Carbon Source-Responsive Elements and gene regulation
by *CAT8* and *SIP4* in the yeast *Kluyveromyces lactis*

DISSERTATION

zur Erlangung des akademischen Grades
doktor rerum naturalium (Dr. rer. nat.)

vorgelegt der

Mathematisch-Naturwissenschaftich-Technischen Fakultät
(mathematisch-naturwissenschaftlicher Bereich)
der Martin-Luther-Universität Halle-Wittenberg

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geb. am 28.06.1970 in Rotterdam

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Halle (Saale), den 30.03.2002

Die Arbeit wurde im Rahmen eines ordentlichen Promotionsverfahrens am 12. Juli 2002 verteidigt.

urn:nbn:de:gbv:3-000005080
[http://nbn-resolving.de/urn/resolver.pl?urn=nbn%3Ade%3Agbv%3A3-000005080]

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1 Introduction

1.1 Regulation of genes: both end and means

In the five years since the complete genome sequence of baker's yeast *Saccharomyces cerevisiae* (Goffeau *et al*, 1996; http://genome-www.stanford.edu/Saccharomyces/) became available, the genome sequences of other eukaryotic model organisms have been completed and made public: the nematode *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium, 1998; http://www.sanger.ac.uk/Projects/C_elegans/), the fruit fly *Drosophila melanogaster* (Adams *et al*, 2000; http://www.fruitfly.org/), the flowering plant *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000; http://www.arabidopsis.org/) and, finally, the human genome (Lander *et al*, 2001; Venter *et al*, 2001; http://www.ncbi.nlm.nih.gov/genome/guide/human/). Comparison of these sequences has taught us that the increase in complexity, particularly from worm to fly to man, is not reflected in the number of genes to the extent it was thought to be previously. The estimates of 30,000 to 40,000 genes in man amount to roughly double to triple the number of *C. elegans* (~14,300) or *D. melanogaster* (~13,600) genes and five to six times as many genes as the unicellular baker's yeast (~6,300). This means that the observed diversity of proteins is created mostly though modifications such as alternative splicing of primary transcripts to mature mRNAs during transcription of a gene. Additionally, co- or post-translational modification of nascent polypeptides, such as cleavage of pre- or preproproteins, methylation or myristoylation to name only a few, serve to form the mature protein. Finally the biological activity of mature proteins can be regulated short-term by phosphorylation/dephosphorylation, differential complex formation and differential localization. Regulated degradation of mRNAs and proteins plays an additional role in determining protein abundance and regulatory response timing.

Even so, the first and prerequisite step leading to production and activity of a given protein is the transcription of the gene that encodes it. As it turns out transcriptional activity is subject to regulation, involving many autoregulatory loops, that is no less complex and intricate than any of the other regulatory mechanisms mentioned above.

1.1.1 Three classes of genes, transcribed by different RNA polymerases

Enzymes of the family of DNA-dependent RNA polymerases carry out the process of transcription, that is the synthesis of a molecule of RNA mirroring the information encoded on the template DNA. Eukaryotes have three different RNA polymerases, RNA pol I, II and III, which transcribe different, specific sets of genes. RNA

polymerase I is responsible for the synthesis of the 18S, 25S and 5.8S ribosomal RNAs, which are cleaved from single precursor RNAs encoded by the highly transcribed rRNA genes (*RDN1* on Chr. XII in *S. cerevisiae*) that are clustered on the chromosomes as tandem repeats. RNA polymerase III synthesizes the 5S ribosomal RNA, the tRNAs and other small, non-protein-encoding RNA molecules (reviewed in Paule and White, 2000). RNA polymerase II finally performs the transcription of all protein-encoding genes (reviewed in Ishihama *et al*, 1998).

1.1.2 Promoter elements of RNA polymerase II

Before transcription can start RNA polymerase II has to be assembled at the transcription start site. This requires both general and specific sequences in the promoter of the gene, the so-called cis-acting factors, and a large set of both general and specific proteins and protein complexes generally called trans-acting factors (reviewed in Hampsey, 1998; Pérez-Martín, 1999; Lee and Young, 2000; Gregory, 2001). The general cis-acting factors are the TATA box and the initiator elements (Inr). The TATA box (consensus $TATA^{A}/_{T}A^{A}/_{T}$) is located 40 to 120 basepairs upstream of the transcription initiation site in yeast and 25 to 30 basepairs upstream in other eukaryotes, while the initiator elements are pyrimidine-rich stretches located around position +1 of transcription. Not all genes contain both elements, but in most promoters at least one is present (Hampsey, 1998). The specific cis-acting sequences are binding sites for transcriptional activators that positively regulate transcription from restricted sets of genes. These sites are collectively called Upstream Activating Sequences (UAS) in yeast and reside around hundred to thousand basepairs upstream of the transcription start site. In metazoans these sequences are called enhancers and may lie up to about a hundredthousand basepairs away from the transcription start site, both upstream and downstream and even inside genes.

