Regulation of the Expression of the Kluyveromyces lactis PDC1 Gene: Carbon Source-Responsive Elements and Autoregulation

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The yeast Kluyveromyces lactis has a single structural gene coding for pyruvate decarboxylase (KlPDC1). In order to study the regulation of the expression of KlPDC1, we have sequenced (EMBL Accession No. Y15435) its promoter and have fused the promoter to the reporter gene lacZ from E. coli. Transcription analysis in a Klpdc1~ strain showed that KlPDC1 expression is subject to autoregulation. The PDC1 gene from Saccharomyces cerevisiae was able to complement the Rag~ phenotype of the Klpdc1~ mutant strain and it could also repress transcription of the KlPDC1–lacZ fusion on glucose. A deletion analysis of the promoter region was performed to study carbon source-dependent regulation and revealed that at least two cis-acting regions are necessary for full induction of gene expression on glucose. Other cis-elements mediate repression on ethanol. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — Kluyveromyces lactis; pyruvate decarboxylase; autoregulation; promoter analysis

INTRODUCTION

The non-conventional yeast Kluyveromyces lactis is receiving increasing attention, not only for its use as a host for the production of heterologous proteins (Rocha et al., 1996), but also, because of its metabolic diversity from Saccharomyces cerevisiae. K. lactis is a respiratory yeast (Gancedo and Serrano, 1989), while in S. cerevisiae fermentation occurs even under aerobic conditions (De Deken, 1966). In spite of these differences in the regulation of fermentative and respiratory metabolism, the two yeasts are closely related and, in general, both the sequence and the function of structural genes are highly conserved (Stark and Milner, 1989; Goffini et al., 1990; Lewis and Pelham, 1990; Bergkamp-Steffens et al., 1992; Jacoby et al., 1993; Prior et al., 1993).

The physiological differences described above underline the importance of studying the metabolism of pyruvate at the branch point between respiration and fermentation in K. lactis, where these pathways seem to be regulated differently from S. cerevisiae.

The first step of the fermentative pathway, i.e. the conversion of pyruvate to acetaldehyde, is catalysed by pyruvate decarboxylase (PDC). In S. cerevisiae, the PDC system is rather complex and consists of three structural genes, PDC1, PDC5 and PDC6 (Schmitt et al., 1983; Kellermann et al., 1986; Schaaff et al., 1989; Seeboth et al., 1990; Hohmann, 1991). PDC1 is responsible for most of the wild-type PDC activity, while PDC5 only becomes active in pdc1Δ strains (Hohmann and Cederberg, 1990). The Pdc1 protein not only exerts a negative regulatory effect on the expression of PDC5, but also autoregulates the expression of its own gene (Liesen et al., 1996). The PDC6 gene does not contribute to PDC activity and becomes active only when fused to an active promoter (Hohmann, 1991). PDC activity in S. cerevisiae is dependent on the regulatory factor Pdc2p (Hohmann, 1993; Raghuram et al., 1994) and on
the common transcriptional regulators of glycolytic gene expression, Ger1p and Rap1p (Butler et al., 1990; Chambers et al., 1995; Liesen et al., 1996). The binding sites for these factors are often found in close proximity (Chambers et al., 1995). It has been suggested that only GCR1 sites located close to RAP1 sites are functionally important in vivo (Bitter et al., 1991; Huie et al., 1992). However, the RAP1–GCR1 box of PDC1 has been identified as an element conferring general promoter activation, but not carbon source-dependent regulation (Butler et al., 1990; Liesen et al., 1996).

We have isolated and sequenced the unique PDC gene from the yeast K. lactis (Bianchi et al., 1996). As opposed to S. cerevisiae, a K. lactis strain, lacking the KIPDCl (formerly KIPDCA) gene, was able to grow at the same rate as the wild-type on glucose media, highlighting the fact that pyruvate channelling through fermentation and respiration in these yeasts is regulated in different ways (Prönk et al., 1996). The transcription of KIPDCl is induced by glucose, repressed by ethanol (Bianchi et al., 1996) and regulated by the RAG3 gene product, which has a high homology to the Pdc2 protein from S. cerevisiae (Prior et al., 1996). In K. lactis the PDC activity is also induced by oxygen shortage (Kiers et al., 1998). In S. cerevisiae, ethanol repression of the PDC1 gene, as well as autoregulation, have been shown to be mediated by a novel cis-acting element, composed of short repetitive sequences called ERA sequences (ethanol repression and autoregulation) and placed downstream of the RAP1–GPR1 box. The ERA sequences have been subsequently found upstream of several glycolytic genes of S. cerevisiae.

