector	Description	Reference or source
Complementation analysis		
YEp*lac*112	*ori,amp^r,lacZ, 2μm,TRP1*	Gietz and Sugino, 1988
YEp*lac*195	*ori,amp^r,lacZ, 2μm,URA3*	Gietz and Sugino, 1988
Sequencing		
pBlueScript® SK+	*ori, f1ori,amp^r,lacZ*	Stratagene
Gene disruption		
YIp*lac*211	*ori,amp^r,lacZ,URA3*	Gietz and Sugino, 1988
In vitro transcription		
pGEM®-4Z	*ori,f1 ori,amp^r,lacZ,* T7/SP6 RNApol. promoters	Promega
Cloning of PCR products		
pGEM®-T easy	*ori, f1ori,amp^r,lacZ,* T7/SP6 RNA pol.promoters	Promega
For protein expression		
pMAL®-c	*ori, f1ori,amp, lacZ, MALE*	New Englands Biolabs

Derived constructs were obtained for different strategies.

Complementation analysis

pMD5.5

A 5.5 kb fragment was transferred from the original clone isolated from the *K. lactis* genomic library (Méndez-Carro, 1992) to YEp*lac*112, cutting with *Bam* HI and *Sal* I.

pMD3.51

Plasmid pMD5.5 was cut with *Sal* I and *Sca* I, and this fragment was inserted in YEp*lac*112 digested with *Sal* I and *Sma* I.

pMD3.52

The same as pMD3.51 but in YEp*lac*195.

pMD2.4

A *Sal* I-*Xba* I fragment from pMD3.5 was subcloned in YEp*lac*112 with the same enzymes.

pMD3.4

A 3.4 kb fragment was excised from pMD5.5 with *Eco* RI and *Xho* I and then inserted in YEp*lac*112 cut with *Eco* RI and *Sal* I.

pMD3.52D

A 0.85 kb *Bgl* II-*Bgl* II fragment was deleted from the insert subcloned in pMD3.52.

Sequencing

pMD3s

The *Sal* I-*Sca* I fragment from pMD5.5 was subcloned in pBlueScript® SK+.

pMD1s

A 1.5 kb fragment was retrieved from pMD3s with *Hind* III and inserted in pBlueScript® SK+ also digested with *Hind* III.

pMD2s

A 2.7 kb *Eco* RI-*Eco* RV fragment from pMD3s was subcloned in pBlueScript® SK+ with the same enzymes.

Gene disruption

pMDI-3

A 0.7 kb *Hind* III-*Eco* RI internal fragment of the gene was ligated into the integrative vector YIp*lac*211.

Protein expression

A *Eco* RI-*Sal* I fragment from pMD3s containing the last 227 amino acids of *Kl*ALAS was fused in frame to the maltose-binding protein sequence of the vector PMAL®-c.

In vitro transcription/translation

***Kl*ALAS**

The 5-aminolevulinic acid synthase of *K. lactis* (*Kl*ALAS) was amplified by PCR using pMD3s as template with primers A and C (added restriction sites are underlined).

Oligo A: 5´- gaga ctcgag (*Ava* I) ggatcc (*Bam* HI) **ATG**GAATCTGTTATTCG-3´

Oligo C: 5´-gaga gagctc (*Sac* I) gtcgac (*Sal* I) CTAGTATCTAATACCAGAAGAGAC-3´

The product was purified and then cloned into pGEM®-4Z under the control of SP6 RNA polymerase using *Ava* I and *Sal* I.

*Kl*ALAS-DHFR

A N-terminal fragment of *Kl*ALAS, encompassing the first ninety amino acids, was fused in frame to the murine DHFR (dihydrofolate reductase). For this purpose, a PCR was performed using pMD3s as template with primers A (see above) and B.

Oligo B: 5´-gaga ctcgag (*Ava* I) ggatcc (*Bam* HI) GACTACATCTGCAGAAGCAG-3´

The product was purified and cloned as an *Ava* I-*Bam* HI fragment into pGEM3-DHFR (a gift from Elena Rojo, Institut Adolf Butenandt, Münich) to be transcribed by SP6 polymerase.

Su9-*Kl*ALAS

A C-terminal fragment of *Kl*ALAS (90-570 residues) was fused in frame after the N-terminus of the subunit 9 of the mitochondrial F1-Fo-ATPase (Su9) from *Neurospora crassa*. The fragment was produced by PCR using pMD3s as template and primers C (see above) and D for its amplification:

Oligo D: 5´-gaga ggatcc (*Bam* HI) GATCATGCCACTAAGGAAGC-3´

The PCR product was purified, digested with *Bam* HI and *Sal* I, and then inserted in front of the Su9 N-terminus of the construct pGEM3-pSu9 (kindly provided by Benedict Westermann, Institut Adolf Butenandt, Münich).

*Kl*ALASmut

Site-directed mutagenesis was performed by PCR on the presequence of *Kl*ALAS as it is outlined in Fig. 2.1. The Cys residues of the HRM in the N-terminal region were replaced by Ala in two steps of PCR, combining appropriate primers. Nucleotide substitutions are in italics.

Oligo mutup:5´-gaga ctcgag (*Ava* I) **ATG**TGTTATTCGTTCTTCTGCCAAGATC*GCT*CCA-3´

Oligo mutdo: 5´-AAATGG*AGC*TTGTTGAGCGAT-3´

The PCR product was purified and then subjected to A-tailing before cloning it into pGEM®-T easy vector. Product of mutagenesis was sequenced to confirm the expected change.

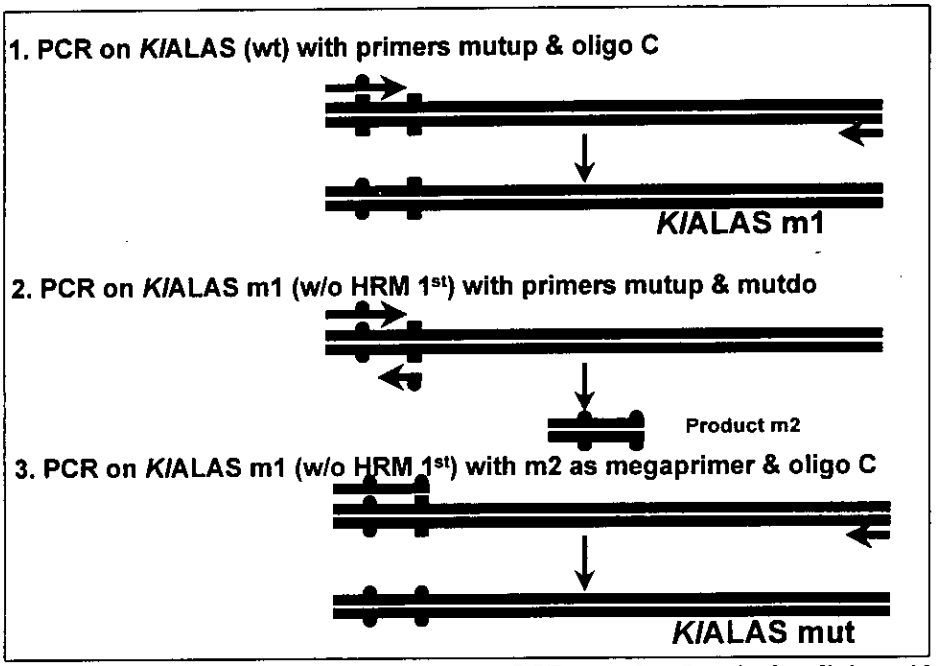

Figure 2.1. Site-directed mutagenesis by PCR on the 5-aminolevulinic acid synthase of *K. lactis*.

2.2.2. Preparation of nucleic acids

2.2.2.1. Bacterial plasmid DNA

i)Small-scale preparation (miniprep)

DNA was obtained by the alkaline lysis method (Sambrook *et al.*, 1989) with minor modifications.

ii)Medium- scale preparation (midiprep).

DNA was isolated from 50 ml cultures. After alkaline lysis of cells, DNA was recovered either by ethanol precipitation or binding to columns. Columns were purchased from QIAGEN (QIAGEN Plasmid Kit), Promega (Wizard® Midipreps DNA Purification System) and Genomed (Jetstart DNA Purification).

2.2.2.2.Yeast genomic DNA

Yeast genomic DNA was prepared following the procedure described by Rose *et al.* (1990).

2.2.2.3.Yeast RNA

Cells were harvested at an $O.D._{600}$ 0.6-0.8 and RNA was extracted according to Zitomer and Hall (1976).

2.2.3. Modification of DNA

2.2.3.1. Cloning

Restriction endonucleases, phosphatase, Klenow polymerase and ligase were used as recommended by the suppliers (Boehringer-Manheim, Promega, Gibco, New Englands Biolabs), or according to standard recombinant methods (Ausubel *et al.*, 1995).

2.2.3.2. Labelling

Probes were labelled by random priming with Klenow polymerase. For Southern experiments, non-radioactive labelling was carried out with the digoxigenin system (Dig DNA Labelling and Detection Kit®, Boehringer Manheim). For Northen experiments, probes were radioactively labelled with [α-P^{32}]dATP (Prime-a-Gene® Labeling System, Promega).

2.2.4. Amplification of DNA by polymerase-chain-reaction

The Polymerase Chain Reaction (PCR) was applied for cloning, directed-mutagenesis and preparation of probes. The reaction mix commonly contained:

- the **template** to be amplified (DNA fragment, plasmid, genomic DNA);
- **primers**: synthetic oligonucleotides at a final concentration of 2 pM;
- **dNTPs:** at a final concentration of 0,25 mM;
- **enzyme:** the high fidelity polymerase *Pwo* was chosen when PCR products were used for cloning, while probes for hybridisation experiments were amplified with *Taq* polymerase;
- the appropriate **buffer** for the corresponding enzyme provided by the vendor (Boehringer Manheim).

The parameters of the cycling for each reaction were conveniently selected in the machine (Gene Cycler BioRad). PCR programmes included the following steps:

-denaturation: 3 minutes / 94°C;

-amplification: 1 minute/ 94°C, 30 seconds-1minute/ t^a annealing (estimated with the *Oligo* programme, Pharmacia) and 1 minute/ 72°C, for 30 cycles;

- final extension: 4 minutes/ 72°C.

2.2.5. Analysis of nucleic acids

2.2.5.1. Agarose gel electrophoresis

i)DNA

Agarose gel electrophoresis was used to separate and purify DNA fragments. Gels contained typically 0.7%-1% agarose in TAE (40 mM Tris·Acetate, 2 mM EDTA-Na_2 ·$2H_2O$). After running, gels were stained with Ethidium Bromide and visualised on an UV-transilluminator. For further purposes, bands were excised from the gel and DNA was recovered with the Gene Clean Kit (BIO101) and QIAquick Gel Extraction Kit (QIAGEN), according to the instructions of the manufacturers.

ii)RNA

RNA was fractionated on 1.5% agarose/formaldehyde gels in 1x MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA-Na_2, pH 7) (Sambrook *et al.*, 1989). Samples were mixed 1:1 (v/v) with formaldehyde buffer (35% formamide, 2x MOPS, 2.3 M formaldehyde). Before loading, samples were denatured at 65 °C for 10 minutes. Gels were stained with Ethidium Bromide after running.

2.2.5.2. Measure of nucleic acid concentration

The amount of DNA was estimated by visualising the bands in minigels stained by Ethidium Bromide on an UV-transiluminator. The concentrations of RNA and DNA in solution were determined by measuring O.D. at 260 nm and 280 nm.

2.2.6. DNA sequencing

Sequencing was carried out with the dideoxy-mediated chain-termination method (Sanger, 1977), using a DNA Sequencing Kit from Amersham. Prior to sequencing, nested deletions were generated with Dnase I in the presence of Mn^{2+} ions, and then subcloned in pBlueScript (Sambrook *et al.*, 1989). Fig. 2.2 summarises an overview of the experimental strategy.

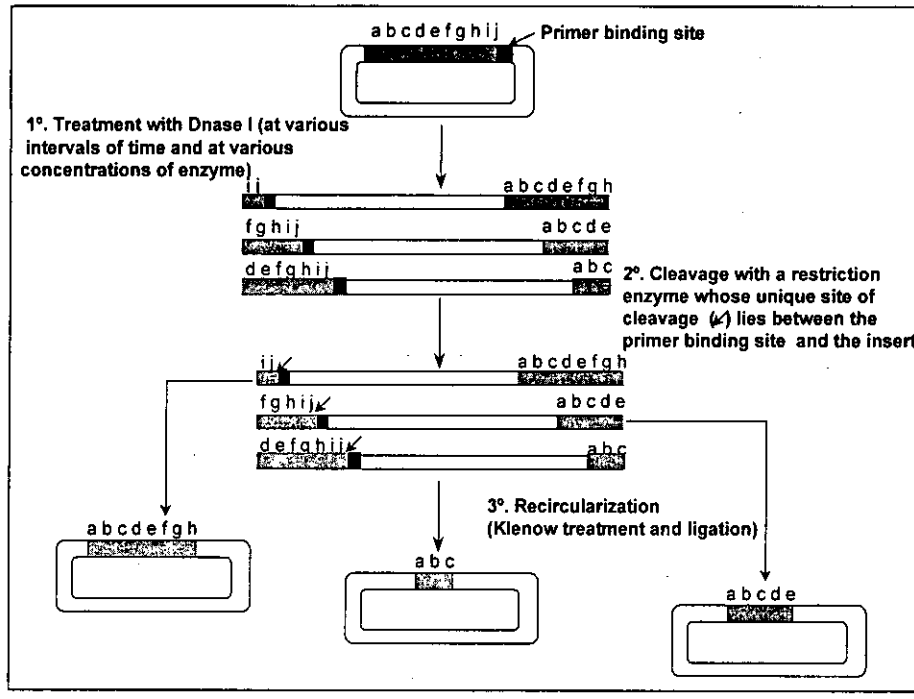

Figure 2.2. Generation of nested deletions with Dnase I in the presence of Mn^{2+}.

2.2.7. Blotting and hybridisation experiments

After electrophoresis, nucleic acids were transferred onto positively charged nylon membranes (Boehringer Manheim) with a vacuum blotter (Vacugene 2016, LKB), according to manufacturer's recommendations. Nucleic acids were later fixed by UV-crosslinking.

DNA blots were hybridised for 5 hours at 52°C with non-radioactive dUTP-digoxigenin labelled probes in hybridisation solution containing 5x SSC (75 mM sodium citrate, 750 mM NaCl, pH 7), 1% blocking reagent (Boehringer Manheim) and 0.02 % SDS. Filters were washed with 2x SSC and 0.1 % SDS for 20 minutes, 10 minutes at 52°C and 10 minutes at room temperature.

RNA blots were commonly hybridised overnight at 65°C with [α-P^{32}]dATP labelled probes in hybridisation buffer containing 0.5 M Na_2HPO_4 pH 7.2, 7% SDS and 1 mM EDTA-Na_2. Filters were washed twice with 0.1 % SDS and 2x SSC, first 15 minutes at 65 °C and then 15 minutes at room temperature.

Concerning detection, non-radioactive signals were optically detected using the colour substrates BCIP/NBT of the Dig DNA Labelling and Detection Kit® from Boehringer Manheim. Radioactive blots were

exposed onto Phosphoimager screens, and signals were quantified by fluorography using the ImageQuant programme (Microsoft).

2.3. PROTEIN PROCEDURES

2.3.1. *In vitro* protein synthesis

Proteins were synthesised by a coupled transcription/translation procedure (Pelham and Jackson, 1976).

Transcription reaction was set up following the manufacturer's instructions, using as RNA polymerases either T7 or SP6 polymerase (Boehringer Manheim, Promega). After transcription, the RNA was precipitated with 95 % ethanol and 10 M LiCl at -70° C for 1 hour, and then recovered by centrifugation (22,000 g, 30 minutes, 2 °C). The pellet was washed once with cold 70% ethanol and recovered by centrifugation. After discarding the supernatant, the RNA pellet was vacuum-dried and resuspended in sterile water with RNasin (Promega). The RNA was stored at -70 °C until use.

Translation of mRNA was accomplished in a nuclease-treated rabbit-reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. Synthesised proteins were aliquoted and stored at -70°C.

2.3.2. SDS-polyacrilamide electrophoresis

Acrylamide gels were prepared according to Laemmli (1970), with an upper stacking gel and a lower separating gel. Samples were resuspended in Laemmli sample buffer 2x (4% SDS, 20% glycerol, 120 mM Tris·HCl pH 6.8, 4.5 % β-mercaptoethanol, 0.1% bromophenol blue) and heated 3 minutes at 95°C prior to loading. Electrophoresis were run in Tris-Glycine buffer (50 mM Tris·HCl pH 8.3, 384 mM glycine, 0.1% SDS) at 28 mA for 2-3 hours. Gels were then stained with Coomassie blue or transferred onto nitrocellulose membranes.

2.3.3. Staining of proteins in acrylamide gels

SDS-PAGE gels were sunk in Coomassie stain solution (10% acetic acid, 30% methanol and 0.1% Coomassie brilliant blue R250) and warmed in the microwave for a few seconds. The gels were shaken in 10% acetic acid/ 30% methanol for destaining.

2.3.4. Transfer of proteins onto membranes

A modified version of the semi-dry blotting protocol was used. For this purpose, a sandwich was built as follows: first, two Whatman 3MM papers saturated with transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.02% SDS) were put on a flat graphite anode; then, the nitrocellulose membrane was lain over, followed by the gel and, finally, two more buffer-soaked Whatman papers. In order to verify the transfer, filters were stained with Ponceau S solution (0.2% Ponceau S, 3% TCA), rinsed with water, and finally dried under an infra-red lamp.

2.3.5. Immunodetection

Blotted proteins were probed with antibodies against mitochondrial proteins. After marking with a pen the positions of the standard-marker proteins on the membranes, the blots were blocked by incubation in a solution of 5% skimmed milk-powder in TBS (10 mM Tris·HCl pH 7.5, 150 mM NaCl) at room temperature for 30 minutes. The first antibody, directed to the mitochondrial protein of interest, was diluted in 5% skimmed milk/TBS at the adequate proportion. Blots were incubated with the first antibody for 1 hour at room temperature and then washed 5 minutes with TBS, 5 minutes with TBS and 0.05% Triton X-100, and finally, 5 minutes with TBS. Filters were incubated with a secondary antibody coupled to horseradish peroxidase (1:5000 in 5% skimmed milk/ TBS) at room temperature for 1 hour, and washed as above. Protein-antibody complexes were detected by chemilumiscence developing reagents (ECL-system, Amersham).

2.3.6. Determination of protein concentration

Protein concentration was determined with the Bio-Rad protein Assay Dye reagent (Bio-Rad), based on the Bradford dye-binding procedure.

2.3.7. TCA precipitation

TCA precipitation was carried out by adding TCA to the protein solution up to 10%. Samples were kept at -20°C for at least 30 minutes and the precipitated proteins were recovered by centrifugation (20,000 g, 15 minutes, 2 °C). The pellet was washed with cold acetone, centrifuged again (20,000 g, 10 minutes, 2°C) and dried at 30°C.

2.4. EXPERIMENTS OF IMPORT INTO MITOCHONDRIA

2.4.1. Isolation of mitochondria from yeast

Mitochondria were prepared from *S. cerevisae* (D273-10B) and *K. lactis* (Y1140) grown in lactate medium, as described by Hermann *et al.* (1994). The first inoculate was started few days in advance of the isolation, and cells were successively passed to higher volumes of fresh medium along the days preceding the last culture (8 liters). When this culture reached an $O.D._{578}$ 1-1.5, cells were harvested, washed with distilled water and weighted. Cells were then resuspended in DTT buffer (100 mM Tris, 10 mM DTT) at 0.5 g cells/ml and incubated with shaking at 30°C for 15 minutes. After washing with 1.2 M Sorbitol, cells were resuspended in zymoliase buffer (1.2 M Sorbitol, 20 mM KH_2PO_4 pH 7.4, 5 mg zymolyase/g cells) at 0.15 g/ml. For zymoliase treatment, samples were kept at 30°C for over 1 hour. To monitor the treatment, 50 µl of the cellular suspension was diluted 1:100 in water (sample A) or in 1.2 M Sorbitol (sample B). When the $O.D._{578}$ of sample A reached 10%-20% of the $O.D._{578}$ of sample B, the reaction was stopped. After digestion with zymoliase, all subsequent operations were carried out at 4°C. Spheroplasts were collected by centrifugation (4,000 r.p.m., 5 minutes) and washed once with 1.2 M Sorbitol. In the next step, spheroplasts were resuspended in a homogenisation buffer containing 0.6 M Sorbitol, 10 mM Tris·HCl pH 7.4, 0.2% BSA fatty-acid free (Sigma, A6003) and 1 mM PMSF at 0.15 g cells/ml, and opened by careful douncing. This homogenate was centrifuged twice at low speed (4,000 r.p.m., 5 minutes), recovering the supernatants. From the last supernatant, mitochondria were pelleted at 12,000 r.p.m. for 12 minutes. The mitochondrial pellet was washed once in SEH buffer (0.6 M Sorbitol, 1 mM EDTA pH 7.4, 20 mM Hepes pH 7.4). Finally, mitochondria were resuspended in an appropriate volume of SEH buffer at a final concentration of 10 mg protein/ml. Aliquots were immediately frozen in liquid nitrogen for storage at -70°C.