1.1.3 Trans-acting factors of RNA polymerase II

The trans-acting factors consist of a large number of proteins that alone or in complexes act at promoters to in- or decrease transcription of genes as required. These factors can be broadly grouped into three classes based on their specificity. The first class, the general transcription machinery, consists of RNA polymerase II itself and its associated general transcription factors (GTFs) and is required for transcription of all RNA pol II-dependent genes. The second class is that of the general transcriptional coactivators and repressors. These are involved in regulation of large groups of genes, mostly through modification of chromatin structure, but are

not required for transcription of all genes. The third and largest class consists of specific transcriptional activators and repressors that strongly and with high specificity bind to their target promoters and regulate transcription of small groups of coregulated genes or even individual genes. This is achieved through enhanced recruitment of factors from the second and first classes to these promoters.
The three classes of trans-acting factors are described in more detail below with a focus on the situation in yeast.

1.1.4 The general transcription machinery

- RNA polymerase II.

  The RNA polymerase II from yeast is a ~550 kDa, 12 subunit enzyme that shows a high degree of homology to all other eukaryotic RNA pol II's. In yeast, RNA pol II shares five subunits with RNA pol I and III. Four other subunits show strong similarity to their pol I and pol III counterparts. Only three are unique to RNA pol II and two of those are the only non-essential components (Hampsey, 1998; Ishihama *et al*, 1998). Directly after TFIIB-binding to the promoter (see below), RNA pol II is recruited to the TBP/TFIID-TATA complex and then forms the center of preinitiation complex (PIC) formation. The main structural feature of RNA pol II for PIC formation is the carboxy-terminal domain (CTD) of its largest subunit, Rpb1p. This structure is remarkable in containing 27 tandem repeats of a highly conserved seven-residue sequence. This sequence, and with it the entire structure of the CTD, is conserved from yeast to mammals, the number of repeats being the only variable. It increases from 27 in yeast through 34 in *C. elegans* and 43 in *D. melanogaster* to 52 in humans. It is the target of a multitude of kinases. Extensive phosphorylation of the CTD is the main structural difference between the unphosphorylated IIA form that enters the PIC and the hyperphosphorylated IIO form that escapes the promoter into elongation (Carlson, 1997; Hampsey, 1998). The alternative CTD phosphorylation has major consequences for the protein-protein interactions it is engaged in during different phases of transcription (Sakurai and Fukasawa, 1998; Bentley, 1999; Hirose and Manley, 2000; Conaway *et al*, 2000). Although the core RNA polymerase II can assemble with the GTFs into a PIC that supports basal transcription *in vitro*, activated transcription requires the presence of transcriptional coactivators. In yeast the major complex fullfilling this role is the SRB/Mediator (see below) rather than the TAF_{II}s. Coimmunoprecipitation experiments using antibodies against Srb proteins, as well as a different approach aimed at RNA pol II-Mediator interaction (at that

time unlinked), led to the discovery that *in vivo* in solution RNA polymerase II occurs mainly in complexes with the SRB/Mediator complex as well as TFIIB, TFIIF and TFIIH or only TFIIB, depending on the method. Less abundant complexes that contain factors different from SRB/Mediator could also be identified (reviewed in Hampsey, 1998; Lee and Young, 2000). These data strongly suggest that RNA polymerase II may be recruited by TBP as a preformed holoenzyme, as the large complex was called. The older model envisioned stepwise assembly on the promter. The purified holoenzyme is capable of mediating activated transcription *in vitro*. The SRB/Mediator complex and TBP contact RNA pol II through the CTD whereas TFIIB contacts other regions of the core polymerase. TFIIH contacts RNA pol II both on the CTD and on the polymerase core (Ishihama *et al*, 1998). After CTD-hyperphosphorylation at the start of elongation, SRB/mediator is displaced. Proteins and complexes instrumental in transcription elongation, mRNA processing and recombination/repair now occupy the CTD (Bentley, 1999; Hirose and Manley, 2000; Conaway *et al*, 2000).

- General Transcription Factors (GTFs) TBP/TFIID and TFIIA.