In this report, we give evidence that autoregulation and oxygen depletion are involved in transcriptional regulation of the KIPDCl gene and that the S. cerevisiae PDC1 gene can substitute KIPDCl gene in function and, partially, in autoregulation. On the other hand, we also show that carbon source regulation relies on multiple cis-acting elements of the promoter region, which allow glucose induction and ethanol repression of KIPDCl transcription.

### MATERIALS AND METHODS

#### Strains, media and general genetic methods

The E. coli strain used was DH5α (p80d/lacZΔM15 Δ(lacZYA-argF) U169 deo rec1 lacI98 lacZΔM15 ΔlacZYA-argF U169 thi XL1 recA1 deo recl thi-1 supF THI-1 supE44 thy-1 thi-1 END1 suF thy-1 thi-1 M15 lacI98 lacZΔM15 ΔlacZYA-argF U169 ara recA1 deo recl thi-1 supF THI-1 supE44 thi-1 thy-1 M15) and the yeast strain used was YPH499 (matα ura3-52 leu2-3,112 his3-11,15 trp1-289 ade2-1 can1-100). K. lactis strains are listed in Table 1. The two KIPDCl strains PM6-7A and MW341-5 have essentially the genetic background of the standard wild-type strain CBS2359 and behave identically as far as genes involved in the Rag− phenotype are concerned (Węsolski-Louvel M., personal communication). The ura3 strain PMI/C1 has been obtained after 5-fluoro-orotic acid selection of resistant clones (McCusker and Davis, 1991) from strain PMI, which is a KlPdc1::URA3 integrative transformant of PM6-7A (Bianchi et al., 1996). Strain MM1-12D has been obtained from a cross between strains PMI/C1 and MW341-5.

Genetic procedures for crossing and sporulation have previously been described (Węsolski et al., 1982). Rich medium was 1% yeast extract and 2% (w/v) peptone (YP). Synthetic minimal (SM) selective medium contained 0.7 g/l of yeast nitrogen base without amino acids (Difco), supplemented with the appropriate amino acids and nucleotides. Glucose and ethanol were added to a final concentration of 2%. Antimycin A was used at a final concentration of 5 μM. Solid medium contained 20 g/l Bacto-agar (Difco).

#### Plasmids and plasmid constructions

All plasmids constructed in this work were obtained using standard techniques (Sambrook et al., 1989).
et al., 1989). A 4·3 kb chromosomal HindIII fragment containing the KlPDC1 gene (Bianchi et al., 1996), was cloned into the unique HindIII sites of the K. lactis multicopy and centromeric vectors KEp6 (Chen et al., 1988) and KCp491 (Prior et al., 1993), respectively. The resulting vectors, harbouring the URA3 gene from S. cerevisiae as a selectable marker, were named KEpPDC and KCpPDC. The 1·2 kb 5\* upstream region from KlPDC1 was sequenced from the 4·3 kb chromosomal HindIII fragment. This promoter region was then amplified by PCR with oligonucleotide primers bearing BamHI and XbaI restriction sites at their 5\* ends. After purification and endonuclease digestion, the promoter fragment has been cloned into the plasmid pFDY180, a pBR322 derivative containing the 3·2 kb lacZ gene from E. coli. The amplified promoter region has been controlled by sequencing. A 4·4 kb BamHI–NheI fragment containing the lacZ gene under the control of the 1·2 kb promoter fragment was then ligated into the centromeric vector KCp491. This plasmid, named pMD12 (Figure 1), was used to construct all further pMD-derivatives, either by using the naturally occurring restriction sites or by amplifying different promoter fragments with oligonucleotide primers carrying the appropriate restriction sites at their 5\* ends to facilitate cloning steps. All amplification steps were performed with the proof-reading VENT-Polymerase (NEB).