2.4.2. Protein import into mitochondria

The import reaction mixture contained buffer A (3% BSA fatty-acid free, 50 mM Hepes pH 7.4), 2 mM NADH, 1 mM ATP, 0.2-0.5 mg/ml mitochondria and 1% radiolabelled precursor. Reactions routinely proceeded for 20 minutes at 25°C (37°C in the case of DHFR construct). Valinomycin and hemin were added when indicated. Import was stopped placing the tubes on ice. After the import reaction, samples were treated with proteinase K under *non-swelling* (sample diluted in SH buffer: 0.6 M

Sorbitol, 20 mM Hepes, pH 7.2) or *swelling* conditions (sample diluted in 20 mM Hepes pH 7.4).

Proteinase K (PK) degrades the precursor that has not been imported and remains outside. For proteinase K treatment, import reaction was split in two halves: one half was transferred to a tube (non-swelling or swelling) without PK; the second half was added to a similar tube but containing PK (20 μg/ml). All tubes were on ice for 15 minutes, and PMSF was finally added to stop the treatment. Samples were centrifuged at 12,000 r.p.m. for 10 minutes (4 °C) and washed once with cold SH buffer/PMSF. As a last step, mitochondrial proteins were denatured in Laemmli buffer for SDS-PAGE analysis.

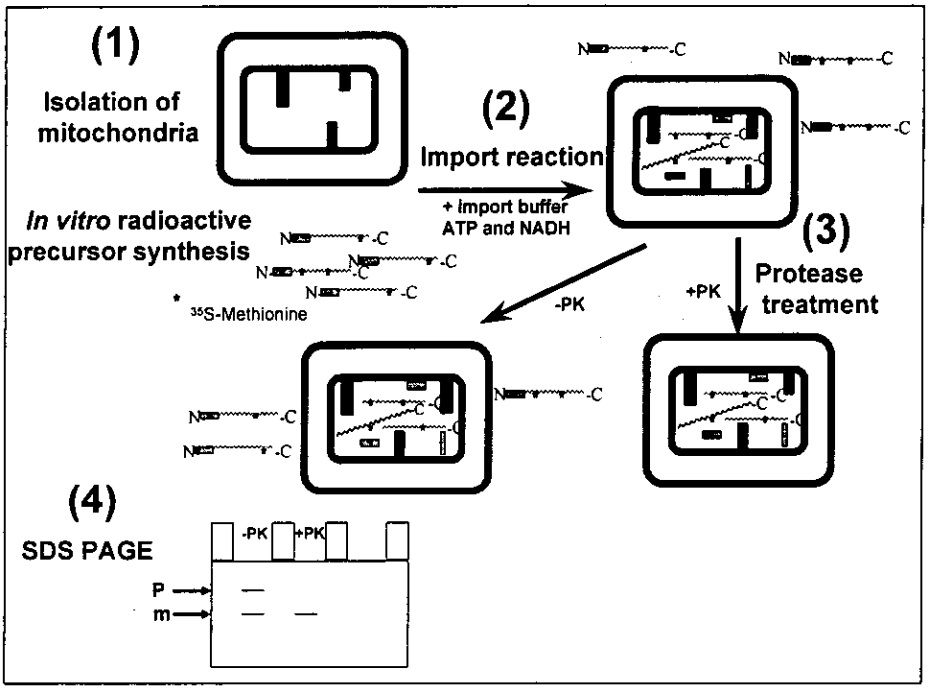

Figure 2.3. An import experiment: schematic overview of the procedure. The radioactive precursor is incubated with isolated mitochondria in the appropriate buffer at the conditions of time and temperature required. For an standard import assay, mitochondria are energised by supplementing the mixture reaction with ATP and NADH. To monitor whether the protein has entered the mitochondria, a treatment with a protease (proteinase K) degrades non-imported precursor which remains outside the mitochondria, and, thus, non-protected against the proteolytical digestion. Since precursors are frequently subjected to proteolytic clevage along the import, the differences in size of bands corresponding to the precursor and the mature forms, resolved by SDS/PAGE, indicate if the import has taken place.

2.4.3. Submitochondrial localisation of imported proteins

2.4.3.1.Conversion to mitoplasts

To ascertain the presence of imported proteins in the intermembrane space, hypotonic swelling of mitochondria was carried out as follows. After the import, mitochondria were centrifuged (12,000 r.p.m., 10 minutes, 4 °C) and resuspended in SH buffer. One half of the mitochondria suspension was diluted 10-fold in 20 mM Hepes pH 7.4 in the presence of PK (swelling). The other half was diluted in SH buffer at the same extent and also PK-treated (non-swelling). Mitochondria/mitoplasts were re-isolated (12,000 r.p.m., 10 minutes, 4 °C), washed once with SH buffer/PMSF, and then denatured in Laemmli buffer for SDS-PAGE analysis.

2.4.3.2. Subfractionation by sonication

Sonication of mitochondria allows to know whether an imported protein is soluble or associated to membrane. Following import, mitochondria were re-isolated, washed and resuspended in SH buffer. Mitochondria were subjected to hypotonic swelling, and the resulting mitoplasts were then sonified (Branson sonifier 250, microtip, step 2, 80% duty, 10x 10 s pulse) on ice. The sonified sample was split in two: one half was TCA-precipitated (total), the other half was centrifuged (22,600 g, 30 minutes, 2°C) and the pellet was resuspended in SH buffer. The pellet suspension and the supernatant were finally precipitated with TCA.

2.4.3.3. Protein extraction with carbonate

Treatment with sodium carbonate extracts proteins which remain peripherally associated to membranes, while integral proteins are not released. To determine if an imported protein was embedded in the membrane or not, after import mitochondria were pelleted and resuspended in 500 µl of 0.1 M CO_3Na_2 for extraction. The sample was incubated for 30 minutes on ice and then split in two: one half was TCA-precipitated (total), the other half was centrifuged (22,600 g, 30 minutes, 2°C). The pellet was resuspended in SH buffer. As the last step, the pellet suspension and the supernatant were TCA-precipitated.

2.5. COMPUTER METHODS

Recording, assembling and analyses of the nucleotide sequences were performed with DNASIS/PROSIS (Hitachi). Searches for DNA and protein homologies were done with FASTA and TFASTA (Pearson and Lipman, 1988). Protein sequences were aligned using the programme CLUSTAL W (Thompson *et al.*, 1994), and the codon usage was determined with CODONS (Lloyd and Sharp, 1992) and PICDI (Rodríguez-Belmonte *et al.*, 1996) software. Promoter analysis was assisted by MatInspector programme (Quandt *et al.*, 1995). Sequences queries were usually sent to NCBI.

OBJECTIVES

OBJECTIVES

Kluyveromyces lactis has become an alternative to the traditional yeast *Saccharomyces cerevisiae*, owing to its industrial applications as well as to studies on mitochondrial respiration. Both yeasts belong to the facultative aerobic group which display the ability to metabolise glucose by oxidative and oxido-reductive pathways. The difference lies in the relative importance of these routes in aerobiosis: while fementation predominates over respiration in *S. cerevisiae* (aerobic fermentative), respiration is prevalent in *K. lactis* (aerobic respiratory yeast). This might result from a distinctive regulation of the energy-yielding metabolism in the two species.

In *S. cerevisiae*, the respiro-fermentative metabolism is controlled by environmental stimuli, including the availability of oxygen and carbon source nature. Oxygen levels determine the expression of many genes related to the oxidative metabolism by mechanisms in which participate heme-modulated transcriptional factors.

The respiro-fermentative metabolism in *K. lactis* has been still less studied, but experimental evidence implies a regulation by oxygen, probably mediated by heme. In this context, the obtention of a heme-deficient *K. lactis* strain was envisaged as a useful tool to investigate the oxygen-dependent regulation and, furthermore, to define the role of heme in the signal transduction of this mechanism. The isolation of the *K. lactis HEM1* gene (*KlHEM1*) had as main goal the construction of a heme-deficient mutant by disruption. On the other hand, the characterisation of *KlHEM1*, coding the first enzyme in the heme biosynthetic pathway (5-aminolevulinic acid synthase), would provide information about the synthesis of heme in *K. lactis*, a metabolic process, functionally linked to the oxidative metabolism, which had not been previously dealt with in this yeast. Although this project was initially conceived to analyse the effect of heme on transcription, the structural features of the 5-aminolevulinic acid synthase prompted us to consider postranscriptional events as well.

Three main objectives were intended:

1°. Characterisation of *KlHEM1* which codes the *K. lactis* 5-aminolevulinate synthase (*Kl*ALAS), and construction of a heme-deficient mutant by disruption of this gene.

2°. Analysis of the role of heme in the regulation of gene expression in response to oxygen in *K. lactis* (transcriptional effect) .

3°. Approach to the protein import into *K. lactis* mitochondria for a further study of the heme effect on the transport of *Kl*ALAS to this organello (postranscriptional effect).

CHAPTER I.
CHARACTERIZATION OF THE *Kluyveromyces lactis* *HEM1* GENE

I.1. THE *Kluyveromyces lactis HEM1* GENE (*KlHEM1*)

I.1.1. Subcloning and localisation

The *KlHEM1* gene was cloned from a *K. lactis* genomic library by complementation of the respiratory deficiency of a *S. cerevisiae hem1* mutant strain (Méndez-Carro, 1992). In order to localise the position of the gene within the isolated 5.5 kb genomic insert, restriction fragments were subcloned in the YEp*lac*112 vector. A derivative carrying an internal deletion was also constructed in the YEp*lac*195 vector. *S. cerevisiae hem1* mutant cells transformed with each subclon were screened for growth on lactate, and the restablishment of the heme biosynthesis pathway in the transformants was tested in the halo assay (Bard and Ingolia, 1984), as it is summarised in Fig. I.1. Subclones **pMD3.5** and **pMD2.4** restored respiration in the *S. cerevisiae hem1* mutant, since transformants with these constructs grew on lactate. These results were confirmed in the halo assay: transformants carrying **pMD3.5** and **pMD2.4** supported the growth of the *S. cerevisiae hem1* mutant as feeder colonies when patched onto top agar containing these mutant cells. From these observations we inferred that a functional *KlHEM1* gene span over a 3.5 kb *Sal* I-*Sca* I fragment in the original clone.

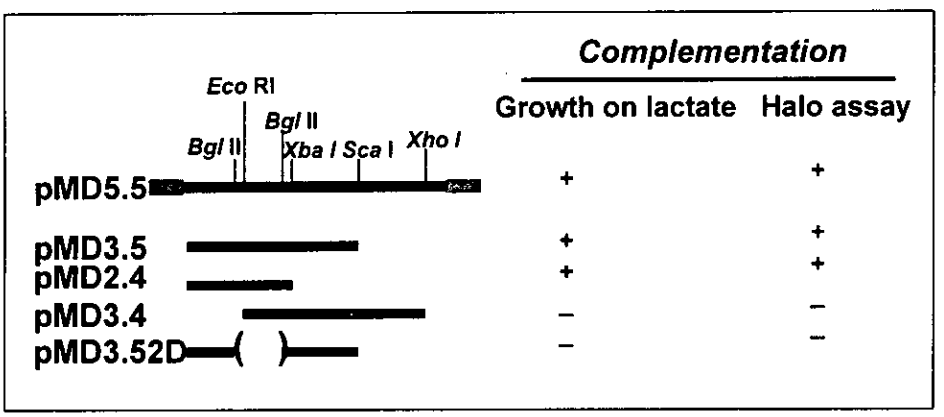

Figure I.1. Localisation of the *KlHEM1* gene within a genomic insert. A partial restriction map of the original clone is given in the upper part. *S. cerevisiae hem1* strain was transformed with different subclones and tested for growth on lactate and in the halo assay (+, denotes complementation; -, denotes lack of complementation).

I.1.2. Nucleotide sequence

The nucleotide sequence of the 3.5 kb *Sal* I-*Sca* I fragment was determined on both strands using a combination of nested deletions and gene walking with synthetic oligonucleotides. This sequence is deposited in the Genebank database under the accession number X92944. Sequence analysis revealed an ORF of 1,713 nt, encoding a putative protein of 570 residues (Fig. I.3). This *K. lactis* gene exhibited strong homology with others coding for ALAS enzymes from different sources, and this held true for the deduced protein sequence. Homology percentages for the best scores obtained from computer searches are presented in Fig. I.2. The highest homology was reached with the *S. cerevisiae HEM1* gene (71% at the DNA level and 72% at the protein level), while lower values were obtained with phylogenetically less related species.

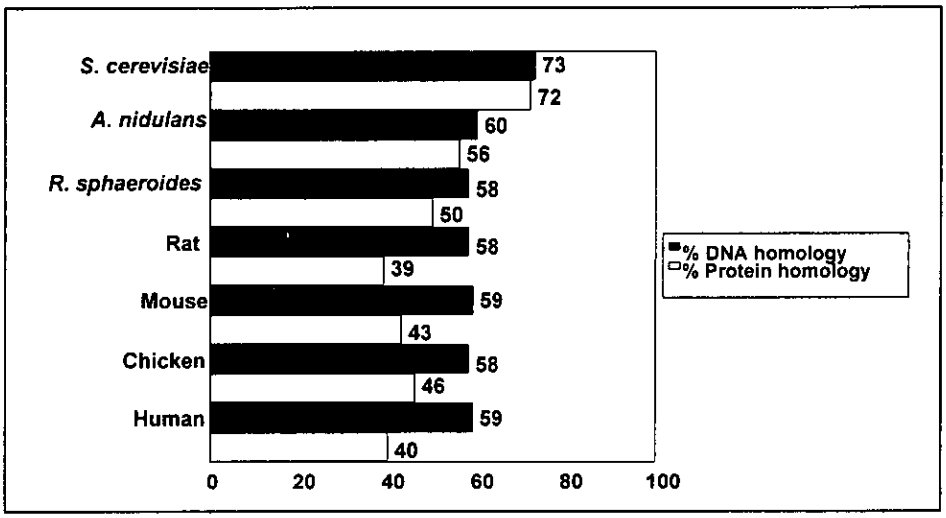

Figure I.2. Comparison of *KlHEM1* with ALAS encoding genes. DNA homology percentage is represented by shaded bars and protein homology percentage is represented by white bars. Sequences were retrieved from GenBank and EMBL databases (see references in Table I.2).