  The TATA-binding protein (TBP) is the first of the "classical" GTFs to bind to the promoter. Together with at least 12 TBP-associated factors (TAF_{II}s) it can form TFIID. Most of the TAF_{II}s identified are essential for viability and required for activated transcription, although not for all genes (Reese *et al*, 1994; Poon *et al*, 1995; reviewed in Pugh, 2000). The TATA-bound TBP or TFIID nucleoprotein complex then functions as a core for assembly of the pre-initiation complex (PIC), consisting of the complete set of GTFs, RNA polymerase II and transcriptional coactivators. Originally it had been found that *in vitro*, purified TBP alone was capable of supporting a low level "basal" transcription that required only RNA pol II and the other purified GTFs and that could not be increased through the action of transcriptional activators (Buratowski *et al*, 1989; Sayre *et al*, 1992; reviewed in Hampsey, 1998; Pugh, 2000). The latter additionally required the presence of the $yTAF_{II}$s (Reese *et al*, 1994; Poon *et al*, 1995), for which reason these were though to be mere transcriptional coactivators allowing the activators to contact the PIC. Recently however it has become clear that the second largest yeast TAF_{II}, $yTAF_{II}145$ that is the homologue of human $TAF_{II}250$ and functions as a scaffold for TFIID assembly through direct contact with TBP, is a histone acetyl transferase (HAT; see below). Moreover 5 essential $yTAF_{II}$s are also integral subunits of the Spt-Ada-Gcn5 acetyltransferase (SAGA) HAT complex (Durso *et al*, 2001;

Gangloff *et al*, 2001; Kirchner *et al*, 2001; Selleck *et al*, 2001) and SAGA can form a complex with TBP. Finally the HATs Gcn5p (SAGA) and yTAF$_{II}$145 (TFIID) are redundant for transcription of at least some genes (Lee *et al*, 2000a) and individual TAF$_{II}$s may be required for transcription of some genes but not others (Durso *et al*, 2001). Whether TBP binds the TATA-box alone or in a complex is in part determined by growth condition (Kuras *et al*, 2000).
TFIIA is a small two-subunit complex important for efficient loading of TBP/TFIID onto the TATA-box. TFIIA has been shown to disrupt TBP dimers, the non-DNA-bound soluble form of TBP, and prevent redimerization. It so accelerates the kinetics of TBP-binding to the TATA-box (Coleman *et al*, 1999). Moreover, it directly contacts one of the TAF$_{II}$s in TFIID and this interaction is important for stability of the TFIID-DNA nucleoprotein complex (Solow *et al*, 1999; Solow *et al*, 2001; Kraemer *et al*, 2001). Finally, TFIIA appears to increase the specificity of TBP/TFIID DNA-binding (Stewart and Stargell, 2001).

- GTFs TFIIB, TFIIE, TFIIF and TFIIH.

The first general transcription factor to bind to the TBP/TFIID-DNA core is TFIIB, a 38 kDa protein that contacts TBP, RNA pol II and DNA adjacent to the TATA-box. It has an important function in proper transcription start site selection by RNA pol II and is a target for recruitment by several transcriptional activators (Pinto *et al*, 1992; Na and Hampsey, 1993; Pinto *et al*, 1994; Roberts and Green, 1994). Next to bind is RNA pol II and stably associated with it, TFIIF.
TFIIF is a heterotrimer in yeast and appears to be the closest to a eukaryotic homologue of the bacterial σ factors that suppress non-specific binding of RNA polymerase to DNA and stabilize the PIC. The latter function is conserved in TFIIF (Henry *et al*, 1992; reviewed in Henry *et al*, 1994). It is performed in part by changing the conformation of the promoter DNA through DNA-binding between TATA-box and transcription start site and upstream of the TATA-box. TFIIF also functions in the elongation phase of transcription by suppressing transient pausing. The third, smallest yeast TFIIF component, the 30 kDa product of the non-essential *TFG3* gene, was shown to be identical to TAF$_{II}$30 of TFIID and the Swp29p subunit of the SWI/SNF complex (Poon *et al*, 1995; Cairns *et al*, 1996b).
TFIIE (Feaver *et al*, 1994a) is assembled into the forming PIC after RNA pol II and contacts the polymerase, TFIIF, TFIIH and probably single-stranded DNA, thereby stabilizing the open complex after promoter DNA-melting.

The final general transcription factor to enter and complete the PIC is TFIIH. Like TFIID it is a large multimeric complex and it is the only GTF apart from TFIID with proven enzymatic activities. It is a DNA-dependent ATPase (Feaver *et al*, 1994b), an ATP-dependent helicase and a CTD kinase. It functions not only in transcription, but also in nucleotide excision repair (NER) of DNA damage (Wang *et al*, 1994; Feaver *et al*, 1997). TFIIH interacts with RNA pol II and TFIIE upon which it is dependent for functionality, probably through stimulation of its enzymatic activities (Ohkuma *et al*, 1995). Functional complementation between *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* only occurs when both TFIIE and H are exchanged (Li *et al*, 1994). The helicase function is required for the role of TFIIH in NER but dispensable for transcription (Bardwell *et al*, 1994; Guzder *et al*, 1994a; 1994b). Transcription requires the CTD kinase function. This activity is mediated by the cyclin-dependent kinase (Cdk)-cyclin pair Kin28-cyclin H. These form a dimer that is also called TFIIK and is less tightly associated in TFIIH than the other subunits. It phosphorylates the C-terminal domain (CTD) of RNA polymerase II around the time when transcription starts (Feaver *et al*, 1994b; 1997).