In order to transform K. lactis with the PDC1 gene from S. cerevisiae and with its pdc1-8 mutant allele (kindly given by Dr Stefan Hohmann, University of Goteborg), two 5 kb fragments, harbouring the whole open reading frames of PDC1 and pdc1-8 alleles together with 1 kb from their 5\* upstream region, were ligated into the centromeric LEU2 vector KCplac13 (kindly given by Dr Wesolowski-Louvel, University of Lyon). The resulting plasmids were named pK3C1 and pK3C1-8, respectively.

Yeast transformation procedures

K. lactis was transformed using the electroporation procedure as described previously in Bianchi et al. (1996).

Northern analysis

400 ml of YE medium in a 1000 ml Erlenmeyer flask were inoculated with wild-type PM6-7A or MW341-5 cells and incubated overnight with shaking (175 rpm) at 28°C to 4 \times 10^7 cells/ml (OD_{600}=1·2). The grown culture was divided into six 50 ml aliquots and transferred to 250 ml flasks; glucose, galactose, fructose, glyceral and ethanol were added to a final concentration of 2%. Carbon source was omitted from one of the six YE aliquots.

Table 2. PDC activity in K. lactis wild-type and Klpdc1\* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>PDC-activity (mU/mg)</th>
<th>Rag phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM6-7A (wt)</td>
<td></td>
<td>215 (± 84)</td>
<td>+</td>
</tr>
<tr>
<td>PMI/C1 (Klpdc1Δ)</td>
<td></td>
<td>&lt;1 (± 10)</td>
<td>–</td>
</tr>
<tr>
<td>PMI/C1 (Klpdc1Δ)</td>
<td>KCpPDC (KIPDC1)</td>
<td>237 (± 69)</td>
<td>+</td>
</tr>
<tr>
<td>PMI/C1 (Klpdc1Δ)</td>
<td>KEpPDC (KIPDC1)</td>
<td>3516 (± 400)</td>
<td>+</td>
</tr>
<tr>
<td>MW341-5 (wt)</td>
<td></td>
<td>212 (± 58)</td>
<td>+</td>
</tr>
<tr>
<td>MW341-5/Δ (Klpdc1Δ)</td>
<td></td>
<td>&lt;1 (± 10)</td>
<td>–</td>
</tr>
<tr>
<td>MW341-5/Δ (Klpdc1Δ)</td>
<td>pK3C1 (ScPDC1)</td>
<td>890 (± 168)</td>
<td>+</td>
</tr>
<tr>
<td>MW341-5/Δ (Klpdc1Δ)</td>
<td>pK3C1-8 (ScPdc1-8)</td>
<td>&lt;1 (± 10)</td>
<td>–</td>
</tr>
</tbody>
</table>

The Klpdc1\* strains were transformed either with the gene coding for pyruvate decarboxylase from K. lactis (KIPDC1) on a centromeric and a multicopy vector, or with the gene from S. cerevisiae (ScPDC1 and Scpdc1-8) on a centromeric vector. Cells from YE medium precultures were grown overnight in selective medium containing 2% glucose or 2% ethanol as carbon source to approximately OD_{600}=1. The transformants with empty vectors (KCp491 and KCplac13) showed no additional PDC activity to the untransformed strains (not shown). Values are averages of three or more independent determinations. Standard errors are reported in parentheses.
of the culture. The flasks were then incubated at 28°C with shaking (175 rpm) for a further 5 h growth. A 50 ml tube was filled up with a further 50 ml aliquot of YP culture, firmly capped, sealed and held at 28°C without shaking for 5 h. This sample was set up to obtain a condition of oxygen shortage. RNAs were then extracted and Northern analysis performed as described in Bianchi et al. (1996).