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TTAGTTATTGGTATTCTCTAGAAGTTAAATTTCTGGCGGTGGTTGGTGTCGCGAAGCGAC -601
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M  E  S  V  I  R  S  S  A  K  I  C  P  F  M  H  S  A  T  G     20
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S  M  Q  S  V  K  A  L  K  N  A  N  L  P  A  I  A  Q  Q  C     40
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P  F  M  G  K  A  M  E  Q  R  R  G  Y  A  S  S  A  S  G  A     60
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CCTGAATTCCGCACCGTTATGGATCGTGTTGATATGATCACTGGTACCTTGGGTAAATCT 1080
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CCGGCGCACAGGTGCGGTTGCTGGCGCCTATATCGCCGGACATCACCG....
```

Figure I.3. Sequence of the *KlHEM1* gene and flanking regions. Nucleotides and amino acids are numbered referring to the translational initiation codon ATG. Putative binding sites for specific transcriptional factors and TATA elements are underlined.

Deviated use of synonymous codons in *KlHEM1* was measured by calculation of the *codon adaptation index*, with the CODONS program (Lloyd and Sharp, 1993) and the *intrinsic codon deviation index*, with the PICDI programme (Rodríguez-Belmonte *et al.*, 1996) (CAI= 0.45; ICDI= 0.21). The values obtained in both approaches for *KlHEM1* are higher than those calculated for the *S. cerevisiae* gene (CAI= 0.21; ICDI= 0.11). Urban-Grimal *et al.* (1986) reported that low codon-bias in the gene *HEM1* of *S. cerevisiae* was consistent with its low expression, since its product only represents 0.01-0.02% of the total protein of the cell. Whether higher codon-bias accounts for higher expression in yeast is still under debate, although evidence from comparison between the *CYC1* of *S. cerevisiae* and *K. lactis* supports this correlation (Freire-Picos *et al.*, 1994).

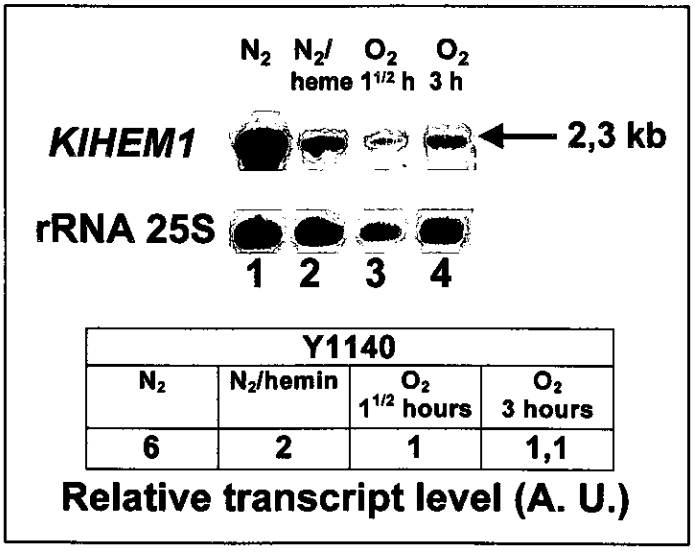
Concerning the 5' non-coding region (Fig. I.3), two putative TATA boxes are situated at -81 and -239 from the translation initiation codon. A pyrimidine-rich element extends between -374 and –411 (McNeil, 1988). A search for transcriptional factor binding sites, using the programme mentioned in section 2.5, found several motifs. Two motifs for Gcr1p lie at positions –475 and –388 respectively. Gcr1p activates genes encoding glycolytic enzymes (Huie and Baker, 1996), but it may be also required for the expression of some other genes (Ciriacy *et al.*, 1991). At position –656 there is a consensus for the binding of the Hap2/3/4/5p complex. This heteromer promotes the transcription of genes mainly in response to heme/oxygen sufficiency and/or growth on non-fermentable substrates (Zitomer and Lowry, 1992). One consensus motif for the Ino2p factor, characteristic of genes related to phospholipid metabolism (Koipally *et al.*, 1996) is present at –530. A site for the glucose-related repressor Mig1p appears at –540 (Cassart *et al.*, 1995). Between –515 to –505 lies a sequence similar to URS1, recognised by Buf (Luche *et al.*, 1992).

the URS1 was first reported in relation to the arginine pathway, it has also been found in the promoters of many other genes involved in carbon source and nitrogen metabolism, meiosis, sporulation or mating-type switching, and in genes encoding components of the respiratory chain (Luche *et al.*, 1993).

I.1.3. The transcription of the *KlHEM1* gene is regulated by heme

The concentration of heme affects a great variety of processes in response to the availability of oxygen in *S. cerevisiae*. Oxygen is necessary for the biosynthesis of heme and has regulatory effect on this pathway as well. We decided to test if the oxygen could modulate the transcription of the *K. lactis HEM1* gene.

Cells were subjected to a shift from hypoxic to aerobic conditions. Changes in *KlHEM1* transcript levels were analysed by Northen blot, using total RNA extracted from cells before and after the shift to aerobiosis. *KlHEM1* mRNA levels decreased as consequence of the oxygen shift (Fig. I.4, compare lanes 1 with 3 and 4). This repression is heme-dependent, as it tends to revert when hemin is added to a hypoxic culture (Fig. I.4, lane 2).



Y1140			
N_2	N_2/hemin	O_2 $1^{1/2}$ hours	O_2 3 hours
6	2	1	1,1

Relative transcript level (A. U.)

Figure I.4. Heme represses the expression of the *KlHEM1* gene. *K. lactis* Y1140 cells were maintained in hypoxia for 4 hours (lane 1) or in the same condition supplemented with hemin (26 μg/ml) (lane 2); and then shifted to aerobic conditions for 1 and an half (lane 3) or 3 hours (lane 4). The blot was hybridised with the internal 0.7 kb *Eco* RI-*Pst* I fragment of the *KlHEM1* gene, and reprobed with the end-terminal labelled RNAr 25S as control of loading. A.U: arbitrary units.

I.2. THE *K. lactis* 5-AMINOLEVULINIC ACID SYNTHASE (*Kl*ALAS)

I.2.1. Sequence analysis

The *KlHEM1* gene sequence predicts a protein of 570 amino acids with an estimated molecular weight of 62.6 kDa and a theoretical isoelectric point of 6.9. The N-terminal region shows the structural features of a mitochondrial-targeting signal (presequence): hydroxylated and basic amino acids with the potential to form an amphipatic helix (Fig. I.5 A). *Kl*ALAS might be synthesised in the cytosol and then imported into mitochondria, similarly to other eukaryotic ALASs. Once in the mitochondria, the *Kl*ALAS precursor might be processed by a specific matrix peptidase which cleaves off the presequence, generating a more hydrophobic N-terminus in the mature form. The N-terminus of the mature ALAS of *S. cerevisiae* is also rich in hydrophobic residues and it could anchor the protein to the mitochondrial inner membrane (Urban-Grimal *et al.*, 1986). Moreover, the *Kl*ALAS presequence contains two HRM (see section 1.1.4.2), a characteristic of other eukaryotic ALASs which is not shared by the *S. cerevisae* homologue (Fig. I.5 B). Hydropathy analysis ruled out the presence of transmembrane segments in any of the two yeast ALAS (Fig. I.6).

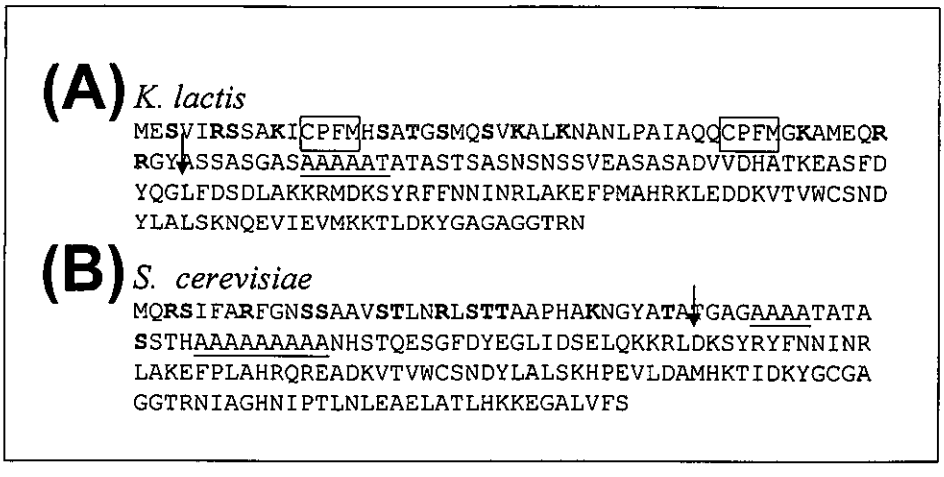


Figure I.5. Structure of the N-terminal region of the ALAS from (A)*K. lactis* and (B)*S. cerevisae* ALAS. Outstanding residues of the putative amphipatic mitochondrial import signal are in bold. The hydrophobic stretch rich in alanine is underlined. The HRM are boxed. The arrows indicate the putative cleavage site of a matrix peptidase.

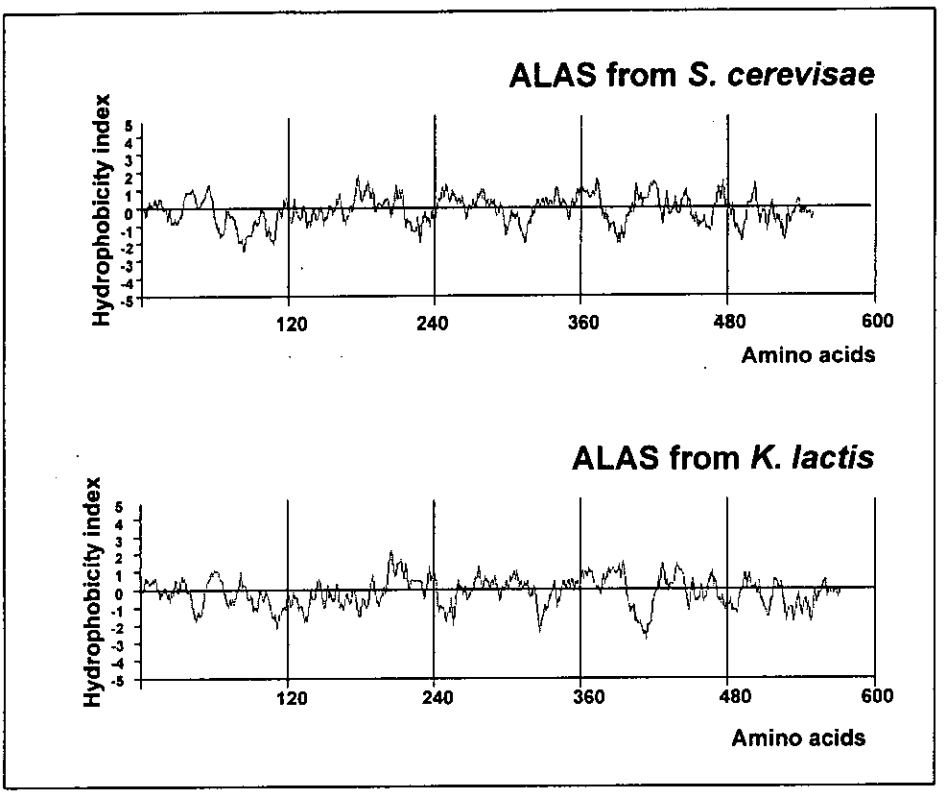


Figure I.6. Hydropathy profile of the ALAS from S. *cerevisae* and *K. lactis*
Evaluation of hydropathy was performed according to Kyte and Doolittle (1982) at a span of 20 amino acids.

Comparison of the *K. lactis* ALAS with ALAS from different origins shows an extensive similarity among them, as previously reported (Cox *et al.*, 1991; Neiddle and Kaplan, 1993; Ferreira and Gong, 1995) (Fig. I.7). This homology is centred in the C-terminal region, while the N-terminus is less conserved. All the ALAS sequences have in common the glycine rich motif GAGAGG, which is thought to participate directly in binding the PLP cofactor. There is also a well-conserved lysine involved in the Schiff-base linkage with PLP (Hunter and Ferreira, 1999).

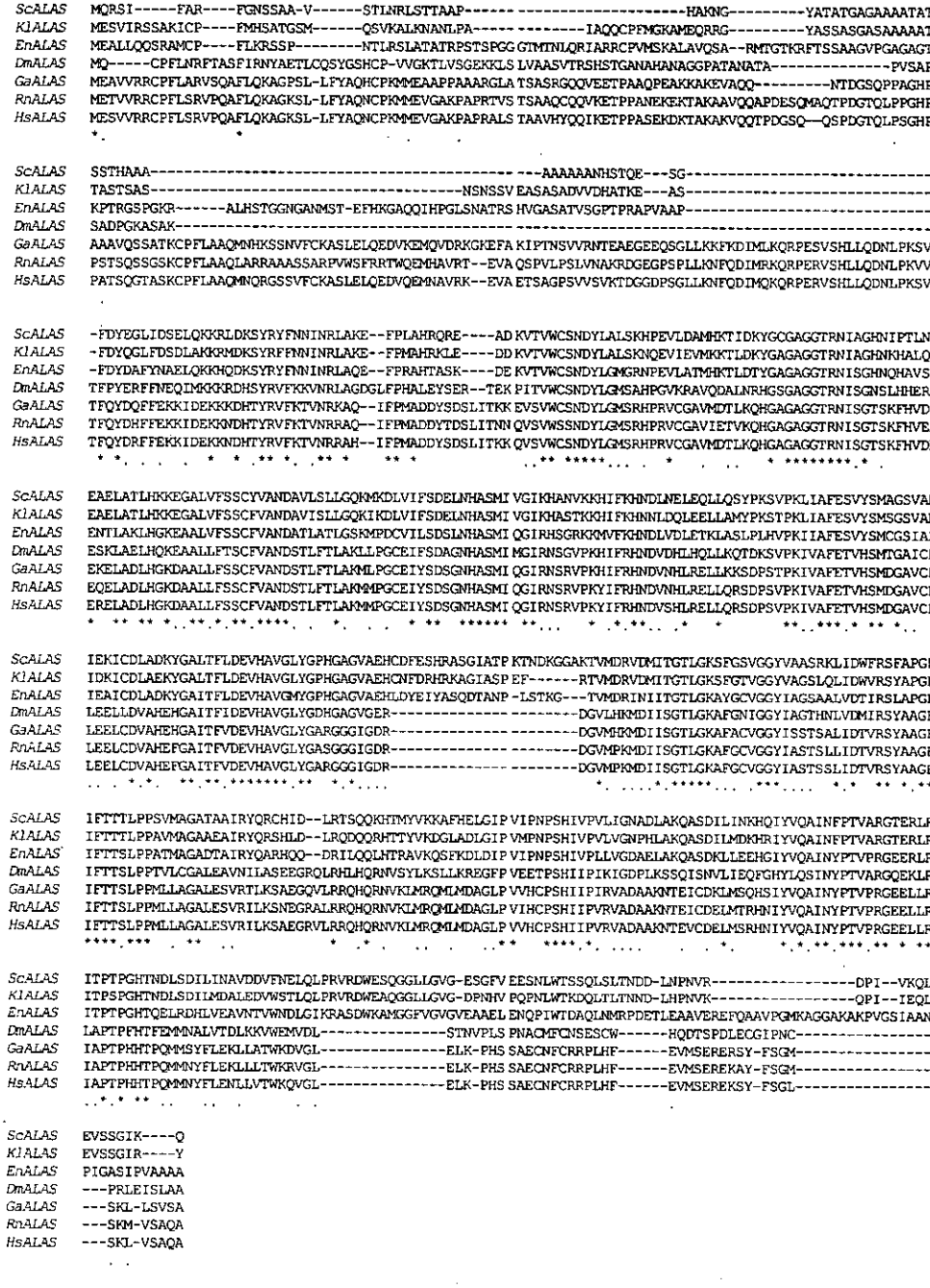


Figure I.7. CLUSTAL alignment of the *K. lactis* ALAS with other eukaryotic homologues. Identical residues are marked with a star and conservative changes are indicated with a dot. Sequences aligned: *ScALAS, S. cerevisiae*; *KlALAS, K. lactis*; *EnALAS, Aspergillus nidulans*; *DmALAS, Drosophila melanogaster*; *GaALAS*, chicken (housekeeping isozyme); *RnALAS*, rat (housekeeping isozyme); and *HsALAS*, human (housekeeping isozyme) (see references in Table 1.2).

I.2.2. Preparation of a polyclonal antibody against the *Kl*ALAS

The availability of a specific antibody against the *Kl*ALAS would be an advantageous tool to purify and characterise the protein. With the goal of raising this antibody, we overexpressed a C-terminal fragment of the *Kl*ALAS in bacteria by means of the maltose-binding fusion protein strategy.

I.2.2.1. Production of the *Kl*ALAS antigen in bacteria

An *Eco* RI-*Sal* I fragment from the clon pMD3s, containing 227 amino acids of the C-terminal moiety of *Kl*ALAS, was fused in frame to the maltose-binding protein sequence of the pMAL-c vector (Fig. I.8). This construct was transformed into and expressed in *E. coli* DH5.

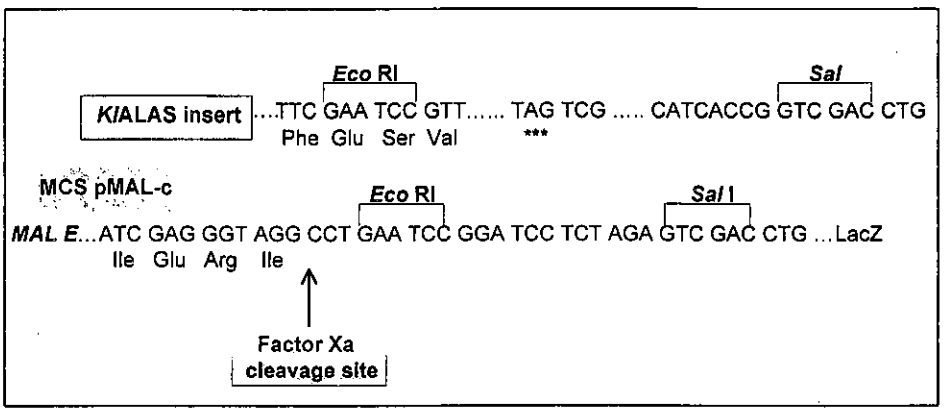


Figure I.8. Strategy for fusing a C-terminal fragment of *K. lactis* ALAS to the pMAL-c vector. The 5'and 3'ends of the coding strand of the cloned fragment are indicated. A linker containing the recognition/cleavage site of the factor Xa lies between the MBP (maltose binding protein) and the *Kl*ALAS insert.

The expression of the fusion was monitored in a pilot experiment. Cells from a 20 ml culture of transformed bacteria were grown at 37°C to an $O.D_{600}$ 0.5. IPTG was then added to a final concentration of 0.3 mM, and incubation pursued at 37°C for 8 hours. To follow the induction, samples were taken before (uninduced) and after the addition of IPTG at regular intervals of time (induced sample), and cells were harvested. Finally, all the samples were denatured, by 5 minutes boiling in Laemli buffer, and analysed by SDS-PAGE (Fig. I.9). Induction with IPTG produced a protein of approximately 66 kDa which was undetectable in non-induced cells. From this preliminary experiment, the conditions for the large-scale procedure were established as follows: 100 ml culture induced with IPTG at a final concentration of 0.3 mM for 6 hours.

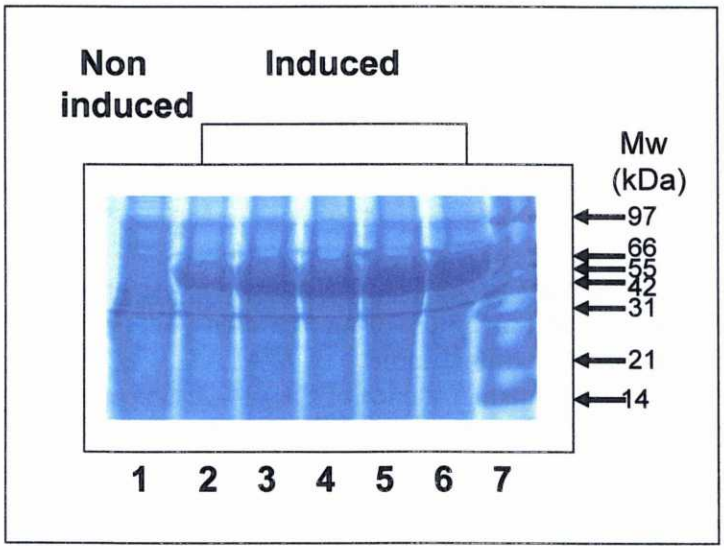


Figure I.9. Expression of MBP-*Kl*ALAS fusion protein in *E. coli*. Samples were taken before (lane 1) and after (lanes 2-6) the induction with IPTG and processed for SDS-PAGE analysis. The gel was stained with Coomassie solution. Molecular weight standards (Mid Ranged Protein Mw Marker, Promega) were loaded in lane 7.

More than 80% of the expressed MBP-*Kl*ALAS formed insoluble aggregates (inclusion bodies) which allowed an easy purification of the fusion protein. Cells were lysed by sonication and inclusion bodies were isolated by low speed centrifugation. The fusion protein was eventually solubilised in a buffer containing 50 mM Tris·HCl pH 6.4, 50 mM DTT and 8 M Urea, with a final yield of 0.5 g protein/l of induced culture.

To separate the *Kl*ALAS antigen from the MBP, the fusion protein was digested with factor Xa protease (New England Biolabs). As the urea of the solubilisation buffer inhibited the enzymatic activity, two steps of dialysis were performed before digestion. The sample, in a final volume of 4 ml, was dialysed against 400 ml of factor Xa reaction buffer (20 mM Tris·HCl pH 8, 1 mM $CaCl_2$ and 100 mM NaCl) at 4°C with 4 changes of buffer over 16 hours. The digestion with factor Xa had to be optimised and was only complete with the addition of SDS to the reaction mixture. The best results were obtained with 0.02%-0.03% SDS (Fig. I.10, lanes 3 and 4).

The digestion of the MBP-*Kl*ALAS fusion resulted in two fragments of 42 kDa (MBP) and 26 kDa (*Kl*ALAS antigen). To confirm the nature of the smaller fragment before immunisation, we carried out a Western blot analysis of the digestion products with an anti-*S. cerevisiae* ALAS antibody (kindly provided by Dr. Labbe-Bois, Institut Jacques Monod, Paris). This

antibody recognised specifically the band corresponding to the *K. lactis* antigen (Fig. I.10, lane 8).

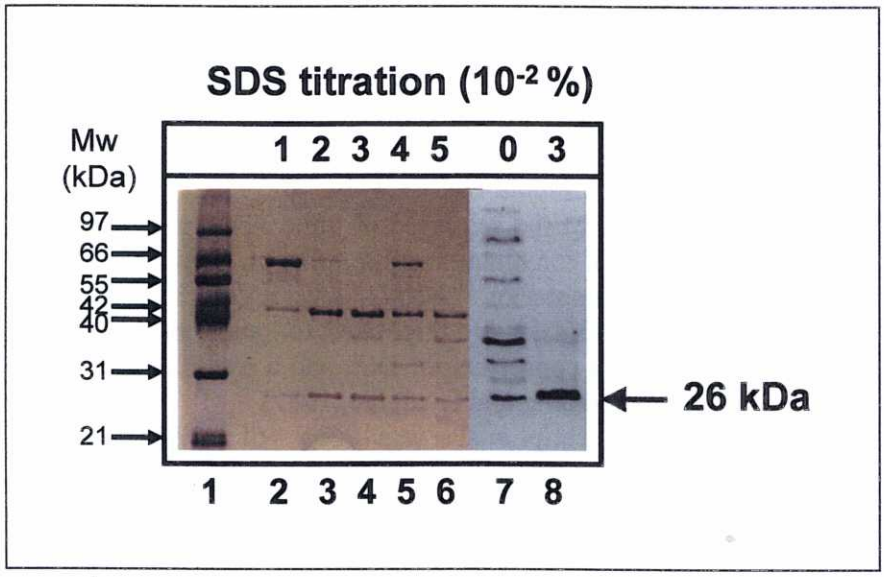


Figure I.10. Cleavage of the MBP-*Kl*ALAS fusion protein by factor Xa in the presence of SDS. 20 µl digestion samples (6 µg of fusion protein; 0,5 µg of factor Xa; 2 µl 10X factor Xa reaction buffer) with SDS at the indicated final concentration were incubated at 37°C for 3 hours. Digestion products were separated by SDS-PAGE. Molecular weight standards (Mid Ranged Protein Mw Marker, Promega) were loaded in lane 1. After electrophoresis the gel was stained with Coomassie blue solution (lanes 1-6), or transferred onto a nitrocelullose filter for Western blot analysis (lanes 7-8).

I.2.2.2. Immunisation and recovery of the mouse antibody

BALB/c mice were intraperitoneally immunised with 0.2 ml of a 1:1 (v/v) mixture of Freund's complete adjuvant (FCA) and PBS containing either 30 µg of the MBP-*Kl*ALAS fusion protein or 30 µg of the *Kl*ALAS fragment. The immunological reaction against the specific *Kl*ALAS fragment was weak after the first bleeding; therefore, the second dose of immunisation was performed only with the fusion protein. Mice were bled two months later and the sera was mixed 1:1 (v/v) with glycerol and stored at –20 °C. The specificity of the mouse antibody against *Kl*ALAS was assessed by Western blot (data non shown), but the low titre of the sera did not allow a good decoration of the filter.