- SRB/Mediator.

The *SRB* (suppressor of RNA polymerase B) genes were identified as suppressors of the growth phenotypes caused by truncation of the CTD to 10 repeats. The Mediator was biochemically identified as a fraction required to support activated transcription in reconstituted systems of RNA pol II and GTFs (Hampsey, 1998; Myers *et al*, 1998; reviewed in Myers and Kornberg, 2000). Only when Mediator was purified as a 20-subunit protein complex and four of the subunits were shown to be Srb proteins, the connection became clear. Identification of the other subunits showed that some were encoded by genes that had previously been found in genetic screens whereas others (*MED* genes) (Myers *et al*, 1998) were new. The individual components will not be discussed here (reviewed in Hampsey, 1998; Myers and Kornberg, 2000; Lee and Young, 2000), Suffice to say that 11 of 20 are essential, that some act genome-wide whereas others affect only restricted sets of genes and that the complex has been shown to be involved both in activation and repression of transcription. Most of these activities result from the interface that SRB/Mediator forms between the core RNA polymerase II, activators, repressors and other proteins involved in regulation of transcription. It integrates and mediates the signals to the CTD of the pol II Rpb1 subunit.

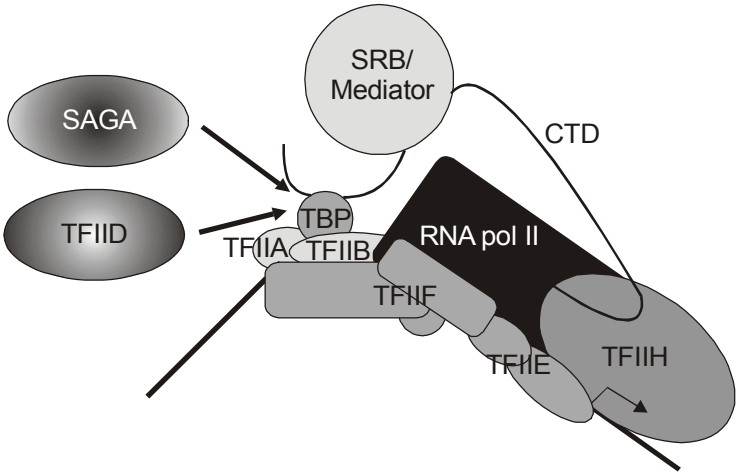


Figure 1-1: Assembly of the pre-initiation complex at the transcription start site.
Rendering of a RNA polymerase II holoenzyme fully assembled on a promoter. TBP may be associated either alone orin complex with TFIID or SAGA.
Adapted from Hampsey, 1998.

1.1.5 General transcriptional coactivators and repressors

- Histone acetyltransferase (HAT) / Histone deacetylase (HDAC)

  In chromatin DNA is wound on octameric histone complexes ($H2A_2\,H2B_2\,H3_2\,H4_2$), forming nucleosomes that in turn can be stacked to form the 30 nm solenoid. This packing serves to compact the DNA in an ordered way, but is also used in regulation of transcription. All histones contain amino-terminal domains that extend from the central globular domains. These can be modified through regulated acetylation, phosphorylation, methylation and ubiquitination (reviewed in Wu and Grunstein, 2000). Regulated acetylation / deacetylation has been shown to play a role in regulation of transcription. Hyperacetylation correlates with increased transcription and *vice versa*, and results from activity of histone acetyltransferases (HATs) (reviewed in Chen *et al*, 2001) and histone deacetylases (HDACs) (reviewed in Hampsey, 1998; Pérez-Martín, 1999; Gregory, 2001), respectively. In yeast the best-studied HAT is SAGA (Spt-Ada-Gcn5-acetyltransferase): a 1.8 megaDalton, 16-subunit protein complex that acetylates histone H3 through the enzymatic activity of its Gcn5p subunit (reviewed in Grant *et al*, 1998; Hampsey, 1998; Pérez-Martín, 1999; Chen *et al*, 2001). Another important yeast HAT is NuA4 (nucleosome acetyltransferase of H4), which acetylates histone H4 (reviewed in Gregory, 2001). This hyperacetylation of histones most likely leads to a loosening of the chromatin, and to some extent of the histone octamer itself, and allowes better access of factors to the nucleosomes and DNA. SAGA and NuA4 have been shown to be recruited to chromatin by acidic activation domains (see below) (Ikeda *et al*, 1999). This implies that they may be recruited to at least some promoters by transcriptional activators already (weakly) bound to their UAS sequences. The NuA4 enzymatic subunit Esa1p is the only essential HAT