Enzyme assays

PDC activity was measured as described by Ullrich (1970) with minor modifications (Bianchi et al., 1996). For the ß-galactosidase assays, yeast cells were inoculated from a preculture, grown in YP medium without any carbon source added, in 50 ml of SM selective medium containing 2% glucose or 2% ethanol and incubated at 28°C overnight in 250 ml Erlenmeyer flasks with vigorous shaking (175 rpm) to OD_{600}. Glass bead extraction of proteins and ß-galactosidase assays were carried out according to Miller (1972) with modifications as described in Rose and Botstein (1983). Protein determination was performed according to Bradford (1976). The plasmids used for the assays contained a centromeric sequence from K. lactis and should therefore be stably present in one to two copies per cell, minimizing artifacts due to multicopy effects (Chen, 1996). Transformed cells were grown in selective medium for monitoring ß-galactosidase activity.

RESULTS AND DISCUSSION

Complementation analysis of the KlpdclΔ strain

In a previous work, we described the isolation of the pyruvate decarboxylase gene from K. lactis (KIPDC1) and its carbon source-dependent expression, i.e. induction on glucose and repression on ethanol. We also showed that K. lactis strains bearing a deletion in the KlPDC1 gene do not grow on glucose media plus antimycin. This inability to grow by fermentation when respiration is blocked has been defined as a Rag phenotype (Goffrini et al., 1990). The ability to grow on glucose in the presence of antimycin A and the carbon-source dependent PDC activity could be restored in a KlpdclΔ strain by transformation with a 4·3 kb chromosomal HindIII fragment, which harbours the KIPDC1 ORF and 1·2 kb of the 5' upstream region, either on a centromeric or on a multicopy vector (KClpPDC and KEpPDC, respectively). These results, reported in Table 2, show that in the KlpdclΔ centromeric transformants the PDC activities were identical to those reported for the single plasmid transformants reported in Table 2.
and its counterpart Pdc1p from S. cerevisiae (Bianchi et al., 1996), the PDC1 gene of the latter yeast was able to complement the Rag“ phenotype of a Klpdc1˜ strain of K. lactis. However, the Klpdc1˜ strain, harbouring the PDC1 gene from S. cerevisiae on a centromeric vector, exhibited a 5–6-fold higher PDC activity than the KlPDC1 transformants. This level of PDC activity was similar to that measured in S. cerevisiae on glucose (Hohmann, 1991) and, in contrast to the PDC activity in S. cerevisiae, was not repressed on ethanol. This indicates important differences between either the regulatory sequences and/or the transcription factors responsible for ethanol repression in these yeasts.

Autoregulation of KlPDC1 expression

In S. cerevisiae, the PDC1 gene product has been shown to have a regulatory influence on the transcription of PDC5 and on that of PDC1 itself (Seebold et al., 1990; Hohmann and Cederberg, 1990; Liesen et al., 1996). To investigate the presence or absence of this effect in K. lactis, we constructed the centromeric vector pMD12, which carried the 1·2 kb promoter region of KlPDC1 on a centromeric vector, exhibited a 5–6-fold higher PDC activity than the KlPDC1 transformants. This level of PDC activity was similar to that measured in S. cerevisiae on glucose (Hohmann, 1991) and, in contrast to the PDC activity in S. cerevisiae, was not repressed on ethanol. This indicates important differences between either the regulatory sequences and/or the transcription factors responsible for ethanol repression in these yeasts.

pMD12 transformants of the Klpc1Δ strain MM1-12D showed activities many-fold higher than the wild-type transformants on both carbon sources, thus revealing a strong repressive effect of the KlPDC1 gene product on the transcription from its own gene promoter. The high ß-galactosidase activity of MM1-12D transformants on ethanol also suggest that in K. lactis KlPdc1p is required for ethanol repression.

To examine whether the PDC1 gene product from S. cerevisiae could restore normal transcriptional regulation of the construct pMD12 in a Klpc1Δ strain, we transformed this strain with pK3C1, a centromeric vector containing the PDC1 gene from S. cerevisiae (see Materials and Methods). The results, shown in Table 3, demonstrated that the Pdc1 protein from S. cerevisiae was able to exert a repressive function on the expression of the lacZ reporter gene. To demonstrate that it was the Pdc1 protein itself and not the absence of PDC activity that caused the repressive effect, we also tested the pdc1-8 gene from S. cerevisiae, which carries a point mutation leading to an enzymatically inactive protein (Schaff et al., 1989). Results also demonstrated that the inactive protein downregulated KlPDC1 promoter-driven expression. The high values observed with the empty vector KCplac13 excluded the possibility.
that the reduced β-galactosidase activity in transformants carrying two centromeric vectors was due to plasmid competition. Neither the Pdc1 nor the Pdc1-8 proteins were able to restore ethanol repression, thus suggesting the involvement in this regulatory circuit of ethanol-specific regulatory protein(s) unable to interact correctly with the heterologous pyruvate decarboxylase.