I.3. DISCUSSION

The *K. lactis HEM1*(*KlHEM1*) gene was cloned by functional complementation of a *S. cerevisiae hem1* mutant (Méndez-Carro, 1992). We localised *KlHEM1* in an approximately 3.5 kb fragment of this genomic insert by subcloning and complementation tests. Sequence analysis revealed an ORF of 1,713 nt with the potential to encode a protein of 570 amino acids, flanked by 957 bp at the 5′ region and 345 bp at the 3′region. *KlHEM1* displays strong homology with other genes encoding ALA synthases from different sources, in particular with the *HEM1* genes from *S. cerevisiae* and *A. nidulans*.

In *silico* analysis of the sequence of the 5′non-coding region of the gene showed a complex combination of putative *cis* regulatory signals. On comparing with the promoter of *ScHEM1*, several coincidences deserve to be remarked: a Hap2/3/4/5p binding site, followed downstream by a negative element with a consensus for Buf, and a pyrimidine-rich element close to the TATA box (Keng and Guarente, 1987).

Concerning transcriptional regulation of *HEM1*, significant differences between *S. cerevisae* and *K. lactis* can be pointed out. The expression of *ScHEM1* is constitutive, regardless the carbon source availability or the heme levels (Keng and Guarente, 1987). By contrast, oxygen affects negatively the *K. lactis HEM1* gene, since this gene is more expressed in hypoxia than in aerobic conditions. Furthermore, the induction observed in hypoxia might be interpreted as an alleviation of the repression in aerobic conditions. Heme can also prevent the transcription of *KlHEM1*, and hence, exerting a feed-back control on its biosynthesis at the first step of the pathway in *K. lactis*. This evidence also suggested that heme could regulate gene transcription in *K. lactis*, what prompted us to deal with this matter as will be presented in chapter II.

The *K. lactis* ALAS bears the structural features required for the activity of the enzyme, such as the glycine-rich to which the PLP cofactor binds (Gong *et al.*, 1996). A mitochondrial-targeting signal at the N-terminal region indicates that *Kl*ALAS is synthesized in the cytosol and goes postranslationally into mitochondria (Neupert, 1997). The requirement of mitochondrial import in the biogenesis of the ALAS represents a point of regulation of the enzymic activity, and, consequently, of the heme biosynthesis as a whole. In higher eukaryotes, heme inhibits the transport of ALAS to the mitochondria through specific motifs (Lathrop and Timko, 1993). This regulation could also affect the *K. lactis* protein, since it contains the necessary HRM, and data concerning this aspect will be discussed in chapter III. The absence of HRM in the *S. cerevisiae* homologue excludes a similar postrancriptional control by heme (Urban-Grimal *et al.*, 1986).

Taken together, while in *S. cerevisiae* heme does not affect the expression of *HEM1*, in *K. lactis* heme seems to regulate the gene both at transcriptional and postranscriptional levels. This contrast might be relevant to understand the differences regarding the respiro-fermentative metabolism between *S. cerevisae* and *K. lactis*, given the central role of heme in the respiration.

CHAPTER II. TRANSCRIPTIONAL REGULATION BY HEME IN *Kluyveromyces lactis*

In *S. cerevisiae* the intracellular level of heme mediates the effect of oxygen on the expression of many genes related to the respiratory metabolism. The involvement of heme in the transcriptional regulation by oxygen has been clearly demonstrated with the use of heme biosynthetic mutants, in which the heme auxotrophy reproduces the pattern of the gene expression observed in the absence of oxygen. While in *S. cerevisiae* the mechanisms controlling the respiratory function at the transcriptional level have been widely studied, in *K. lactis* the information available is short. In order to gain a deeper insight on this subject, we analysed in *K. lactis* the expression of three genes which are oxygen-responsive in *S. cerevisiae*. We asked whether these *K. lactis* genes would be regulated by oxygen, and if so, whether heme would intervene in this regulation, as it has been demonstrated in *S. cerevisiae*. To answer the second question we constructed a *K. lactis* heme-deficient mutant by disruption of the *KlHEM1* gene.

II.1. CONSTRUCTION AND CHARACTERISATION OF A *K. lactis hem1* NULL MUTANT

The *KlHEM1* gene disruption strategy is depicted in Fig. II.1 A. An internal 0.7 kb *Hind* III-*Eco* RI fragment of the gene was cloned in the integrative plasmid YIp*lac*211 (Gietz and Sugino, 1988). This construct was linearized with *Asp*718 and then used to transform the *K. lactis* haploid strain MW190-9B. Prototroph candidates for uracil were subsequently screened in CM-lactate, and those which did not grow on lactate plates were chosen. Southern analysis of the candidate *Klhem1*::URA3 confirmed the correct replacement (Fig. II.1 B).

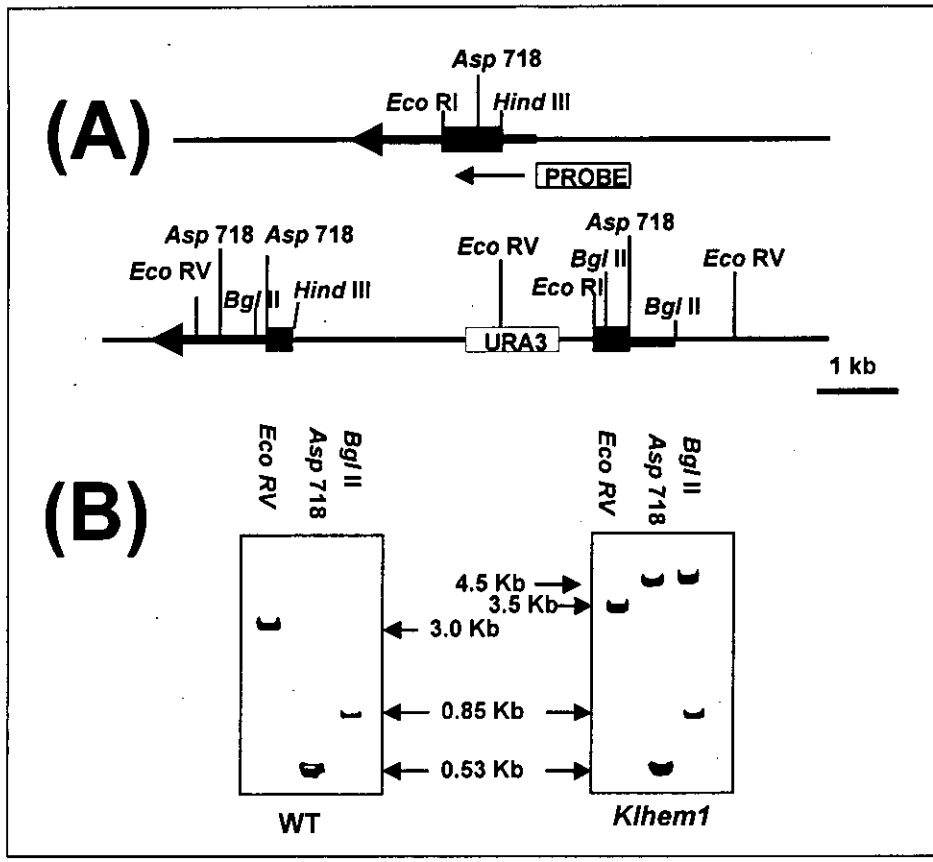


Figure II.1. Disruption of the *KlHEM1* gene. (A)Schematic representation of the disruption strategy. (B)Analysis of the disruption by Southern blot. Genomic DNA from MW190-9B (wt) and the null mutant (*Klhem1*::URA3) were digested with the enzymes *Eco* RV, *Asp* 718 and *Bgl* II. The hybridisation was carried out at 52°C for 5 hours, using a probe containing a 0.85 kb *Bgl* II-*Bgl* II fragment of *KlHEM1*.

The functional efficacy of the gene replacement in the null mutant was also confirmed by the absence of the 2.3 kb *KlHEM1* transcript of the wild type (Fig. II.2 A). Since disruption of *KlHEM1* prevents heme biosynthesis, the *Klhem1*::*URA3* strain lacks functional cytochromes, and therefore, it is not able to grow on non-fermentable media such as lactate (Fig. II.2 B).

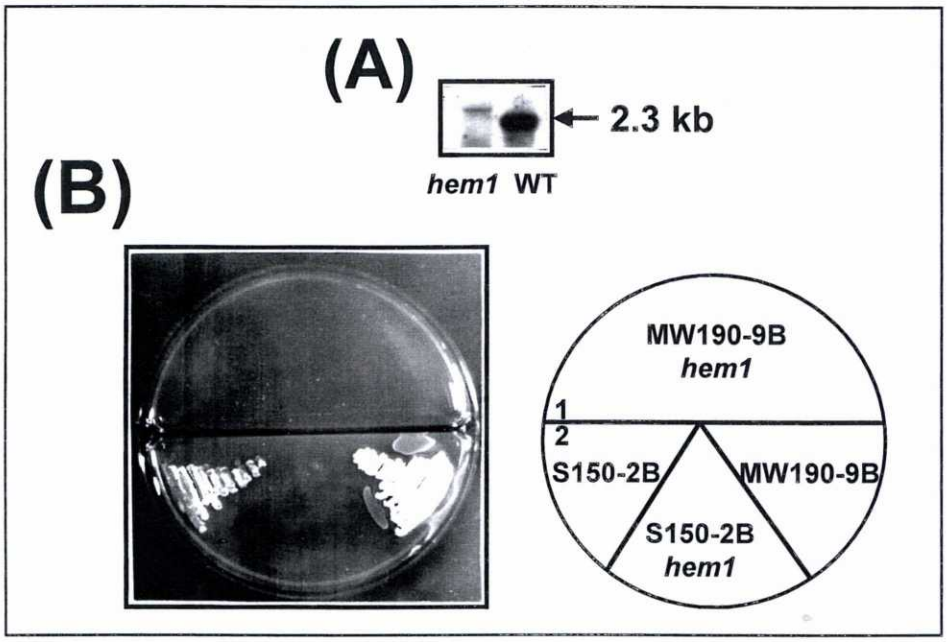
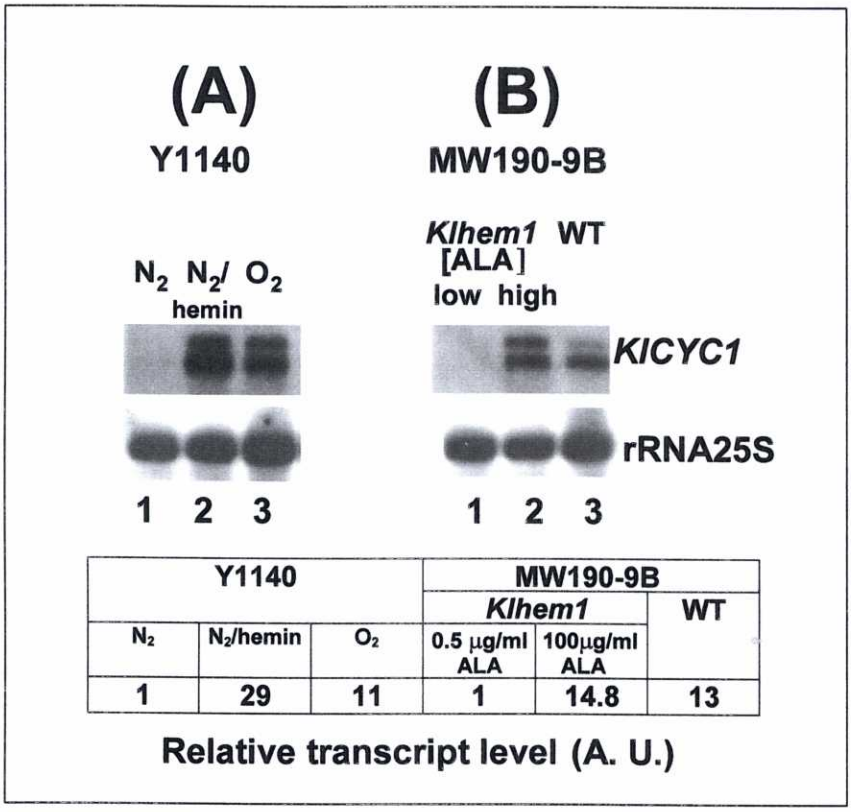


Figure II.2. Characterisation of the *K. lactis hem1* null mutant. (A)Northern blot analysis. Total RNA was extracted from the wild type and mutant strains for hybridisation with a 0.7 kb *Eco* RI-*Pst* I fragment of *KlHEM1*. **(B)Phenotypic test for respiratory growth**. *K. lactis* MW190-9B and *S. cerevisiae* S150-2B together with the derivatives *hem1* mutants were spread onto a lactate plate (**1**, CM-uracil; **2**, CM) and photographed after two days.

II.2. THE OXYGEN/HEME EFFECT ON THE *K. lactis CYC1* GENE (*KlCYC1*)

In our aim to determine the involvement of heme in the transcriptional regulation by oxygen in *K. lactis*, *KlCYC1* was a gene of choice, as a well-studied oxygen/heme-responsive gene in this yeast (Freire-Picos *et al.*, 1995), to test the accuracy of the experimental approach. The strategy consisted of: 1)an analysis of the response to oxygen, when cultures were shifted from hypoxic to aerobic conditions; 2)an analysis of the heme effect with the *K. lactis hem1* mutant (*in vivo*), or by adding hemin to hypoxic cultures. For experiments carried out with the mutant strain, cultures were supplemented with either low or high levels of 5-aminolevulinic acid to generate respectively heme-deficient or heme-sufficient conditions inside the cells. For shift experiments, *K. lactis* Y1140 strain was grown in hypoxia either with or without hemin for 4 hours and then, the culture without hemin was further incubated under aerobic conditions for 3 hours.

Data showed that the transcription of the *KlCYC1* gene is induced in the presence of oxygen and not in hypoxia, as had been already reported (Fig. II.3 A, compare lanes 3 and 1). The availability of heme determines the levels of expression, since *KlCYC1* transcription could be restored in hypoxia when hemin was added to the culture (Fig. II.3 A, lane 2). The involvement of heme in *KlCYC1* expression was demonstrated *in vivo* using the *hem1* mutant: in heme-sufficient conditions *KlCYC1* was optimally expressed (Fig. II.3 B, lanes 2 and 3), while the expression decayed drastically in heme-deficient conditions (Fig. II.3 B, lane 1).



Y1140			MW190-9B		
			Klhem1		WT
N_2	N_2/hemin	O_2	0.5 µg/ml ALA	100µg/ml ALA	
1	29	11	1	14.8	13

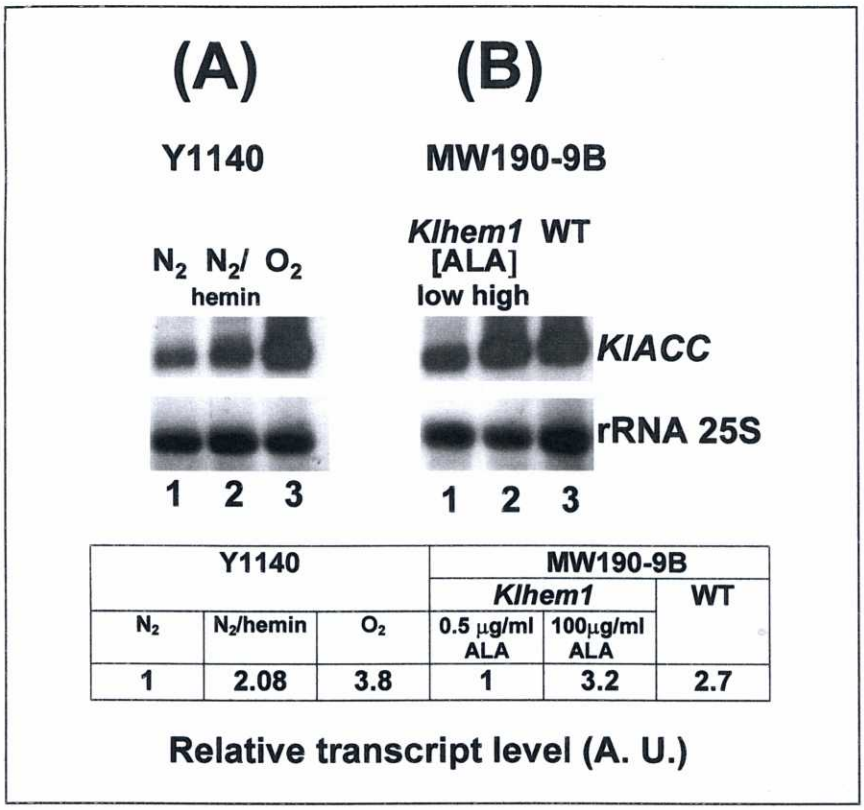
Relative transcript level (A. U.)

Figure II.3. Transcriptional effect on *KlCYC1*. (A)*KlCYC1* is an aerobic gene. The *K. lactis* Y1140 strain was grown under hypoxia for 4 hours in the absence (lane 1) or presence (lane 2) of 26 µg/ml hemin, and then shifted to aerobic conditions for 3 hours (lane 3). Total RNA was extracted, fractionated in a 1,5 % agarose/formaldehyde gel and transferred to a nylon filter. The filter was hybridised with a 1.9 kb *Pst* I fragment from the plasmid pART10 containing the *KlCYC1* gene (upper panel), and then reprobed with rRNA as a control of loading (lower panel). **(B)*In vivo* effect of heme on *KlCYC1* transcription.** Northern analysis as specified in (A), using for RNA extraction cultures of *K. lactis* MW190-9B *hem1* mutant grown in the presence of 0.5 µg/ml (lane 1) or 100 µg/ml of ALA (lane 2), and of the wild-type isogenic strain (lane 3). A.U.: arbitrary units.

II.3. THE OXYGEN/HEME EFFECT ON THE *K. lactis ACC* GENE (*KlACC*)

Viola *et al.* (1995) isolated the *K. lactis ACC* gene, homologue to the *ACC2* which encodes the most abundant ADP/ATP translocator isoform in *S. cerevisiae* (Lawson *et al.*, 1988). Given the role of this protein in ATP synthesis during oxidative metabolism, *KlACC* was also included in this analysis. *ACC2* is preferentially expressed in the presence of oxygen and heme (Betina *et al.*, 1995). We studied the behaviour of *K. lactis ACC* gene in response to oxygen levels, as well as the involvement of heme in that effect.

Northern blot analyses were performed according to what stated in section II.2, and the probe was prepared by PCR from *K. lactis* genomic DNA. As shown in Fig. II.4 A, the *K. lactis* gene was more expressed under aerobic conditions (lane 3), though the transcription was not completely abolished in hypoxia (lane 1); the addition of hemin to hypoxic cultures induced the expression, revealing that heme exerts a positive effect on *KlACC* transcription (lane 2). This positive action of heme was also observed when comparing the *KlACC* levels in heme-sufficient cultures (Fig. II.4 B, lanes 2 and 3) to those in heme-deficient cells (Fig. II.4 B, lane 1).



Y1140			MW190-9B		
			Klhem1		WT
N_2	N_2/hemin	O_2	0.5 µg/ml ALA	100µg/ml ALA	
1	2.08	3.8	1	3.2	2.7

Relative transcript level (A. U.)

Figure II.4. Transcriptional effect on *KlACC*. (A)*KlACC* is moderately induced in the presence of oxygen. Northern blot analysis was essentially carried out as described in the legend to Fig. II.3. The filter was hybridised with a 0.5 kb *ACC* fragment amplified by PCR from *K. lactis* genomic DNA (upper panel), and reprobed with rRNA as control of loading (lower panel). **(B)*KlACC* transcription depends on heme.** Northern analysis as specified in (A), using for RNA extraction cultures of *K. lactis* MW190-9B *hem1* mutant strain grown in the presence of 0.5 µg/ml (lane 1) or 100 µg/ml of ALA (lane 2), and of the wild-type isogenic strain (lane 3). A.U.: arbitrary units.

II.4. THE OXYGEN/HEME EFFECT ON THE *K. lactis HEM13* GENE (*KlHEM13*)

In *S. cerevisiae* the regulation of the heme biosynthesis pathway lies mainly in *HEM13* (*ScHEM13*), a hypoxic gene which is only expressed at low oxygen tension. Heme modulates the *ScHEM13* transcription, although it is not the only factor involved (Amillet *et al.*, 1995). Taking into account these considerations, we found it interesting to examine the transcriptional regulation of the *HEM13* in *K. lactis*. As the gene had not been isolated yet, we proceeded to clone an internal fragment of the ORF to prepare a suitable probe.

II.4.1. Cloning a fragment of the *K. lactis HEM13* gen by PCR

Using genomic DNA from *K. lactis* as template and primers designed from conserved regions of the *HEM13* gene from diverse sources (Fig. II. 5 A), we obtained two fragments (Fig. II.5 B). The shortest fragment, which fitted to the expected length of the product, was subcloned into pGEM®-T easy vector and sequenced. A homology search of this sequence performed in the *Saccharomyces* Genome Database by FASTA analysis revealed a high degree of homology to the *S. cerevisae HEM13* gene, with a 77.1% identity in a 425 nucleotides overlap (Fig. II.6).

(A)