identified so far and NuA4 has been shown to be recruited to promoters of ribosomal protein genes. Massive expression of these genes is required in dividing cells and loss of Esa1p function leads to cell cycle defects (reviewed in Gregory, 2001).
HAT activity is counteracted by complexes that have histone deacetylase activity (HDACs) and remove acetyl moieties from histone tails (reviewed in Ayer, 1999; Ng and Bird, 2000). In yeast five HDACs were identified; Rpd3p, HDA1, *HOS1*, *HOS2* and *HOS3*. Rpd3p and HDA1 are the best studied. Activity appears not to be global but restricted to defined sets of genes. Deletion leads to hyperacetylation of histones H3 and H4 (Rundlett *et al*, 1996). Histone deacetylation is involved in regulation of transcription of specific genes. The Rpd3p-Sin3p complex was shown to be recruited to the promoters of *INO1* and several other genes by the DNA-binding factor Ume6p and to repress transcription (Kadosh and Struhl, 1998; Elkhaimi *et al*, 2000). However, together with a co-activator Ume6p can also act as an activator for different genes. Rpd3p-Sin3p also represses some genes independent of Ume6p. HDA1 was recently shown to be recruited to the *ENA1* promoter by Tup1p (see below) and to mediate repression (Wu *et al*, 2001).

- Chromatin remodeling complexes.

  This second class of chromatin modifying complexes consists of DNA-dependent ATPases that perform physical movement of nucleosomes. In yeast the 2 megaDalton, 8- to 11-subunit SWI/SNF complex (for switch = defective in mating type switching / sucrose non fermenting = defective in invertase *SUC2* gene expression) can disrupt histone octamers without dislodging them from the DNA, move nucleosomes along the DNA strand they are on and remove octamers from one DNA strand and place them on another strand (reviewed in Hampsey, 1998; Pérez-Martín, 1999; Sudarsanam and Winston, 2000; Peterson and Workman, 2000). So can the related RSC complex (remodels the structure of chromatin) (Cairns *et al*, 1996a). The access of SWI/SNF to nucleosomes is increased, and its activity is therefore higher, on chromatin that has been loosened up through HAT activity. It, like SAGA, can physically interact with the activation domains of several transcriptional activators and might require recruitment to its target promoters. The fact that retention of SWI/SNF on target promoters is increased after HAT-mediated histone acetylation, even in the absence of transcriptional activators, argues for an ordered recruitment and activity at least on some promoters (Hassan *et al*, 2001). Finally, SWI/SNF has been shown to interact with RNA

polymerase II itself as a part of SRB/Mediator (see below) (Wilson *et al*, 1996a). Whereas SWI/SNF appears to act only on some classes of genes, RSC seems to be more general in its activity and more important than SWI/SNF, for loss of RSC leads to a similar phenotype as does loss of HAT NuA4 (reviewed in Gregory, 2001). Although little is known about repression by chromatin remodeling complexes, yeast Isw2 has been shown to repress early meiotic genes (Kent *et al*, 2001). It does so in parallel to Rpd3p-Sin3p and has to be recruited by Ume6p (Goldmark *et al*, 2000).