Transcription of KIPDC1 in wild-type strains

Oxygen shortage might become a critical condition when measuring the activity or the transcription of the fermentative gene KIPDC1 in cells grown to high density in flasks. In fact, it has been recently demonstrated that PDC activity is induced in K. lactis cells grown in a chemostat at low oxygen supply (Kiers et al., 1998). However, no data on KIPDC1 transcription in cells grown under oxygen limitation have been reported to date.

In order to exclude any interference by oxygen on our subsequent analysis on carbon source regulation of KIPDC1 transcription, we performed a Northern analysis as described in Materials and Methods. The results, shown in Figure 2 for strain PM6-7A, confirmed that KIPDC1 transcription is induced by glucose and repressed by ethanol. Identical results were obtained with strain MW341-5. Glycerol and sugars, such as galactose and fructose, could also induce KIPDC1 transcription, suggesting that the onset of one or more signals triggering the induction of this gene might depend on glycolytic steps downstream of sugar phosphorylation and/or steps involving C3 compounds, as reported by Boles and Zimmermann (1993) for the induction of ScPDC1.

Northern analysis also showed that KIPDC1 transcription was induced by oxygen limitation in the absence of any added carbon source, but that this regulatory effect was absent in cells grown in shake flask up to OD600=1.2. This finding allowed us to perform the carbon source-dependent transcriptional analysis of the KIPDC1 promoter, described below, while no attempt has been made up to now to identify elements involved in oxygen regulation.

Nucleotide sequence of the KIPDC1 5’ upstream region

The nucleotide primary structure of the 1.2 kb upstream region from the pyruvate decarboxylase structural gene KIPDC1 was determined by sequencing (EMBL Nucleotide Sequence Database; Accession No. Y15435).

A computer analysis of the promoter sequence revealed several putative binding sites for common fungal transcription factors (Quandt et al., 1995). Three binding sites for Gcr1p could be identified in the farther upstream region, respectively at positions -766, -865 and -1172 upstream from the ATG start codon. Two additional Gcr1p binding sites at positions -585 and -591 were found to form a UASRPG (Larson et al., 1994), together with a putative Rap1p binding site located at -623. To date, little is known about the regulation of glycolytic gene expression in K. lactis. However, several transcription factors from K. lactis have been isolated by functional complementation of their S. cerevisiae counterparts (Jakobsen and Pelham, 1991; Gonçalves et al., 1992; Na and Hampsey, 1993; Mulder et al., 1994), demonstrating the close relationship between these yeasts. The KIRAP1 homologue, on the contrary, was unable to complement a rapα mutant strain from S. cerevisiae, even if a binding of the Rap1p to the consensus sequence could be observed (Larson et al., 1994; Ishii et al., 1997).

Immediately downstream from the RAP1–GCR1-binding site, there are five repetitive sequences, T/GGAAATGG/T, showing a slight homology with the ERA sequences from S. cerevisiae. Two potential TATA boxes were found at the positions -177 and -108.

Identification of glucose and ethanol-dependent regulatory regions in the upstream sequence of KIPDC1

In order to study the importance of cis-acting sequences in KIPDC1 expression, we generated a series of 5’ and internal promoter deletions, starting from the plasmid pMD12, and we introduced the different constructs in a wild-type strain. The effect of the deletions on expression was measured by assaying the levels of β-galactosidase activity in the transformants grown on glucose- or ethanol-containing media. The results are reported in Figure 3.