```
ScHEM13   GAACCAAGATGG-GACCCCACAAACTTGGTGGTTTGGTGGTGGTGCTGATTTGACACCTTCTTACTTATACGAAGAAGACGGTCAATTATTCCACCAACT
HsHEM13   GAAG-AAGCTGATGGCCACAAGCAGT-GGTGGTTTGGTGGTGGATGTGACCTCACTCCAACATACTTGAATCAAGAAGACGCTGTCCATTTTCACAGAAC
MsHEM13   GAGG-AAGCTGACGGTAACACACACT-GGTGGTTTGGGGGTGGCTGTGACCTCACACCGAGATACTTGAACCAAGAGGATGCTGTCCATTTCCACCGTAC
          **   *** **  *   **  * * ********** *****  ***  * ** **    *****  *  **** ** * *      ** ***

ScHEM13   GCACAAGGATGCCTTGGACAAGCACGACACTGCTTTGTACCCACGTTTCAAGAAATGGTGTGATGAGTACTTCTACATTACCCACCGTAAGGAAACACGT
HsHEM13   TCTGAAGGAGGCTTGTGACCAGCATGGTCCAGATCTCTACCCCAAATTTAAAAAATGGTGTGATGATTACTTCTTTATAGCCCATCGTGGAGAGCGGCGG
MsHEM13   TCTAAAGGAAGCTTGCGATCAGCATGGGCCAGACATCTACCCAAAGTTTAAAAAATGGTGTGACGACTACTTCTTTATAGTTCACCGCGGGGAGCGGAGG
          *  ***** ** *  **  **** *   * *   * *****    ** ** ********** ** *******  **    ** **    **     *

ScHEM13   GGTATTGGTGGTATATTCTTTGACGATTATGATGAACGTGACCCACAAGAAATATTGAACATGGTTGAAGACTGTTTCGATGCTTTCTTGCCATCCTACT
HsHEM13   GCCATTGGTGGTATCTTTTTTGATGATCTTGACTCTCCGTCCAAGGAGGAGGTGTTTCGCTTTGTACAGAGCTGTGCCAGGGCTGTAGTTCCTTCTTACA
MsHEM13   GGCATCGGCGGCATCTTTTTTGACGATCTTGACTCCCCCTCCAAGGAGGAGGCTTTCCGCTTCGTGAAGACGTGTGCTGAGGCTGTGGTCCCTTCCTATG
          ** ** ** ** ** ** ***** ***  ***    *   *   * **    **  * * **  *    ***      *** *  * ** ** **

ScHEM13   TGACTATCGTCAAGAGAAGAAAAGATATGCCATATACAAAGGAAGAACAACAATGGCAGGCCATTAGACGTGGTAGATACGTTGAATTCAACTTAATCTA
HsHEM13   TTCCCCTTGTGAAAAAGCACTGTGATGACTCATTCACCCCCCAGGAGAAGCTGTGGCAGCAGCTCAGAAGAGGACGGTATGTAGAATTTAATCTGCTGTA
MsHEM13   TTCCCATTGTGAAGAAGCACTGCGATGACTCCTACACCCCCAGGGACAAGCTGTGGCAGCAGCTGAGGAGAGGGCGGTATGTGGAGTTTAATCTGTTGTA
          *  *  * ** ** *        ***    * *  **       **  * *  ******    * **  * **  * ** ** ** ** **  *  * **

ScHEM13   CGATAGAGGTACCCAATTCGGTTTGAGAACCCCAGGCTCTAGAGTTGAGTCAATTTTGATGAGTTTGCCTGAACATGCTTCATGGTTATACAACCACCAC
HsHEM13   TGATCGGGGCACAAAGTTTGGCCTCTTCACTCCAGGATCCAGAATTGAAAGTATCTTGATGTCTTTACCTCTAACTGCCCGATGGGAGTACATGCATTCA
MsHEM13   TGATCGGGGCACCAAGTTTGGCCTCTTTACTCCAGGATCCAGGATCGAAAGTATCTTGATGTCTTTACCTCTAACAGCAAGATGGGAGTACATGCATTCT
          *** * ** **  * ** **  *    ** ***** ** **  * **    ** ******  *** ***  *   **   ****   ****  **
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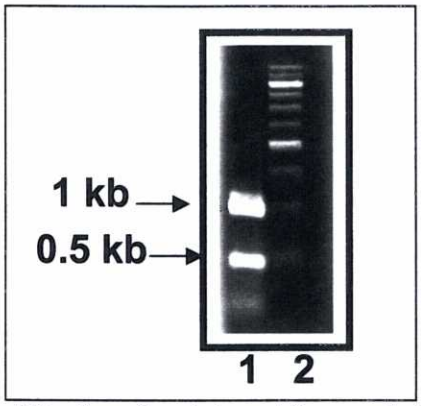


Figure II.5. Amplification of an internal fragment of the *K. lactis HEM13* gene by PCR. (A)Design of the primers for *HEM13* PCR. CLUSTAL alignment of a central region of *HEM13* gene sequences from different sources (*ScHEM13*: *S. cerevisae*, Zagorec *et al.*, 1988; *HsHEM13*: *H. sapiens HEM13*, Martasek *et al.*, 1994; *MmHEM13*: *Mus musculus HEM13,* Taketani *et al.*, 1994). Sequences from which PCR primers were designed are in bold face. **(B)Products of *HEM13* PCR.** Ethidium-bromide stained 0.8% agarose gel. Lane 1: PCR products (genomic DNA from *K. lactis* Y1140 was used as template for PCR amplification: 94°C/3 minutes; 30 cycles of 94°C/30 seconds, 50°C/1 minute, 72°C/ 1 minute; 72°C/ 4 minutes); lane 2, Mw (500 bp ladder, Boehringer Manheim).

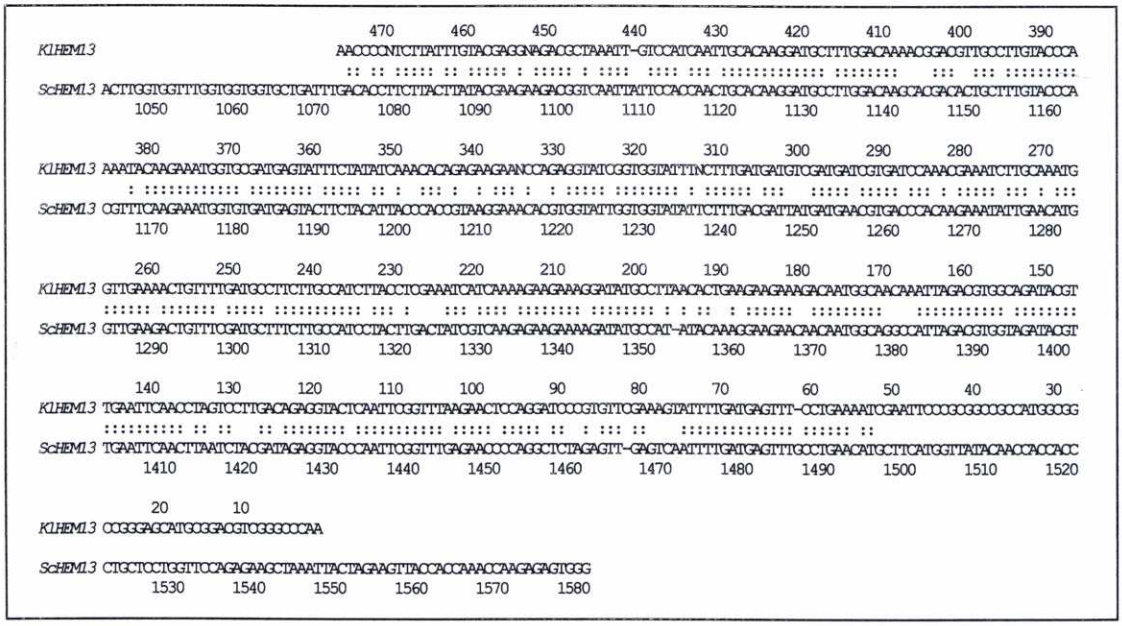
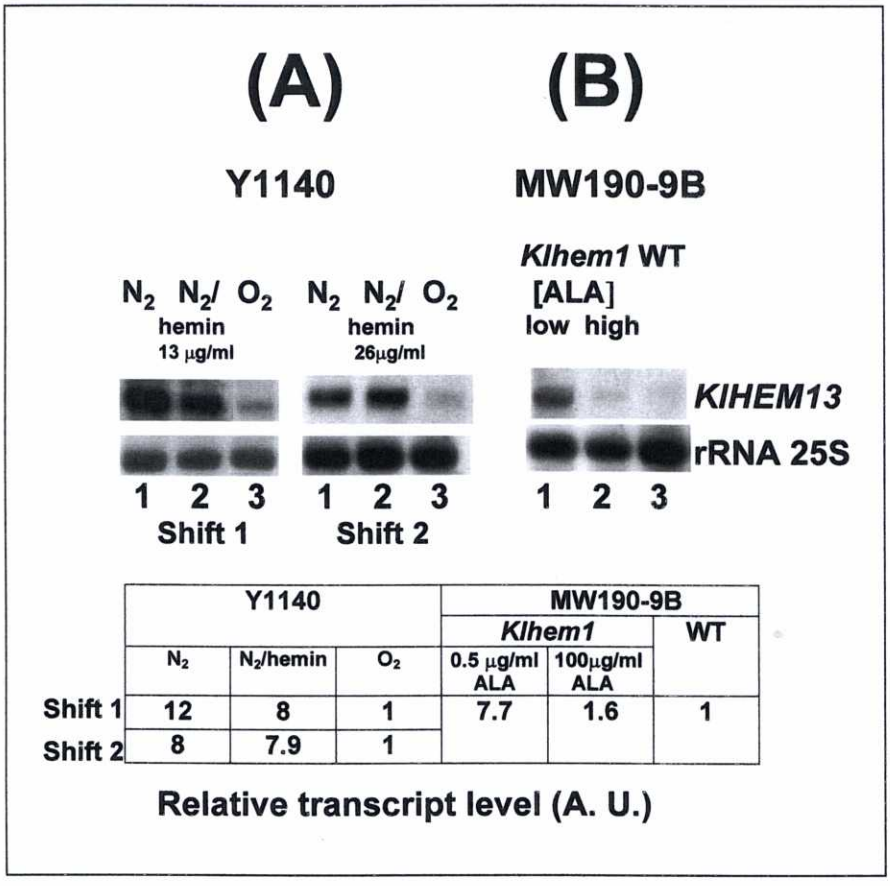


Figure II.6. FASTA analysis of the *K. lactis HEM13* PCR fragment. Alignment of the amplified fragment from *KlHEM13* and the *ScHEM13* gene. Analysis on one of the DNA strands sequenced is shown.

II.4.2. Analysis of the *K. lactis HEM13* gene expression

The expression of *HEM13* was studied by Northern analysis in the same conditions described in section II.2. *KlHEM13* mRNA levels were higher in hypoxia than in the presence of oxygen (Fig. II.7 A; in shift 1 and 2, compare lanes 1 and 3). When hemin (26 μg/ml) was added to a hypoxic culture, the *KlHEM13* levels remained essentially unaffected (Fig. II.7 A; in shift 1, compare lanes 1 and 2), although supplementation with hemin only up to 13 μg/ml caused a slight decay in the hypoxic induction of the gene (Fig. II.7 A; in shift 2, compare lanes 1 and 2). The results obtained using the *Klhem1* mutant are more conclusive suggesting an involvement of heme in the transcriptional regulation of the gene (Fig. II.7 B, compare lane 1 to lanes 2 and 3).



	Y1140			MW190-9B		
				Klhem1		WT
	N_2	N_2/hemin	O_2	0.5 µg/ml ALA	100µg/ml ALA	
Shift 1	12	8	1	7.7	1.6	1
Shift 2	8	7.9	1			

Relative transcript level (A. U.)

Figure II.7. Transcriptional effect on *KlHEM13*. (A) *KlHEM13* is mainly expressed in hypoxia. Northern blot analysis was performed according to conditions described in legend to Fig. II.3. Two concentrations of exogenous hemin were tested as indicated in shift 1 and 2. The filter was hybridised with a 0.5 kb fragment from *K. lactis HEM13* amplified by PCR (upper panel), and reprobed with rRNA as control of loading (lower panel). **(B)Involvement of heme in the aerobic repression of *KlHEM13*.** RNA was extracted from *K. lactis* MW190-9B *hem1* mutant cells grown in the presence of either 0.5 µg/ml (lane 1) or 100 µg/ml of ALA (lane 2), and from the wild-type isogenic strain (lane 3). A. U.: arbitrary units.

II.5. DISCUSSION

In this chapter we have considered the putative function of heme as effector of the oxygen signal in the transcriptional regulation of *K. lactis* genes. The mechanisms of gene regulation by oxygen have been well documented in *S. cerevisae* (for revision, Zitomer and Lowry, 1992; Pinkham and Keng, 1994; Kwast *et al.*, 1998), though they have not been tested in other yeast metabolic types. A large set of genes in *S. cerevisiae* are affected by oxygen in a heme-dependent manner, such as those encoding proteins related to mitochondrial respiration and other processes requiring oxygen (see Table I.3). In *K. lactis* only a few of the homologue counterparts of these *S. cerevisiae* oxygen/heme responsive genes have been isolated so far, among them *KlCYC1* (Freire-Picos *et al.*, 1993) and *KlACC* (Viola *et al.*, 1995), involved both in the maintenance of the oxidative metabolism. Our analysis comprised these two genes and *KlHEM13*, this latter being representative of another oxygen-dependent pathway, the biosynthesis of heme. This small set, being far away from covering all the categories affected by oxygen, included two aerobic genes (*KlCYC1* and *KlACC*) and one hypoxic (*KlHEM13*), the three under heme-mediated regulation in *S. cerevisiae*.

To analyse *in vivo* the effect of heme, we constructed a heme-deficient strain by disrupting the *KlHEM1* gene. In the absence of ALA, the product of the enzyme encoded by *HEM1*, the mutant cannot grow on non-fermentable media, because the heme auxotrophy prevents the synthesis of cytochromes. The respiratory-deficient phenotype exhibited by the *Klhem1* mutant precludes the existence of a second ALAS enzymatic activity, a duplicity very common among the higher eukaryotes and even in some prokaryotes. The heme auxotrophy of the mutant can be rescued by supplementing the culture with ALA. The amount of this supplement determines the intracellular level of heme and represents an accurate and versatile way to control the intracellular levels of the effector.

The *K. lactis* genes show an expression pattern in response to oxygen comparable to their *S. cerevisiae* homologues: *KlCYC1* and *KlACC* are expressed to higher levels in aerobic conditions, while *KlHEM13* is preferentially transcribed in hypoxia. This behaviour is in accordance with the functions of their products. Since *KlACC* and *KlCYC1* encode components of the energetic machinery of mitochondria, they are aerobically-induced genes. The hypoxic induction of *KlHEM13* could serve to sustain the synthesis of heme at expenses of residual levels of oxygen, as has been suggested for the *S. cerevisiae* gene (Labbe-Bois and Labbe, 1990).

Our results indicate that heme mediates the oxygen signal and has a regulatory effect on the transcription of both aerobic and hypoxic genes in *K. lactis*. The degree of expression estimated by comparing the transcript

levels in the *K. lactis* wild-type strain in hypoxia *versus* aerobic conditions correlates acceptably well with data obtained in the *hem1* mutant comparing low and high ALA supplementation in all cases (see quantification presented in Figs. II.3, II.4 and II.7). Addition of hemin to hypoxic cultures mimics the expression pattern obtained in aerobiosis for the *KlCYC1* and *KlACC* genes, but this would not apply to *KlHEM13*. For this later, there is a striking difference between the slight effect caused by the addition of hemin to hypoxic cultures of the wild-type cells and the more acute effect observed when the heme-deficient mutant is supplemented with high levels of ALA. This discrepancy could admit different explanations. We can speculate about a direct repressor effect of ALA, or another intermediary down-stream to ALA in the biosynthetic pathway acting on *KlHEM13* transcription. Other alternative could mean that the regulation of *HEM13* in hypoxia is partly heme-dependent, and other positive factors would also contribute to the induction, as has been described in *S. cerevisiae* (Amillet *et al.*, 1995). Further research is necessary to clarify this point.

How heme mediates the action of oxygen in *K. lactis* is another question to be answered. Similar mechanisms involving specific heme-modulated transcriptional factors to those described in *S. cerevisiae* may probably operate in *K. lactis*, although this model awaits to be confirmed. In the analysis of the promoters of *KlCYC1* (Freire-Picos *et al.*, 1995; Ramil *et al.*, 1998) and *KlACC* (Viola *et al.*, 1995) binding sites for *S. cerevisiae* Hap1p and Hap2/3/4/5p complex have been found. Moreover, *KlHAP2* (Nguyen *et al.*, 1995), *KlHAP3* (Mulder *et al.*, 1994) and *KlHAP4* (Bourgarel *et al.*, 1999) have been already cloned, but direct experimental evidence of the implication of these factors in transcriptional regulation by heme is not available.

With regard to *KlCYC1*, in heterologous expression experiments, Freire-Picos *et al.* (1995) showed that the heme-dependent factors Hap1p and Hap2p from *S. cerevisiae* participate in the regulation by oxygen and carbon source of this *K. lactis* gene. The functional significance of the consensus for Hap1p binding has been also demonstrated by a deletion analysis of the gene promoter (Ramil *et al.*, 1998).

The *KlACC* promoter contains a Hap2p binding consensus between nucleotides 624-631. The complex Hap2/3/4/5p determines the regulation of the *S. cerevisiae ACC* counterpart by oxygen and carbon source, and allows the activation of the gene in non-fermentable media and aerobic conditions (Betina *et al.*, 1995). Nevertheless, *KlACC* transcription escapes from Hap2p control (personal communication). In both *ACC2* and *KlACC* genes, the expression seems heme-dependent only in part.

Concerning *KlHEM13*, there is still no information about the upstream sequence of the gene to better understand the current data in

terms of *cis*-regulatory regions. The *S. cerevisae HEM13* gene is subjected to a complex transcriptional regulation in which take part Hap1p and Rox1p. The aerobic repression of *ScHEM13* relies mostly on the action of Rox1p (Keng, 1992). More recently, it has been demonstrated that the hypoxic induction would not only depend on the release from Rox1p repression, but also on additional positive factors which are not still known (Amillet *et al.*, 1995).

Summing up, heme controls the transcription of aerobic and hypoxic genes in *K. lactis*, an aerobic respiratory yeast, as it occurs in the aerobic fermentative *S. cerevisiae*. This heme-dependent regulation might be a basic mechanism for adapting the balance respiration-fermentation in response to oxygen levels, even when the respiratory capacity is unlimited like in *K. lactis* (González-Siso *et al.*, 1996). Finally, we remark the existence of genes induced under hypoxia in *K. lactis*, suggesting that this respiratory yeast has also developed adaptative mechanisms to grow in low oxygen-tension conditions.

CHAPTER III. POSTRANSCRIPTIONAL REGULATION BY HEME IN *Kluyveromyces lactis*

The vast majority of mitochondrial proteins are encoded by nuclear genes and synthesised on cytoplasmic ribosomes as precursors containing specific signals in the N-terminal region, which target them to the organello: presequences (Neupert, 1997). Although presequences are not restricted to a consensus, they are rich in positively charged and hydroxylated residues and might form an amphiphilic α-helix (or possibly β-sheets) across membranes or in membrane-like environments (von Heijne, 1986). During the transport of proteins into mitochondria, presequences interact successively with the import machinery of the outer membrane (**TOM**, **t**ranslocase of the **o**uter **m**embrane) and the inner membrane (**TIM**, **t**ranslocase of the **i**nner **m**embrane) (Pfanner and Meijer, 1997). Once the precursor enters the matrix, presequences are cleaved off by a specific processing peptidase (MPP) (Luciano and Geli, 1996). Many components participating in mitochondrial protein import have been identified in last years, and they have provided us with a coarse picture of the molecular mechanisms involved. These achievements have come mainly from studies carried out in *S. cerevisiae* and in *Neurospora crassa* (Neupert, 1997), but recent evidence indicates that the transport to mitochondria in mammals occurs by similar reactions, what increases the interest of these studies (Komiya and Mihara, 1996).

The N-terminal region of of *K. lactis* 5-aminolevulinic acid synthase contains the canonical determinants of a mitochondrial-targeting signal. Moreover, the presequence of *Kl*ALAS carries two motifs through which heme could inhibit the mitochondrial import of this protein. To address the question of whether heme regulates the transport of *Kl*ALAS to mitochondria, we undertook the present study. Herein, we report general aspects of mitochondrial protein import in *K. lactis* and how this process can be regulated by heme.

III.1. PROTEIN IMPORT INTO ISOLATED MITOCHONDRIA FROM *Kluyveromyces lactis*

III.1 .1. Isolation of mitochondria from *K. lactis*

Mitochondria from the *K. lactis* were isolated by the procedure routinely used for *S. cerevisiae* in Dr. Stuart's laboratory (Institut Adolf Butenandt, Münich) (Hermann *et al.*, 1994). Mitochondria were obtained in parallel from *S. cerevisiae* to evaluate the efficiency of the method. To test whether the preparation from *K. lactis* was adequate for import experiments, mitochondria were incubated with the precursor of the β-subunit of F1-ATPase (F1β) from *S. cerevisiae*. The same import was performed with mitochondria from *S. cerevisiae*.

the precursor of the β-subunit of F1-ATPase (F1β) from *S. cerevisiae*. The same import was performed with mitochondria from *S. cerevisiae*.