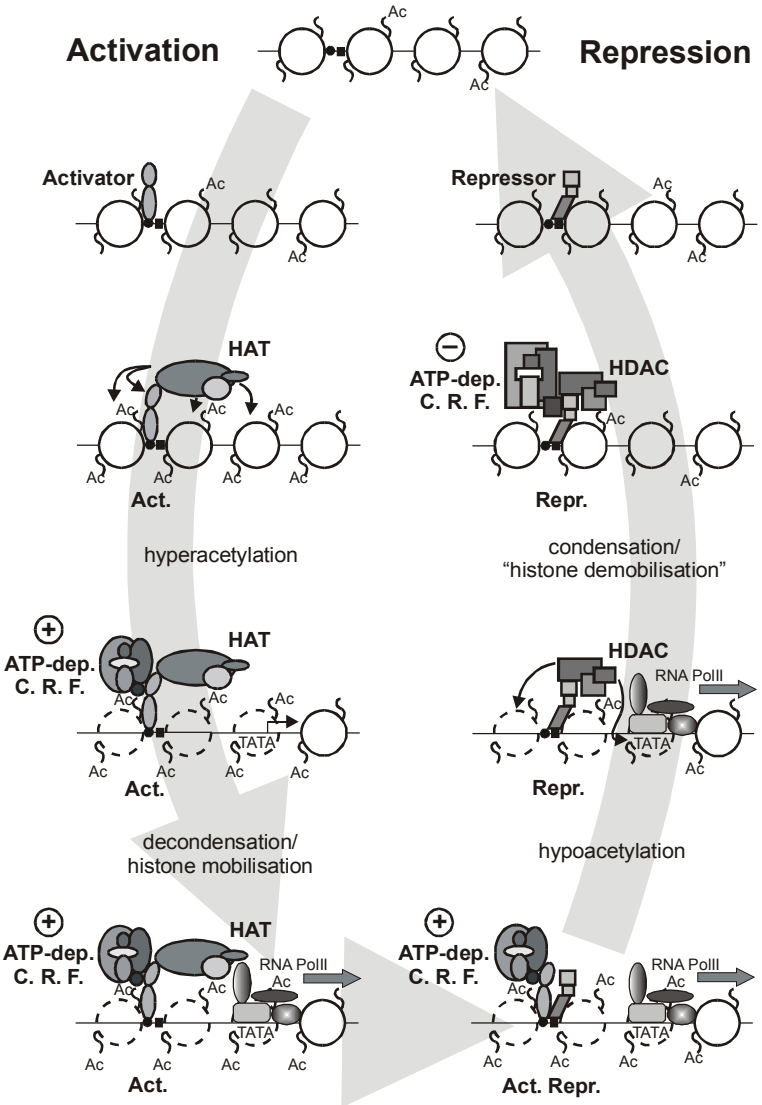


Figure 1-2: A model of the role of chromatin remodeling in regulation of transcription. To activate transcription of a gene, its activator binds to the promoter which is packed on hypoacetylated histone octamers in condensed chromatin. A HAT recruited by the activator hyperacetylates the nucleosomal histones and facilitates recruitment of a positively acting ATP-dependent chromatin remodeling factor (+ ATP-dep. C.R.F.) that decondenses the promoter chromatin. On the now accessible transcription start site the RNA polymerase II holoenzyme assembles and transcription is initiated. Adapted from Gregory, 2001.

One class of transcriptional repressor proteins and complexes interacts directly with the transcriptional machinery. In this class belongs the Mot1 protein that disrupts the TBP/DNA complex at promoters (Darst *et al*, 2001). The NC2 complex probably destabilizes or disrupts interaction of TFIIA and TFIIB with the TBP/DNA nucleoprotein, thereby destabilizing TBP-TATA interaction (Lemaire *et al*, 2000). Another complex called Ccr4-NOT appears to function by a similar mechanism (reviewed in Hampsey, 1998; Lee and Young, 2000).

1.1.6 Specific transcriptional activators and repressors

The general transcription machinery, made up of the general transcription factors, RNA polymerase II and SRB/Mediator as described, is capable of sustaining low level basal transcription. This can be modulated to some extent by the action of HATs like SAGA, their HDAC counterparts and chromatin remodeling complexes such as SWI/SNF or RSC.
The often much larger differences in transcription (up to the 1000-fold range) of many genes, caused by changing growth conditions or progression through the cell cycle, require the action of transcriptional activators and/or repressors. Since activation and repression are not mere structural or mechanistic mirror images, I will in the following summarize present knowledge about activators first and about repressors second.
As mentioned before, activators show some common features:

- Domain structure

  In contrast to most enzymes, transcriptional activators are modular. The different functions of transcriptional activators (DNA-binding, activation) are generally organized in separate structural features, the so-called domains, within the protein as a whole (reviewed in Lee and Young, 2000). As a consequence, individual functions can usually be altered or destroyed by mutation without affecting the other function(s) of the protein, which is very difficult or impossible for most enzymes. This allows highly informative domain-swap experiments in which domains from several activators are combined to form new, artificial activators. Almost any combination is possible. The strength of a weak activator can be increased, for example, by adding copies of its own activation domain or substitution by the very strong herpes simplex virion protein 16 (VP16) or adenovirus E1A activation domains. Domain structure even allows reconstitution of functional activators through protein-protein interaction if one protein contains a DNA-binding domain and the other an activation domain. This is the principle of the two-hybrid system (Fields and Song, 1989; Chien *et al*, 1991). One protein (bait) is fused to a DNA-binding domain (DBD), the other to an activation domain (AD). Both constructs are expressed in the same (yeast) cell and when the proteins interact a functional activator is formed that activates expression of a reporter gene under control of the cognate UAS. This functions for a multitude of combinations of DBDs plus cognate UAS, ADs and reporter genes and in basically every organism.

- Sequence-specific DNA binding.