Deletions in the farther 5’ promoter region, removing one or two of the single Gcr1p putative binding sites (vectors pMD10 and pMD8), progressively reduced the activity on glucose. More extended deletions, which also removed the putative RAP1–GCR1 box and the downstream...
**Figure 3.** β-galactosidase activities of different pMD-transformants. The sequences indicated were cloned into the centromeric vector KCp491 and introduced either in the *Klpdc1* strain MW341-5 or in the *Klpdc1Δ* strain MM1-12D. β-galactosidase activities were determined after growth of the transformed cells to OD₆₀₀=1.0 on SM selective medium with the indicated carbon source (Glc=glucose, EtOH=ethanol). Each value represents an average of measurements from at least three different transformants. Putative controlling promoter elements of *Klpdc1* are schematically depicted in the upper part of the figure. The striped ovals indicate the positions of the putative GCR1 consensus sequences, while the squared box marks the positions of the RAP1 binding site. The dotted grey box indicates the TATA box. **ERA**-like sequences are marked by arrows (see text for details). G/E, ratio of activities measured in glucose grown cells to ethanol grown cells; 'n.d.', not determined.
region containing the ERA-like repetitive sequences (vectors pMD4 and pMD2), led to further reduced activity on glucose. The deletion of only the repetitive sequences in vector pMD12R had no effect on β-galactosidase activity of the transformants on glucose, suggesting that these sequences are not involved in glucose induction of KlPDC1. On the other hand, when the region containing the RAP1–GCR1 box was also absent (vector pMD12RR), a sharp decrease of the activity on glucose could be observed. These results suggest the existence of multiple cis-acting regulatory elements important for induction on glucose, which should be located between positions -577 and -1172. Site-directed mutagenesis of the KlPDC1 promoter structure of pdc1 genes are concerned. However, important differences have also been found, as one might expect for two yeasts in one of which fermentation is largely dispensable, as is the expression of pyruvate decarboxylase in K. lactis is modulated by two mutually non-exclusive effects of induction by glucose and repression by ethanol. It should be noted, however, that the construct bearing only the active TATA box (vector pMD2) still led to carbon source-dependent β-galactosidase activity. Again, intermediate values (200 ± 30 β-galactosidase units) of the reporter gene activity were observed on both glucose and ethanol media, suggesting that, in K. lactis, the basal level of transcription might also depend on the presence of carbon source-specific regulatory factors.

Promoter elements mediating autoregulation

As shown above, the KlPDC1 expression is subjected to autoregulation. The transformation of the lacZ fusions into a Klpdc1Δ strain allowed us to indicate promoter regions involved in autoregulation.

Results reported in Figure 3 showed that all of the tested constructs, with the exception of vectors pMD4 and pMD2, led to higher activities in a Klpdc1Δ background than in the wild-type strain, indicating that autoregulatory factor(s) should require sequences upstream from position -444. However, two distinct regions seemed to mediate autoregulation with different efficiency. In fact, the deletion of the farther region, between -1172 and -800, had a stronger effect on the decrease of β-galactosidase activity (vectors pMD10 and pMD8) than the deletion of the region including the ERA-like sequences, between positions -577 and -444. A similar situation was observed when the transformants were grown on ethanol medium, although transcriptional activity on ethanol was in all cases higher than on glucose. This result supports the conclusion that the autoregulatory factors are the major repressing agents in KlPDC1 expression. This differs from the situation in S. cerevisiae, where the β-galactosidase values, obtained on ethanol media in a pdc1Δ strain, never exceeded the values measured on glucose (Liesen et al., 1996).

In conclusion, our results reveal some similarities between the two closely related yeasts K. lactis and S. cerevisiae, as far as autoregulation, carbon source-dependent expression and the promoter structure of PDC1 genes are concerned. However, important differences have also been found, as one might expect for two yeasts in one of which fermentation is largely dispensable, as is the
case of \textit{K. lactis}, or is the prevalent metabolic pathway, as is the case of \textit{S. cerevisiae}. It is interesting to note that the putative binding sites for all of the transcription factors isolated from \textit{K. lactis} so far are remarkably conserved, while the factors that ensure the different regulation of channeling of metabolites through either the fermentative or the respiratory pathway might rely on the diversity of specific regulatory agents.

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