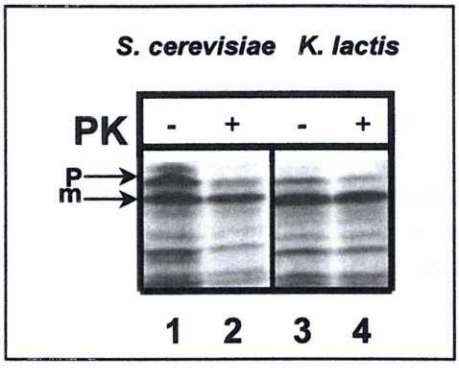


Figure III.1. Import of the *S. cerevisiae* F1β into yeast mitochondria. [^{35}S] methionine labelled F1β was synthesised in reticulocyte lysate and imported into mitochondria from *S. cerevisae* (lanes 1 and 2) and *K. lactis* (lanes 3 and 4) at 25°C for 20 minutes. After import, samples were either treated with proteinase K (20 μg/ml) or not, as indicated. Mitochondria were then re-isolated and all samples were analysed by SDS-PAGE and fluorography. **p**, precursor; **m**, mature.

The precursor F1β was efficiently imported and processed in mitochondria isolated from both yeasts. As protein import requires membrane potential, we conclude that the procedure chosen is a suitable and reproducible method for the isolation of functionally intact mitochondria from *K. lactis*.

III.1.2. The import of the 5-aminolevulinic acid synthase of *K. lactis* depends on the membrane potential

Radiolabelled *Kl*ALAS precursor of apparent Mw 55 kDa was imported efficiently into mitochondria from both *K. lactis* and *S. cerevisiae* (Fig. III.2). In the presence of the membrane potential (lanes +ΔΨ), import occurred into a protease-resistant location (Fig. III.2, compare lane 4 with 5 and 6) and was accompanied by proteolytic processing to a 50 kDa mature form. Import did not take place in the absence of mitochondrial membrane potential (lanes -ΔΨ).

*Kl*ALAS reached the matrix after import, since mature form is protected against protease digestion in mitoplasts (Fig. III.2, compare lanes 5 and 6).

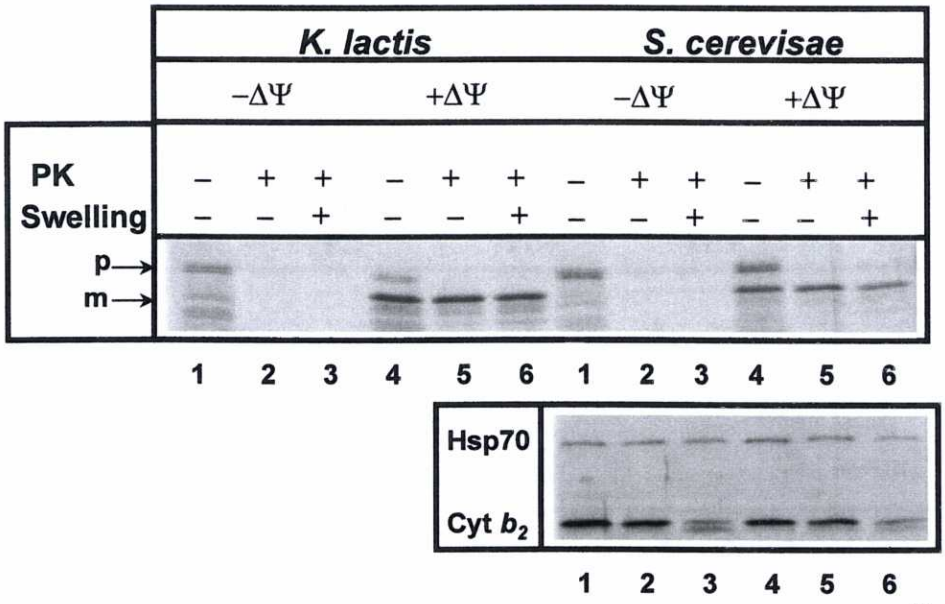


Figure III.2. Import and sorting of *Kl*ALAS into yeast isolated mitochondria. [^{35}S] methionine labelled *Kl*ALAS was imported into mitochondria either in the absence (lanes -ΔΨ, 1-3; +Valinomycin) or in the presence (lanes +ΔΨ, 4-6; +NADH) of membrane potential. After import mitochondria were re-isolated and treated with PK where indicated, under non-swelling (lanes 1, 2, 4 and 5) and swelling conditions (lanes 3 and 6). Samples were analysed by SDS-PAGE and fluorography. In import with isolated mitochondria from *S. cerevisiae*, cytochrome b_2 and mtHsp70 were immunoblotted as controls for intermembrane space and matrix destinations respectively. **p**, precursor; **m**, mature.

III.1.3. The N-terminal region of the 5-aminolevulinic acid synthase of *K. lactis* contains a functional presequence

Mitochondrial targeting signals have been shown to be functional by joining them to a non-mitochondrial passenger protein, such as the dihydrofolate reductase (DHFR), and observing the import. In order to ascertain the role of the N-terminal presequence of *Kl*ALAS in mitocondrial-targeting, a chimeric protein comprising the first ninety residues from *Kl*ALAS fused in frame to the reporter DHFR was constructed.

Radiolabelled *Kl*ALAS-DHFR was imported into isolated mitochondria both from *K. lactis* and *S. cerevisiae* (Fig. III.3), where it became processed and inaccessible to exogenously added PK (Fig. III.3, compare lanes 4 and 5). Import was ΔΨ–dependent, since it did not occur when the membrane potential was dissipated by the addition of the uncoupler valinomycin (lanes -ΔΨ). *Kl*ALAS presequence led the DHFR

to the mitochondrial matrix where the protein was protected against PK added to mitoplasts (Fig. III.3, compare lanes 5 and 6).

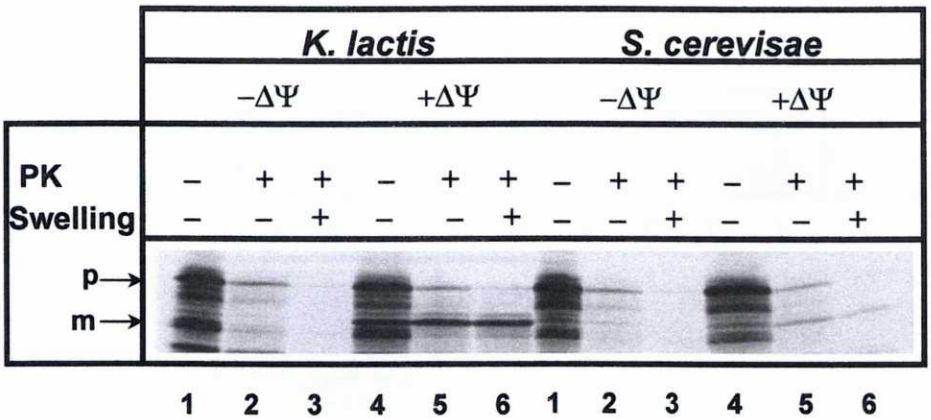


Figure III.3. Import and sorting of *Kl*ALAS-DHFR into yeast isolated mitochondria. Import reaction and PK-treatment were essentially carried out as detailed in legend to Fig. III.2. The import of *Kl*ALAS-DHFR required 37°C.

III.2. SUBMITOCHONDRIAL LOCALISATION OF THE 5-AMINOLEVULINIC ACID SYNTASE OF *K. lactis*

It has been proved that the *Kl*ALAS presequence targets the precursor from the cytoplasm to the mitochondria where it undergoes a proteolytic maturation. Subfractionation by hypotonic swelling revealed that the *Kl*ALAS is a matrix protein (section III.1.2), either soluble or membrane-bound. To further investigate the final destination and topology of *Kl*ALAS inside mitochondria, additional analyses were done.

III.2.1. Subfractionation by mild sonication: the *Kl*ALAS is associated to the inner membrane

If an imported protein is soluble in the matrix, it should fractionate in the supernatant of broken mitoplasts. Following import, mitochondria were re-isolated to obtain mitoplasts by hypotonic swelling. The membrane of mitoplasts was disrupted by mild sonication (Glick, 1995), and this suspension was fractionated by centrifugation, as it was explained in section 2.4.3.2. Total, supernatant (soluble proteins) and pellet (membrane-bound proteins) fractions were analysed by SDS-PAGE and fluorography.

*Kl*ALAS mostly appeared in the pellet fraction, suggesting that the protein is somehow associated to the inner membrane. (Fig. III.5, sonication lanes).

III.2.2. Carbonate extraction: the *Kl*ALAS is associated peripherally to the inner membrane

Carbonate extraction treatment releases proteins which are peripherally associated to a membrane, leaving those which are embedded. Following import, mitochondria were pelleted and then resuspended in a carbonate solution as was indicated in section 2.4.3.3. Total, supernatant (peripheral proteins) and pellet (embedded proteins) fractions were analysed by SDS-PAGE and fluorography.

After carbonate treatment, *Kl*ALAS was recovered in the supernatant, what is in accordance with a peripheral association of the protein to the inner membrane (Fig. III.5, CO_3Na_2 lanes).

The scheme herein summarises the steps of subfractionation.

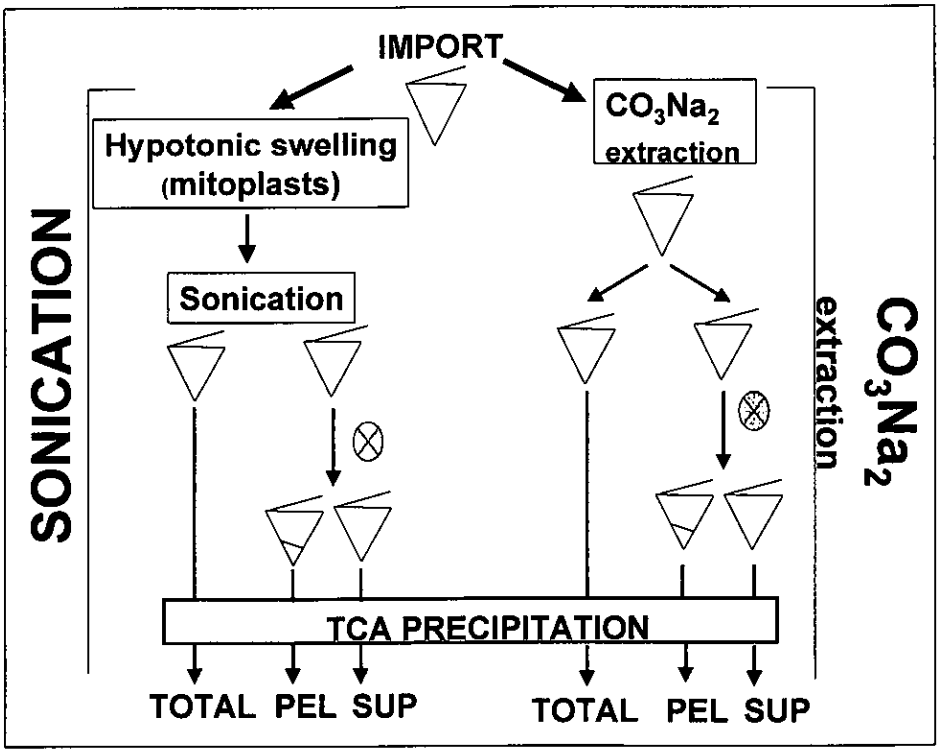


Figure III.4. Experimental scheme for the submitochondrial localisation of the *Kl*ALAS.

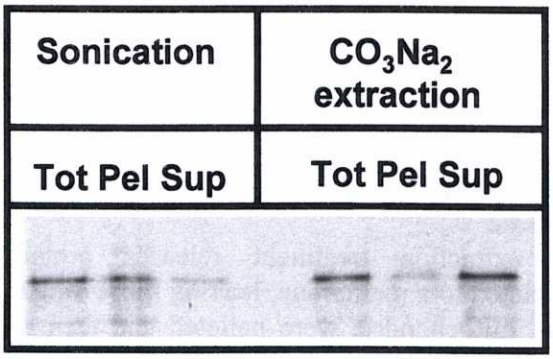


Figure III.5. Submitochondrial topology of *Kl*ALAS. Radiolabelled *Kl*ALAS was imported into isolated mitochondria from *K. lactis*. After import, mitochondria were re-isolated, then resuspended in SH buffer and divided into two equal aliquots. The first aliquot was used to isolate mitoplasts, PK-treatment and sonication. The second aliquot was subjected to carbonate extraction. Subfractionation proceeded as was illustrated in Fig. III.4. All fractions were analysed by SDS-PAGE and fluorography.

III.3. REGULATION OF MITOCHONDRIAL IMPORT OF *K. lactis* 5-AMINOLEVULINIC ACID SYNTHASE BY HEME

III.3.1. Heme inhibits the import of *Kl*ALAS into mitochondria

The presequence of the *Kl*ALAS contains two HMR which are also present in other eukaryotic ALAS (Table III.1). It had been demonstrated that HMRs mediate a heme-dependent inhibition on the import of the ALAS from rat into mitochondria (Lathrop and Timko, 1993). With this precedent, we investigated the potential control that heme might exert on the *K. lactis* protein.

To study the effect of heme, import reactions were performed with increasing concentrations of hemin up to 20 μM. To ascertain the role of the *Kl*ALAS N-terminal region, three precursors were imported: 1)*Kl*ALAS: full-length protein, bearing two HRMs; 2) *Kl*ALAS-DHFR: a fusion of the N-terminal region from *Kl*ALAS, containing the HRM motifs, with the DHFR sequence; and, 3)Su9-*Kl*ALAS: fusion of the the N-terminus of the subunit 9 of the mitochondrial F1-Fo-ATPase from *Neurospora crassa*, without HRMs, and the C-terminal region of *Kl*ALAS (Fig. III.6). F1-ATPase β-subunit from *S. cerevisiae* (F1β) was used as a control unaffected by heme, and co-imported together with each of the mentioned precursors. Results from these experiments are summarised in Fig. III.7.

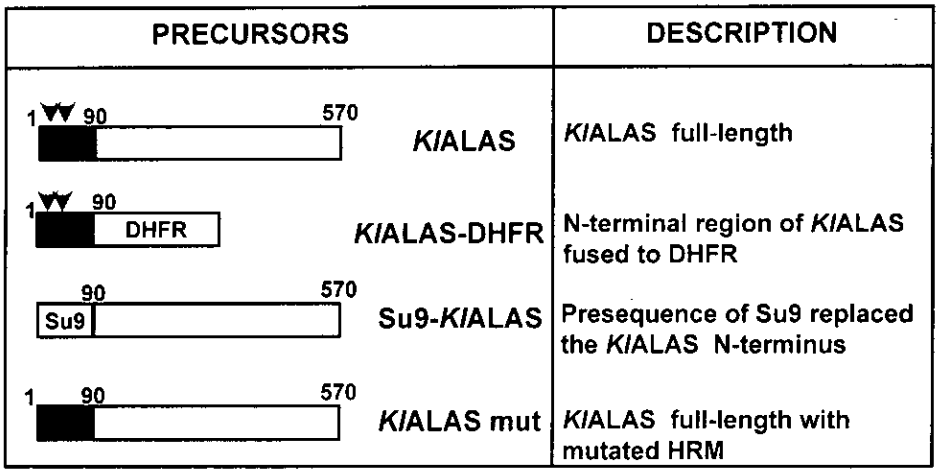


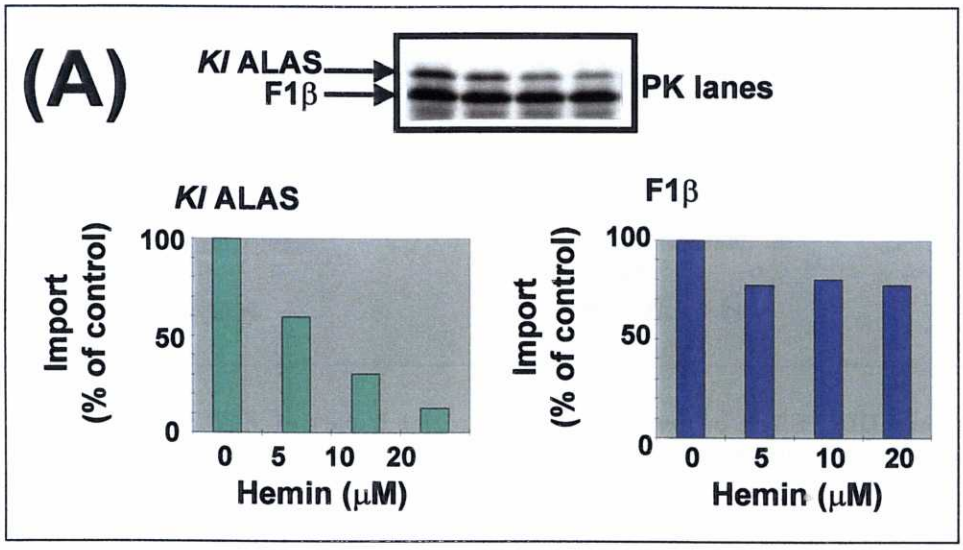
Figure III.6. Proteins imported in this study. Schematic representation of the *Kl*ALAS protein and fusion derivatives. The arrows indicate the HRM motifs. Numbers correspond to *Kl*ALAS amino acid sequence.

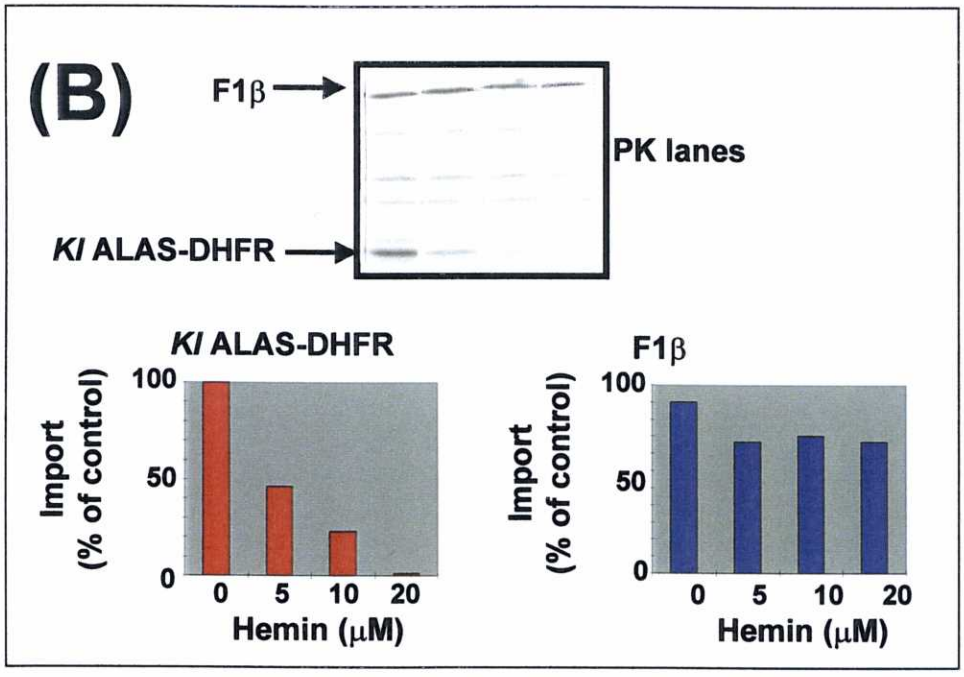
ALAS	N-TERMINAL REGION	POSITION
K. lactis	MESVIRSSAKI**CP**FMHSATGSMQSVKALKNANLPAIAQ Q**CP**FMGKAMEQRRGYASSASGASAAAAATATASTSASN	12, 40
A. nidulans	MEALLQQSRAM**CP**FLKRSSPNTLRSLATATRPSTSPGG GTMTNLQRIARR**CP**VMSKALAVQSARMTGTKRFTSSAA	12, 51
P. falciparum	MRKKRTLKVSINEIKKY**CP**FVKNIQFLYNTNEKKNNLV LSVMSDL**CP**VGKAINEKHFIIIDNKSKINIIKILKQAN	18, 46
D. melanogaster	MQ**CP**FLNRFTASFIRNYAETLCQSYGSH**CP**VVGKTLVS GEKKLSLVAASVTRSHSTGANAHANAGGPATANATAPV	3, 29
Rat ALA-H	METVVRR**CP**FLSRVPQAFLQKAGKSLLFYAQN**CP**KMME VGAKPAPRTVSTSAAQCQQVKETPPANEKEKTAKAAVQ QAPDESQMAQTPDGTQLPPGHPSPSTSQSSGSK**CP**FLA	8, 33, 110
Rat ALA-E	MVAAAMLLRS**CP**VLSKGPTGLLGKVAKTYQFLFGIGRC PILATQGPTCSQIHLKATKAGADSPSWTKSH**CP**FMLSE	11, 38, 70
Mouse ALA-E	MVAAAMLLRS**CP**VLSQGPTGLLGKVAKTYQFLFSIGRC PILATQGPTCSQIHLKATKAGGELQDRKSKIVQRAAPE	11, 38
Chicken ALA-H	MEAVVRR**CP**FLARVSQAFLQKAGPSLLFYAQH**CP**KMME AAPPAAARGLATSASRGQQVEETPAAQPEAKKAKEVAQ QNTDGSQPPAGHPPAAAVQSSATK**CP**FLAAQMNHKSSN	8, 33, 101
Chicken ALA-E	MAAFLRC**P**LLARHPPLARAFATGAR**CP**FMGFAHRAAPE LQEDVERPQIPAVEVLEELLRDGGAALNRTVRDCMDED	7, 26
***Opsanus* ALA-H**	MDVIVRR**CP**FLARVPQAFFQQSKKSLAVYAQR**CP**FMME LASKPMAPSLARALCSSSSSQQKIEDTMSTGEVLKPKA EAKLPVGLATPPSNEAVAPK**CP**FLAAEMGQNNSNVVRQ	8, 33, 97
***Opsanus* ALA-E**	MAAFLHH**CP**FLKSMPKPALRRRVPALLSLADRC**P**VIVH QVCISRLHILETKLDVSPTQPKRQRLSLLDQKRLFAQT ATQVAVSVSKG**CP**FVSSQIGMVRASPEVQEDVQADLKS	8, 33, 88
Human ALA-H	MESVVRR**CP**FLSRVPQAFLQKAGKSLLFYAQN**CP**KMME VGAKPAPRALSTAAVHYQQIKETPPASEKDKTAKAKVQ QTPDGSQQSPDGTQLPSGHPLPATSQGTASK**CP**FLAAQ	8, 33, 108
Human ALA-E	MVTAAMLLQC**CP**VLARGPTSLLGKVVKTHQFLFGIGRC PILATQGPNCSQIHLKATKAGGDSPSWAKGH**CP**FMLSE	11, 38, 70

Table III.1. Localisation of HRM in the N-terminal regions of the eukaryotic ALAS. The Cys-Pro dipeptide of the motif is in bold. Sequences were retrieved from the NCBI protein database (see references in Table 1.2).