  Activators show high-affinity, high-specificity binding to DNA sequences in the promoters of the genes they regulate. These sequences are called Upstream Activating Sequence (UAS) and are usually located some 100 to 1000 basepairs upstream of the transcription start site (reviewed in Hampsey, 1998; Lee and Young, 2000). Several conserved motifs mediating DNA-binding have been identified (reviewed in Patikoglou and Burley, 1997).
  The helix-turn-helix class of activators is based on a constellation of α-helices that insert into the major groove of the DNA double helix. These factors bind as monomers (heat shock factors), homo- or heterodimers (Matα1/Matα2).
  The members of the class of fibrous activators are elongated and bind DNA as homo- or heterodimers. Dimerization is mediated by a leucine zipper (Gcn4p), a helix-loop-helix region or both. C-terminal acidic sequences reshape into α-helices after making contact with their cognate DNA sequences in the major groove, positioning the dimer on the DNA like a pair of tweezers.
  The third large class is that of the metallo-proteins. All metallo-protein activators characterized to date complex zinc and most do so through one of three mechanisms. One zinc ion is coordinated by two cysteines and two histidines (Cys^2His^2 zinc finger; TFIIIA) or by four cysteines (Cys^4 double loop-zinc-helix, homo- or heterodimeric binding; steroid/nuclear receptors).
  Coordination of two zinc ions by six cysteines (Baleja *et al*, 1992) is found only in fungi and defines the $Zn(II)_2Cys_6$ binuclear zinc cluster transcriptional activators (reviewed in Schjerling and Holmberg, 1997). These bind DNA as homodimers and co-crystal structures of the zinc cluster domains of Gal4p, Ppr1p, Put3p and Hap1p bound to DNA were produced (Marmorstein et al, 1992; 1994; Swaminathan *et al*, 1997; King *et al*, 1999a; 1999b).
  Due to the high specificity of recognition only minor deviations from the consensus sequence are tolerated. Identification of a consensus binding sequence for an activator in a promoter is an indication that this regulator may regulate the gene. Even so, in the context of multi-megabase genomes such sequences occur plentifull. An important question is whether each consensus sequence, found for example in the +1 to -1000 regions of all open reading frames in a genome, is actually bound by its cognate regulator. Recently it was shown for the yeast transcriptional activators Gal4p and Ste12p that this is not so (Ren *et al*, 2000), indicating that determinants additional to sequence-specificity regulate actual binding; perhaps accessibility of the site in chromatin, as yet unidentified structural features of the promoter context or interactions with the general transcription machinery.

- Activating potential

  Transcriptional activators all contain at least one activation domain. Clear consensus protein sequences are lacking in the regions identified as activation domains in eukaryotes. Therefore, these domains were rather arbitrarily classified as acidic, glutamine-rich or proline-rich, based on overrepresentation of these amino acids (reviewed in Nelson, 1995; Triezenberg, 1995). The by far largest class is that of the acidic activation domains. These are characterised by a high content of acidic residues interspersed with hydrophobic/aromatic ones. It has become clear that the latter are at least as important in the overall structure as the acidic ones. Although mutational changes in acidity strongly correlate with activator potency, mutation of the hydrophobic/aromatic residues strongly impairs activating potential of the domains (Young *et al*, 1998). One problem in studying activation domains is that they appear unstructured in solution, only adopting their protein-interaction-competent shapes after initial contact with their interaction partner(s): a form of induced fit. These shapes may be amphipatic α-helices or, as had been shown for Gal4p, β-sheets (Leuther *et al*, 1993; Van Hoy *et al*, 1993). In either case an acidic and hydrophylic face opposite the hydrophobic face that performs the actual protein-protein interaction might stabilize the structure. Activators containing at least one activation domain of this type are often (very) strong and function over large distances. They function through recruitment of co-activators and components of the general transcription machinery and accordingly have been shown to contact components of HAT complexes (SAGA, NuA4) (Ikeda *et al*, 1999), chromatin remodeling complexes (SWI/SNF, RSC), GTFs (TBP (Kotani *et al*, 2000), TFIIB (Wu *et al*, 1996), TFIIE, TFIIF, TFIIH) and/or components of SRB/Mediator (Hampsey, 1998). Glutamine-rich activation domains do not occur in yeast and metazoan domains of this type (Sp1) were originally shown not to be functional in budding yeast (in contrast to fission yeast *S. pombe* (Remacle *et al*, 1997)). This seemed to correlate with the absence in the *S. cerevisiae* genome of a counterpart to the human $TAF_{II}110$, which is the target of glutamine-rich activation domains. Recently, however, it was shown that glutamine-rich activation domains do function in *S. cerevisiae* if the promoter structure is comparable to the metazoan model (Xiao and Yeang, 1998; Escher *et al*, 2000). A glutamine-rich activator bound close to the TATA-box, inactive by itself, shows strong synergy with distally bound acidic activators. The proline-rich activation domains take a position somewhere in between. They are able to weakly activate transcription in yeast by themselves from promoter-proximal

locations and show a synergistic effect with distal acidic activation domain factors (Tsai-Pflugfelder *et al*, 1998; Li, 1999).

- Regulated activity.