Hemin inhibits the mitochondrial import of the *Kl*ALAS: the import rate was approximately 12% of the control without hemin at 20 μM (Fig. III.7 A). Import of *Kl*ALAS-DHFR was also inhibited even to a greater extent, owing to a faster translocation of this precursor (Fig. III.7 B). By contrast, the fusion Su9-*Kl*ALAS did not show any reduction on import in the presence of hemin (Fig. III.7 C). As expected, import of the control F1β remained essentially unaltered in all tested conditions. In conclusion, hemin prevents the import of *Kl*ALAS into mitochondria, and this effect

hemin prevents the import of *Kl*ALAS into mitochondria, and this effect should be attributed to specific elements at the N-terminal region of the protein.





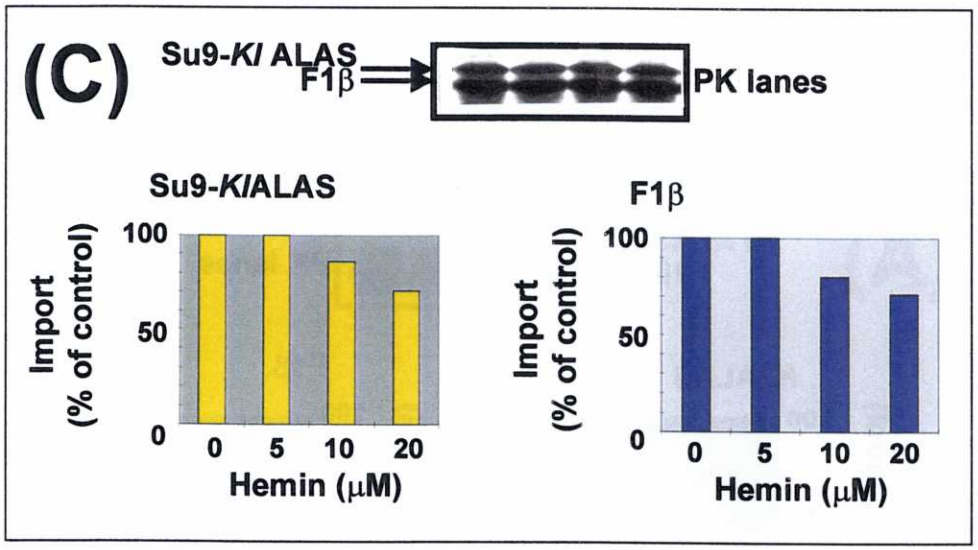


Figure III.7. Effect of hemin on mitochondrial import of *Kl*ALAS full-length protein and derived fusions. Radiolabelled proteins were imported into isolated mitochondria from *K. lactis* in the presence of hemin. Reactions with *Kl*ALAS and Su9-*Kl*ALAS were carried out at 25°C for 20 minutes; *Kl*ALAS-DHFR was imported at 37°C. Following import, samples were treated with PK (20 μg/ml). Mitochondria were re-isolated and samples were analysed by SDS-PAGE and fluorography. Quantification of fluorographs was performed with Tina system (Fujitsu). Data were normalised to the import in the absence of hemin. **(A)Hemin effect on *Kl*ALAS import; (B)Hemin effect on *Kl*ALAS-DHFR import; (C)Hemin effect on Su9-*Kl*ALAS import.**

III.3.2. The HRM are required for the inhibition of mitochondria import of *Kl*ALAS by heme

To prove the involvement of the HRMs in the inhibition on *Kl*ALAS import by heme, the sequence of motifs was changed by site-directed mutagenesis (see experimental procedure in Fig. 2.1), converting the Cys residues to Ala. Changes were verified by sequencing after mutagenesis (Fig. III.8 A), and subsequently, mutant *Kl*ALAS was imported in the presence of hemin as has been already specified in the previous section.

Substitutions of Cys by Ala in the presequence are compatible with import, since the mutated protein entered and was processed inside the mitochondria. Hemin, however, had no effect on the import of mutated *Kl*ALAS lacking HRMs (Fig. III.8 B). These findings demonstrate that hemin causes the inhibition through the HRMs.

(A)

*Kl*ALAS WT

```
ATG GAA TCT GTT ATT CGT TCT TCT GCC AAG ATC TGT CCA TTT ATG CAC TCT GCC
Met Glu Ser Val Ile Arg Ser Ser Ala Lys Ile Cys Pro Phe Met His Ser Ala

GCT CAA CAA TGT CCA TTT ATG GGT AAG GCT ATG GAA CAA CGT AGG GGT TAT GCT
Ala Gln Gln Cys Pro Phe Met Gly Lys Ala Met Glu Gln Arg Arg Gly Tyr Ala
```

*Kl*ALAS mut

```
ATG GAA TCT GTT ATT CGT TCT TCT GCC AAG ATC GCT CCA TTT ATG CAC TCT GCC
Met Glu Ser Val Ile Arg Ser Ser Ala Lys Ile Ala Pro Phe Met His Ser Ala

GCT CAA CAA GCT CCA TTT ATG GGT AAG GCT ATG GAA CAA CGT AGG GGT TAT GCT
Ala Gln Gln Ala Pro Phe Met Gly Lys Ala Met Glu Gln Arg Arg Gly Tyr Ala
```

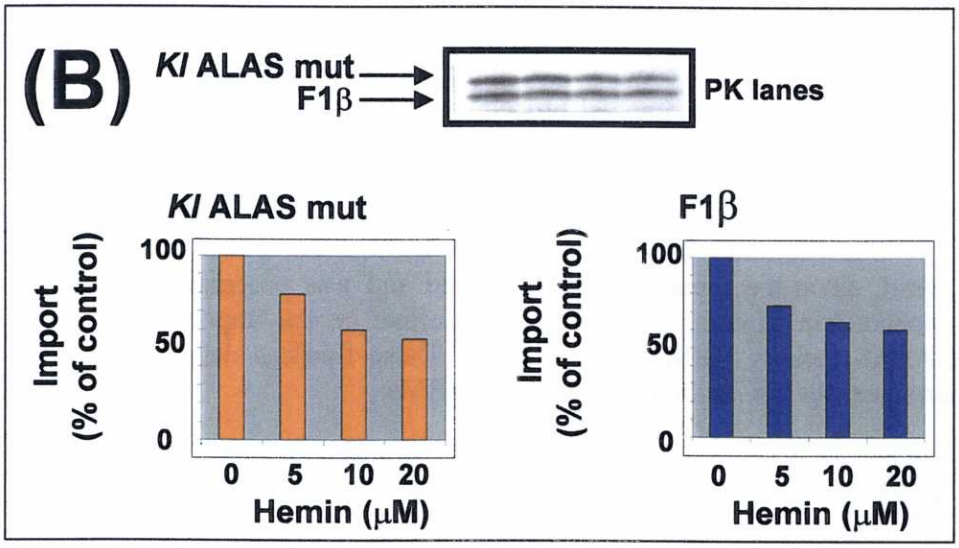


Figure III.8. Role of HRM in the inhibition by heme. (A)Site-directed mutagenesis at HRM motifs. Sequence of the wild-type and mutant protein are indicated. Changed codons are shown in bold type. **(B)Effect of hemin on mutated *KI*ALAS import.** Import ad quantification were performed as described in legend to Fig. III.7.

III.4. DISCUSSION

Mitochondria are unique organelles which harbour numerous metabolic pathways and supply cells with energy. Throughout the eukaryotic kingdom, mitochondria exhibit a high degree of conservation in their morphology and protein composition. Most of the mitochondrial proteins (99%), both structural or enzymatic, are postranslationally imported into the organello. Significant advances in understanding mitochondrial protein import have derived from research on *S. cerevisiae* and *N. crassa* (Neupert, 1997). Although the mechanisms of protein targeting and translocation across biological membranes seem rather similar (*conservative hypothesis*), there are also a number of differences and not less exceptions. It would be worthwhile to gain more evidence in other organisms to know if mitochondrial import is conserved. The research has already started in *Schizosaccharomyces pombe* (Moore *et al.* 1992), but no information was available in *K. lactis* prior to this work. In this context, the study of *Kl*ALAS import and its regulation deserved attention.

The isolation of mitochondria from *K. lactis* by the procedure developed to obtain mitochondria from lactate-grown *S. cerevisiae* cells gave a good rate of quality and yield. Reliable methods for isolating mitochondria must preserve the integrity of organelles, and preparations are usually tested for the intactness of the the submitochondrial compartments by immunoblot, before use (Glick, 1995). A pilot import experiment is also commonly performed, since import requires the membrane potential and, hence, the presence of intact membranes. Given that specific antibodies against mitochondrial markers of *K. lactis* had not been still raised, we directly tested mitochondrial preparations in imports. Isolated mitochondria from *K. lactis* imported not only the precursor of the *Kl*ALAS (homologous import), but also this of the β-subunit of F1-Fo ATPase from *S. cerevisiae* and a fusion protein containing a presequence of the subunit 9 of the F1-Fo-ATPase from *N. crassa* (heterologous import). All precursors became processed as expected. These results support the widely accepted idea of the conservation of mitochondrial import mechanisms throughout eukaryotes. Although our approach has been restricted to the *Kl*ALAS, a matrix-targeted protein, once import conditions have been set up, it would be of interest to extend this study to precursors directed to other compartments for a more complete overview of the traffic to mitocondria in *K. lactis*.

The N-terminal region of *Kl*ALAS presents the structural features of a mitochondrial presequence. We have shown that the precursor is imported into isolated mitochondria from both *K. lactis* and *S. cerevisae*, emphasising the conservation of the molecular machinery necessary for the import process. Furthermore, the role of this presequence has been

confirmed, since it drives the import of the otherwise non-mitochondrial DHFR. Although more studies are required to define precisely the structural determinants in the functionality of this targeting signal, comparison with other mitochondrial presequences anticipates the importance of the amphipatic α-helix for interaction with the receptor of the outer membrane (Cyr, 1997). Concerning the putative cleavage site, preliminary studies (data non shown) suggest that the precursor of *Kl*ALAS is processed only once by an activity sensitive to EDTA and o-phenantroline which might be similar to the matrix-processing-peptidase (MPP).

With regard to the submitochondrial location of the mature ALA, the enzyme has been found attached to the matrix surface of the inner membrane in mammal cells (May *et al.*, 1986). In *S. cerevisiae*, however, data are quite ambiguous, and while Urban-Grimal *et al.* (1986) propose an interaction between *Sc*ALAS and the inner membrane, Volland and Grimal (1988) insiste that the enzyme is in the matrix as a soluble protein. Our current results favours an association of the *Kl*ALAS to the inner membrane, probably at the hydrophobic stretch Ala-rich in the new N-terminal of the protein after processing.

The primary objective of this work was to find out if heme could regulate the import of the *K. lactis* ALAS into mitochondria. This aspect was of interest in terms of comparison between *K. lactis* and *S. cerevisiae*. As previously remarked, while the ALAS of *S. cerevisiae* lacks of HRMs, the protein of *K. lactis* bears two of these motifs in the N-terminal region. We have demonstrated that heme inhibits the mitochondrial import of the *K. lactis* ALAS, and that this inhibition depends on the HRMs of the presequence. A similar effect had been previously reported for rat ALAS (Lathrop and Timko 1993) or the *S. cerevisiae* CCHL (Steiner *et al.*, 1995). How heme exerts this postranscriptional control at the level of import has not been fully investigated yet. *K. lactis* could be a feasible model system to unravel this mechanism, which seems fundamental in the regulation of eukaryotic ALAS.

CONCLUDING REMARKS AND PERSPECTIVES

Heme mediates basic processes in biological systems utilising oxygen. The functional relationship between heme and oxygen has been demonstrated both in prokaryotes and in eukaryotes (for revision, Bunn and Poyton, 1996). Not only are the heme functions intimately entwined with those of oxygen, but the oxygen itself determines the production of heme by cells, being substrate in the biosynthesis. In the absence of oxygen, cells cannot produce heme and, in turn, the lack of heme does not allow cells the oxygen consumption.

The functional correspondence heme/oxygen and the pathways of oxygen sensing and heme signalling have been extensively investigated in *S. cerevisiae*, a simple eukaryotic model. It is accepted that oxygen regulates the respiratory metabolism in *S. cerevisiae* through the intracellular heme, affecting the expression of respiration-related genes. Heme exerts the oxygen action on gene expression by activating specific transcriptional factors (Zitomer and Lowry, 1992; Pinkham and Keng, 1994; Kwast *et al.*, 1998). Nevertheless, yeasts do not form a homogenous group as far as the respiro-fermentative metabolism is concerned. To what extent the regulation by oxygen and heme described for *S. cerevisiae*, an aerobic fermentative yeast, applies to other metabolic types is an open question. One of the objectives of this work intended to provide answers in this respect for *K. lactis*, an aerobic respiratory yeast. The unlimited respiratory capacity reported for *K. lactis*, in contrast to *S. cerevisiae*, might come from distinctive mechanisms governing the respiro-fermentative metabolism.

As observed in *S. cerevisiae*, those *K. lactis* genes involved in the respiratory function are aerobically induced; such is the case of *KlCYC1*, encoding the cytochrome *c* (Freire-Picos *et al.*, 1995) and *KlACC*, which codes the mitochondrial ADP/ATP translocator (Viola *et al.*, 1995). Nevertheless, in *K. lactis*, respiration-related genes might display higher levels of expression than their *S. cerevisiae* counterparts, on the basis of codon usage (Freire-Picos *et al.*, 1994; Luani *et al.*, 1994). This finding points directly to differences in gene expression between these two yeasts. In *S. cerevisae*, as remarked above, the transcriptional regulation by oxygen has been already drawn up, and it is orchestrated by heme. In *K. lactis*, we have confirmed that heme also mediates the action of oxygen on gene expression, and moreover, the existence of heme-modulated transcriptional factors appears highly probable (Ramil *et al.*, 1998). Indeed, expression analyses of *KlCYC1* in *S. cerevisiae* mutants have shown heterologous activation by Hap1p (Freire-Picos et al., 1995). With regards to Hap2/3/4/5p, genes for subunits Hap2p, Hap3p and Hap4 have been cloned already, although the role of this complex might be different to that described in *S. cerevisiae* (Nguyen, *et al.*, 1995; Mulder *et al.*, 1994, Bourgarel *et al.*, 1999). Future research will unravel the circuitries and

factors involved in *K. lactis* gene regulation by oxygen, to find at the molecular level the basis of the metabolic differences between this yeast and *S. cerevisae*. The *hem1* mutant constructed in this work will be of help to extend the study of the heme effect to other *K. lactis* genes, and to complete the oxygen/heme-responsive repertoire of this yeast.

Concerning the heme biosynthesis, our results suggest noticeable differences with *S. cerevisae*. In *S. cerevisiae*, oxygen regulates the heme biosynthetic pathway at the step of the coproporphyrinogen oxidase (CPO), the product of the *HEM13* gene. The oxygen is not only the substrate of this reaction, but also affects the transcription of *HEM13*. Excepting *HEM13*, the other *S. cerevisiae* genes encoding heme biosynthetic enzymes display a constitutive transcription. This expression pattern meets the steady requirements for heme in cells, so as to maintain the synthesis of essential metabolites (sterols or methionine) regardless the environmental conditions.

The transcriptional pattern of *KlHEM13* resembles that of *ScHEM13*, since both genes are induced in hypoxia. Nevertheless, in our approach, the induction degree of the *K. lactis* gene (8-12 fold) seems not to be as high as that reported for *S. cerevisiae* (40-fold) (Zagorec *et al.*, 1988). The question arises whether this lesser induction of *KlHEM13* has a physiological significance, or it is merely casual. It is worthwhile to gain more insight on this aspect, to assess the contribution of *KlHEM13* to the regulation of the heme biosynthesis in this yeast.With regard to *HEM1*, the *K. lactis* gene is moderately induced in hypoxic conditions, what may probably reflect an alleviation of the repression exerted on *KlHEM1* by the abundance of heme in aerobiosis. This feed-back control also operates at a postrancriptional level, since heme inhibits the import of the *K. lactis* 5-aminolevulinic acid synthase into mitochondria. Neither such a transcriptional nor a postranscriptional heme effect have been constated for the *S. cerevisiae HEM1* gene (Keng and Guarente, 1987; Urban-Grimal, *et al.*, 1988). Are these features of *KlHEM1* expression relevant to the regulation of the heme biosynthesis in *K. lactis*? From our results, we can anticipate that the first reaction might constitute an important point of regulation by heme and oxygen, but, whether this is the most important and rate-limiting step in the pathway has to be confirmed yet. Could this tight regulation on *KlHEM1* have a particular significance in the metabolism of *K. lactis*?

The postrancriptional regulation on the ALAS mitochondrial import by appears a general feature in eukaryotes, as predicted by the occurrence of the HRM motifs in the sequence of all proteins known so far, from protozoa to human. This regulation has been proved to operate in *K. lactis*, but curiously, it seems not to exist in *S. cerevisae* (Urban-Grimal *et al.*,

1986). Could this striking aspect have evolutionary implications, or even, metabolic significance?

It is plausible to think that *K. lactis* would require a more intense synthesis of heme to sustain a higher respiratory activity than *S. cerevisae*. The characterization of other *KlHEM* genes could help to validate or rectify this statement. Functional complementation of *S. cerevisae* heme-deficient mutants is a straightforward strategy for cloning the *KlHEM* remainder. For a preliminary approach to expression analyses, amplification by PCR, using primers designed from the *S. cerevisae* counterparts, can work out. Now that a *K. lactis* heme-deficient mutant is available, it will also feasible to assess the regulatory effect of heme at each step of its biosynthetic pathway.

The main conclusions drawn from this study are now summarised in the following lines:

1°. We have characterised the gene *HEM1* of *K. lactis* (*KlHEM1*) encoding the first enzyme of the heme biosynthetic pathway in this yeast: the 5-aminolevulinate synthase (*Kl*ALAS). The trancription of *KlHEM1* is reduced in the presence of oxygen and heme.

2°. The disruption of *KlHEM1* determines the loss of the respiratory metabolism in the mutant, which rules out the existence of a second ALAS in *K. lactis*, a quite common duplicity among other eukaryotes.

3°. The expression analysis of *K. lactis* respiration-related genes reveals that the response to oxygen can be mediated by heme. In *K. lactis*, heme would be a pivotal effector in the mechanisms of transcriptional regulation by oxygen, as it was shown in *S. cerevisiae*.

4°. Not only does heme modulate transcription in *K. lactis*, but also extends its regulatory actions to further events: heme inhibits the import of the *Kl*ALAS into mitochondria.

5°. Therefore, in *K. lactis* heme exerts a double feed-back control on the first step of its biosynthesis, by acting both at transcriptional and postrancriptional levels.

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SUMMARY (spanish version)

INTRODUCCIÓN: IMPORTANCIA BIOLÓGICA DEL GRUPO HEMO EN LEVADURAS

El grupo hemo ostenta una gran importancia en todos los sistemas biológicos que utilizan oxígeno, desde bacterias a mamíferos. Además de su papel estructural como grupo prostético de globinas, citocromos y otras proteínas enzimáticas, hemo regula numerosos procesos relacionados con el consumo de oxígeno (Padmanaban *et al.*, 1989). En su acción reguladora, hemo es un efector multifuncional que modula la expresión génica, desde la transcripción a otras etapas postranscripcionales. Así en la levadura *Saccharomyces cerevisiae*, hemo media el control transcripcional que ejerce el oxígeno sobre muchos genes relacionados con el metabolismo oxidativo.

Por su condición de anaerobio facultativo, *S. cerevisiae* puede crecer tanto en presencia como en ausencia de oxígeno, utilizando la respiración o la vía fermentativa respectivamente. Esta versatilidad fisiológica deriva de unos mecanismos de regulación transcripcional que, en respuesta a los niveles de oxígeno, activan o reprimen genes relacionados con el metabolismo respiro-fermentativo. Los genes sometidos a esta regulación se agrupan en dos categorías: 1)genes aeróbicos: se expresan en presencia de oxígeno, y codifican componentes de la cadena de transporte electrónico mitocondrial y sistemas que protegen de los radicales oxidantes; 2)genes hipóxicos: se expresan cuando los niveles de oxígeno son bajos, y codifican para proteínas relacionadas con una mejor adaptación a la hipoxia (Zitomer y Lowry, 1992).

Numerosos trabajos han abordado a nivel molecular la regulación que el oxígeno ejerce sobre el metabolismo de *S. cerevisiae* (para una revisión pormenorizada, Zitomer y Lowry, 1992; Pikham y Keng, 1994; Kwast *et al.*, 1998), describiendo los mecanismos y los factores implicados. Uno de los modelos más aceptados para explicar esta regulación establece que el oxígeno promueve la síntesis de hemo, con el concomitante incremento en la concentración de este efector. Así, hemo a su vez activa factores transcripcionales específicos, que, en último término, promoverán o reprimirán la transcripción de los genes diana. Tres factores fundamentales participan en los circuitos de regulación por oxígeno y hemo en *S. cerevisiae*: 1)Hap1p, activador de genes aeróbicos; 2)Rox1p, que en presencia de oxígeno reprime la expresión de los genes hipóxicos; y 3)Hap2/3/4/5p, complejo activador que responde a dos estímulos, la presencia de oxígeno y el crecimiento en fuentes de carbono no fermentables.

La importancia del grupo hemo en *S. cerevisiae* justifica el interés que suscitó conocer su síntesis, un proceso que debe atender las necesidades de este metabolito en la célula, tanto para funciones estructurales como de regulación. Los estudios sobre la biosíntesis de hemo

en *S. cerevisiae* se desarrollaron paralelamente a la investigación sobre el metabolismo oxidativo y la caracterización de citocromos. Estos estudios contribuyeron a definir en eucariotas esta ruta metabólica, con los mecanismos de reacción para cada etapa y su regulación. En *S. cerevisiae* los genes relacionados con la biosíntesis de hemo ya han sido clonados y caracterizados en mayor o menor medida. Sin embargo, este no es el caso para otras levaduras, donde hemo podría tener una importancia funcional comparable a la asignada en *S. cerevisiae*.

MATERIALES Y MÉTODOS

Las líneas celulares de bacterias y levaduras fueron cultivadas y mantenidas en los medios apropiados, según indicaciones de Sambrook *et al.* (1989) y Rose *et al.* (1990). Para las técnicas básicas de Biología Molecular, se aplicaron los protocolos descritos por Sambrook *et al.* (1989) y Ausubel *et al.* (1995), con las adaptaciones pertinentes según la práctica habitual del laboratorio. Respecto a los experimentos de importe mitocondrial y análisis de proteínas, se utilizaron los protocolos optimizados a tal efecto en el grupo de la Dra. Rosemary Stuart (Instituto Adolf Butenandt, Münich).

OBJETIVOS

En la levadura *S. cerevisiae* el oxígeno regula el metabolismo respiro-fermentativo, controlando la transcripción de los genes que participan en esta función. El grupo hemo interviene en esta regulación como mediador entre la señal de oxígeno y factores transcripcionales específicos que actúan directamente sobre los genes diana.

Kluyveromyces lactis pertenece al grupo de levaduras anaerobias facultativas, como *S. cerevisiae*. No obstante, existe una importante diferencia entre ambas especies: en aerobiosis, *K. lactis* desarrolla una mayor actividad respiratoria que *S. cerevisiae*. Esta propiedad de *K. lactis* podría tener su origen en una regulación del metabolismo respiro-fermentativo distinta a la que opera en *S. cerevisiae*. La regulación génica por oxígeno en *K. lactis* no ha sido tan estudiada como en *S. cerevisiae*, y de ahí el interés en investigar estos aspectos. Por ello, se inició este trabajo con el objetivo de definir el papel que hemo podría desempeñar en la regulación transcripcional por oxígeno. La clonación del gen *HEM1* de *K. lactis* (*KlHEM1*), que codifica el primer enzima de la biosíntesis de hemo

(5-aminolevulinato sintasa), permitiría construir por disrupción un mutante hemo-deficiente, muy útil para los subsiguientes análisis de expresión. Por otra parte, la caracterización de *KlHEM1* proporcionaría información sobre la síntesis de hemo en *K. lactis*, una ruta que también depende del oxígeno, y que no había sido previamente estudiada esta levadura. Aunque era objetivo prioritario examinar el efecto de hemo sobre la transcripción, las características de la 5-aminolevulinato sintasa de *K. lactis* permitieron analizar también efectos a nivel postranscripcional.

Se enumeran a continuación los objetivos propuestos para la realización de esta Tesis:

1°. Caracterización de *KlHEM1* que codifica el enzima 5-aminolevulinato sintasa de *K. lactis* (*Kl*ALAS), y obtención de un mutante hemo-deficiente por disrupción del gen.

2°. Análisis del papel de hemo en la regulación de la expresión génica por oxígeno en *K. lactis* (efecto transcripcional).

3°. Estudio del importe mitocondrial de proteínas en *K. lactis*, para examinar el efecto de hemo sobre el transporte de *Kl*ALAS a la mitocondria (efecto postranscripcional).

CAPÍTULO I. CARACTERIZACIÓN DEL GEN *HEM1* DE LA LEVADURA *K. lactis* (*KlHEM1*)

El gen *KlHEM1* había sido clonado a partir de una librería genómica por complementación en una línea *hem1* de *S. cerevisiae* (Méndez-Carro, 1992). A partir del clon aislado se inició este trabajo, delimitando en primer lugar la extensión del gen, para proceder a su secuenciación.

El gen *HEM1* de *K. lactis*

Análisis de la secuencia

El fragmento secuenciado contiene una fase de lectura abierta de 1.710 nt (nucleótidos), precedida de una región no codificante 5´de 957 nt, y una región no traducida 3´ de 345 nt. La secuencia ha sido depositada en la base de datos del EMBL (Laboratorio Europeo de Biología Molecular) con el número X92944.

La búsqueda de homologías con otros genes *HEM1* de diferentes especies reveló un alto grado de conservación, tanto en la secuencia de ADN como en la composición de aminoácidos de la proteína traducida. La mayor homología se obtuvo al comparar *KlHEM1* con los genes *HEM1* de especies filogenéticamente próximas, como la levadura *S. cerevisiae* (73% ADN; 72% proteína) o el hongo *Aspergillus nidulans* (60% ADN; 56 % proteína).

Respecto al uso de codones, los valores obtenidos para *KlHEM1* son más elevados que los calculados para el gen de *S. cerevisiae*. Esta característica diferencial ha sido descrita para otros genes de *K. lactis* relacionados con el metabolismo respiratorio (Freire-Picos *et al.*, 1994; Luani *et al.*, 1994).

Respecto a la región 5', el promotor de *KlHEM1* contiene sitios de unión para diversos factores transcripcionales: Gcrp1p (-475 y –388); Mig1p (-540); Hap2/3/4/5p (-656); Ino2p (-530) o la proteína Buf (-515). Dos cajas TATA putativas aparecen en las posiciones –81 y–239, y un elemento rico en pirimidinas se extiende desde –378 hasta –414 (González-Domínguez *et al.*, 1997).

Regulación transcripcional del gen KlHEM1

A diferencia de gen *HEM1* de *S. cerevisiae* que presenta una expresión constitutiva con independencia de los niveles de oxígeno o la fuente de carbono (Keng y Guarente, 1987), *HEM1* de *K. lactis* responde a los niveles de oxígeno. En efecto, *KlHEM1* se induce en hipoxia (6 veces). Por otra parte, la adición de hemina a un cultivo hipóxico reduce la inducción esperada en esas condiciones, hecho que prueba la participación de hemo en la regulación del gen. La inducción de *KlHEM1* en hipoxia puede resultar de la desaparición de una represión que actuaría sobre el gen en presencia de oxígeno y hemo.

La proteína 5-aminolevulinato sintasa de *K. lactis*

La secuencia del gen *KlHEM1* predice una proteína de 570 aminoácidos, con un peso molecular de 62.6 kDa y un punto isoléctrico de 6.9: la 5-aminolevulinato sintasa de *K. lactis*.

La región N-terminal de *Kl*ALAS presenta las características estructurales de una señal para el importe de proteínas a la mitocondria, o presecuencia. En esta región cabe destacar también un sitio putativo para una peptidasa mitocondrial, que eliminaría la presecuencia durante el transporte a la mitocondria. Tras la escisión de la presecuencia, el nuevo extremo N-terminal que aparece es más hidrofóbico, con abundancia de restos de alanina, y podría servir como anclaje de la proteína madura a la membrana mitocondrial interna. Además, en la presecuencia existen dos

elementos HRM ("heme regulatory motifs"), que también han sido identificados en otras proteínas reguladas por hemo (Zhang *et al.*, 1995). Los HRMs constan de un núcleo conservado cisteína-prolina, flanqueado por un residuo hidrofóbico anterior y un aminoácido cargado positivamente posterior. Hemo se une de manera reversible a estos motivos a través del residuo de cisteína (Zhang *et al.*, 1995).

CAPÍTULO II. REGULACIÓN TRANSCRIPCIONAL POR HEMO EN *K. lactis*

Se analizó el efecto del oxígeno y de hemo sobre la expresión de genes relacionados con el metabolismo oxidativo en *K. lactis*. Para observar la respuesta a oxígeno, un cultivo de *K. lactis* Y1140 fue sometido a hipoxia con burbujeo continuo de nitrógeno durante cuatro horas, y a continuación, mantenido en aerobiosis durante tres horas más. Para valorar el efecto de hemo, dos fueron las estrategias: 1)añadir hemina a un cultivo hipóxico; 2)suplementar el cultivo de un mutante hemo-deficiente *hem1* con distintas concentraciones de ácido 5-aminolevulínico (ALA); las células cultivadas en presencia de 0.5 µg/ml de suplemento sintetizarán menos hemo que aquellas suplementadas con 100 µg/ml, consiguiendo así distintos niveles intracelulares de este metabolito. Se extrajo ARN de los mencionados cultivos para análisis por *Northen blot* con sondas específicas de los genes considerados en este estudio:

1)*KlCYC1* (citocromo *c*, Freire-Picos *et al.*, 1994)

Este gen fue incluido como control de las condiciones experimentales ensayadas, pues su regulación por hemo y oxígeno ya había sido demostrada (Freire-Picos *et al.*, 1995).

2)*KlACC* (translocasa ADP/ATP, Viola *et al.*, 1995)

Este gen está relacionado con el metabolismo oxidativo, ya que el transportador que codifica permite el mantenimiento de la fosforilación oxidativa acoplada al transporte electrónico.

3)*KlHEM13* (coproporfirinógeno oxidasa)

Se incluyó como gen representante de la biosíntesis de hemo, otra ruta metabólica que depende de oxígeno. Como el gen *HEM13* no había sido aislado en *K. lactis*, se procedió a clonar por PCR un fragmento interno de su ORF para utilizar como sonda.

Construcción de una línea hemo-deficiente de *K. lactis*

Para observar el efecto de hemo "in vivo", se obtuvo un mutante hemo-deficiente de *K. lactis* por disrupción del gen *KlHEM1*. El análisis fenotípico confirmó la hemo-deficiencia de ese nulo: la línea construida es incapaz de crecer en lactato, debido a la falta de citocromos respiratorios en ausencia de hemo. Además, la disrupción fue verificada por *Southern* y *Northen blot*.

Efecto de oxígeno/hemo en la transcripción del gen *CYC1* de *K. lactis*

Estudios previos habían demostrado que *KlCYC1* se induce en presencia de oxígeno y hemo (Freire-Picos *et al.*, 1995). Se confirmó esta regulación "in vivo", al tiempo que se verificó la idoneidad de las condiciones experimentales utilizadas para analizar el efecto de hemo en este abordaje.

Efecto de oxígeno/hemo en la transcripción del gen *ACC* de *K. lactis*

El gen *KlACC* se expresa más en presencia de oxígeno, pero mantiene una transcripción basal en hipoxia. Hemo ejerce un efecto positivo sobre la regulación de *KlACC*, aunque la inducción del gen en aerobiosis depende probablemente de más factores.

Efecto de oxígeno/hemo en la transcripción del gen *HEM13* de *K. lactis*

Clonación del un fragmento del gen KlHEM13

Para obtener una sonda específica del gen *HEM13* de *K. lactis*, se preparó una PCR con ADN genómico de la línea silvestre de *K. lactis* Y1140 y cebadores diseñados con la secuencia de *HEM13* de *S. cerevisiae*. El producto de PCR utilizado como sonda fue previamente verificado por secuenciación. El análisis por FASTA de la secuencia obtenida reveló una elevada homología con el gen *HEM13* de *S. cerevisiae* (77.1% identidad en 425 nt de solapamiento).

Análisis transcripcional

Como su homólogo en *S. cerevisiae* (Labbe-Bois y Labbe, 1990), *KlHEM13* se expresa preferentemente en hipoxia. De los experimentos realizados con el mutante *hem1*, se desprende que hemo participa en la represión del gen *KlHEM13*.

CAPÍTULO III. REGULACIÓN POSTRANSCRIPCIONAL POR HEMO EN *K. lactis*: EFECTO SOBRE EL IMPORTE DE DE LA 5-AMINOLEVULINATO SINTASA

En eucariotas el enzima ALAS se sintetiza inicialmente en el citosol como un precursor que después se dirige a la mitocondria, donde alcanza su conformación y topología definitivas. Estudios realizados en los años 80 constataron que hemo inhibía la entrada de ALAS en la mitocondria (Yamauchi *et al.*, 1980; Yamamoto *et al.*, 1983; Srivastava *et al.*, 1983; Hayashi *et al.*, 1983). Lathrop y Timko (1993) identificaron los elementos responsables de ese efecto, al observar que unos motivos de secuencia en la señal de importe mitocondrial de ALAS de rata eran necesarios para la inhibición del importe: HRM ("heme regulatory motifs"). La proteína de *K. lactis* presenta dos HRMs que podrían causar un efecto similar. Con este trabajo se pretendía estudiar esa posible acción, completando así el estudio del papel regulador de hemo. A este interés, se añadía el hecho de iniciar en *K. lactis* los estudios sobre el importe mitocondrial de proteínas, proceso que, hasta entonces, se había investigado exhaustivamente en *S. cerevisiae* y en *Neurospora crassa* .

Importe de proteínas en mitocondrias aisladas de *K. lactis*

Aislamiento de mitocondrias de K. lactis

Se obtuvieron mitocondrias de *K. lactis* con un protocolo utilizado para el aislamiento de mitocondrias de *S. cerevisiae*, y que resultó ser adecuado tanto en rendimiento como en calidad de la preparación.

Mitocondrias aisladas de *K. lactis* importaron y procesaron eficientemente el precursor de *Kl*ALAS. Esta capacidad no se limita a una proteína propia. En efecto, las mitocondrias de *K. lactis* importaron también la subunidad 9 de la ATPasa de *N. crassa*, o la subunidad β de la ATPasa de *S. cerevisiae* (F1β).

*El importe de Kl*ALAS *requiere potencial de membrana*

El precursor *Kl*ALAS (Pm: 55 kDa) fue importado y procesado en mitocondrias aisladas de *K. lactis* y *S. cerevisiae*, rindiendo una forma madura de 50 kDa que alcanzó la matriz. Este importe requiere potencial de membrana pues se inhibe al añadir valinomicina, un agente desacoplante.

*La región N-terminal de Kl*ALAS *contiene una presecuencia funcional*

La región N-terminal de esta proteína contiene una señal funcional de importe a la mitocondria, como se demuestra al observar que la fusión de esa zona a la dihidrofolato reductasa, proteína no mitocondrial, alcanza también el orgánulo.

Localización submitocondrial de *Kl*ALAS

El importe a la mitocondria de *Kl*ALAS finaliza con su emplazamiento en la membrana mitocondrial interna. Varios resultados sugieren esta topología:

1°. En mitoplastos -mitocondrias desprovistas de membrana externa- la proteína no es sensible a digestión con proteinasa K. Este hecho indica una posición interior respecto a la membrana mitocondrial interna, bien soluble en la matriz, bien asociada a esta membrana por la cara matricial.

2°. En subfraccionamiento de mitoplastos sonicados, la proteína se concentra en el sedimento, lo que descarta su presencia en la matriz como proteína soluble.

3°. Por último, la extracción de la proteína con carbonato sódico indica una asociación periférica a la membrana interna.

Regulación del importe mitocondrial de ALAS de *K. lactis* por hemo

*Hemo inhibe el importe de Kl*ALAS *a la mitocondria*

Los experimentos de importe se realizaron en presencia de concentraciones crecientes de hemina, hasta 20 μM. Para determinar la función de la presecuencia en el posible efecto de hemo se sintetizaron tres precursores: 1)*Kl*ALAS: 5-aminolevulinato sintasa íntegra con los dos HRMs; 2) *Kl*ALAS-DHFR: proteína de fusión, que contiene noventa aminoácidos de la región N-terminal de la *Kl*ALAS precediendo a la DHFR murina, provista también de los HRMs; 3)Su9-*Kl*ALAS: proteína de fusión, con la región N-terminal de la subunidad 9 de la ATPasa de *N. crassa*, sin HRMs, precediendo una región C-terminal de la *Kl*ALAS. Cada uno de los precursores fue importado junto con una proteína control cuyo importe no es afectado por hemo (F1β, la subunidad β de la ATPasa de *S. cerevisiae*)

La hemina inhibió el importe de *Kl*ALAS: la tasa de importe se redujo a un 12% respecto al control, con una concentración de hemina de 20 μM. Una inhibición similar se observó en el importe de la fusión *Kl*ALAS-DHFR. Sin embargo, el importe de la fusión Su9-*Kl*ALAS no resultó afectado. Estos resultados indican la importancia de la región N-terminal de *Kl*ALAS en el efecto inhibidor de hemo sobre el importe.

La inhibición del importe por hemo requiere los HRM

Para probar la implicación de los HRMs en la inhibición del importe por hemo, se analizó el comportamiento de una *Kl*ALAS desprovista de esos motivos. Con ese fin, los restos de cisteína, imprescindibles para la funcionalidad del HRM, fueron sustituidos por alanina mediante mutagénesis dirigida.

La hemina no inhibe el importe de la proteína mutada, confirmando la importancia de los HRMs en la acción de hemo, como se había demostrado en trabajos precedentes (Lathrop y Timko, 1993; Steiner *et al.*, 1995).

CONCLUSIONES

En el cuadro adjunto se resumen brevemente las conclusiones más destacadas obtenidas en este trabajo:

1°. Se ha caracterizado el gen *HEM1* de *K. lactis* (*KlHEM1*), que codifica el primer enzima de la ruta de biosíntesis de hemo en esta levadura: la 5-aminolevulinato sintasa. La transcripción de *KlHEM1* se reduce en presencia de oxígeno y hemo.

2°. La disrupción de *KlHEM1* determina la pérdida de la capacidad respiratoria en el mutante, descartándose la existencia de una segunda ALAS en *K. lactis*, duplicidad muy común en otros eucariotas.

3°. El análisis transcripcional de genes de *K. lactis* relacionados con el metabolismo oxidativo revela que la respuesta a oxígeno puede ser mediada por hemo. Por tanto, en *K. lactis*, hemo intervendría como efector de la señal de oxígeno en mecanismos de control transcripcional, al igual que sucede en *S. cerevisiae*.

4°. En *K. lactis* hemo no sólo actúa sobre la expresión génica a nivel de la transcripción, sino que también tiene un efecto regulador en etapas posteriores: hemo inhibe el importe mitocondrial de la 5-aminolevulinato sintasa.

5°. Así pues, en *K. lactis*, hemo ejerce un doble control "feed-back" sobre la primera etapa de su biosíntesis, actuando tanto sobre la transcripción como postranscripcionalmente.

BIBLIOGRAFIA (véase en páginas 105-115)

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