  For a protein to be a regulator, its own activity must be regulated. Many ways to achieve this regulation of the regulator have been identified. The most direct way is to in- or decrease synthesis as required. Both transcription and translation can be modified to this end. Regulation of transcription is employed for most regulators at least to some extent, but since it is a rather slow and unprecise response, additional mechanisms of regulation are usually superimposed. Regulation of translation is not very common but for example in the case of transcriptional activator Gcn4p, transcription of the *GCN4* gene is increased less than two-fold whereas a strong increase in translation leads to about ten-fold increase in protein concentration upon amino acid starvation (Mueller *et al*, 1998; Hinnebusch, 1997; 1984; Thireos *et al*, 1984; Hope and Struhl, 1986).
  Some activators require phosphorylation for activity, for example Cat8p, which is required for expression of gluconeogenic and glyoxylate cycle genes on poor carbon sources as glycerol or ethanol (Randez-Gil *et al*, 1997). The nitrogen source-regulated activator of proline utilization genes *PUT1* and *PUT2*, Put3p, is constitutively bound to the DNA, but is increasingly phosphorylated (and activated) with decreasing nitrogen source quality (Huang and Brandriss, 2000). Phosphorylation has the advantage of being very fast and readilly reversible.
  Some factors require interaction with organic or inorganic molecules to unfold their activity. The transcriptional activator Hap1p activates transcription of *CYC1* and *CYC7* (encoding cytochrome *c* isoforms required for respiration), amongst others, in response to oxygen. It depends on interaction with heme, which requires oxygen for synthesis, to be released from a high molecular weight complex before it can bind DNA and activate transcription (Zhang *et al*, 1998; Hon *et al* 1999; Hach *et al*, 1999; 2000; Ramil *et al*, 2000; reviewed in Zhang and Hach, 1999). The activator of the metallothionein gene *CUP1* and superoxide dismutase gene *SOD1*, Ace1p, cannot bind to DNA unless complexed with copper(I) (Gralla *et al*, 1991).
  A different method of regulation is alternate localization. The transcription factors Msn2p and Msn4p, which activate numerous genes in response to a number of stresses (Boy-Marcotte *et al*, 1998), are actively retained in the cytoplasm. This retention requires high cAMP and protein kinase A (PKA)

activity. In response to stress cAMP and PKA activity are reduced and Msn2p/Msn4p migrate to the nucleus to bind stress response elements (STREs) and activate transcription. This relocalisation is reversible by renewed cAMP-stimulated PKA activity (Gorner *et al*, 1998).
A different mechanism for regulating location has been found for the Spt23p and Mga2p transcriptional activators. These related factors are involved in regulation of unsaturated fatty acid-content of membranes. They are themselves retained at the endoplasmatic reticulum and the cytoplasmic face of the nuclear envelope through C-terminal sequences inserted in the membrane. Activation, dependent on fatty acid composition of the membrane, requires ubiquitination followed by proteasome-mediated partial proteolysis of the membrane anchor. This releases the "mature" transcription factors in the cytoplasm so they can migrate to the nucleus and activate transcription (Hoppe *et al*, 2000).

Summarizing, transcriptional activators are highly specific, DNA binding proteins that regulate defined sets of genes by recruiting components of chromatin remodeling complexes and/or the general transcription machinery to enhance preinitiation complex formation in response to a stimulus.

On the repression side of regulation the picture is not so clear and ordered. One reason for this is the difficult experimental accessibility of transcriptional repression. Also, influence on transcription and DNA binding are not as closely linked in repressors as in activators. Finally, repressors appear not to act as gene-specific as activators, which leads to pleiotropic effects of mutations in repressors and sometimes to inviability of the mutants. Nevertheless a lot has been learned about mechanisms of repression. Genes may be repressed through DNA-bound factors, although repressors with specific DNA-binding and repressing function in one protein are rare. The best characterized such protein, Ume6p, binds URS sequences of genes required for mitosis and sporulation, amongst others, and recruits the Sin3-Rpd3 histone deacetylase and/or Isw2 chromatin remodeling complex (Elkhaimi *et al*, 2000; Goldmark *et al*, 2000). More often DNA-binding proteins involved in repression function as adaptors for accessory repressing factors. The best studied of such complexes is the Ssn6 (Cyc8)-Tup1 complex. The functional complex is an Ssn6-$(Tup1)_4$ heteropentamer (Varanasi *et al*, 1996; Jabet *et al*, 2000) with the main function of mediating protein-protein interactions. Neither protein is DNA-binding but both function in repression when artificially targeted to promoters through fusion with a DNA-binding domain (Keleher *et al*, 1992; Tzamarias and Struhl, 1994). In such experiments Tup1p alone is sufficient for repression whereas Ssn6p requires Tup1p

for repression. Ssn6p contains ten 34-residue tetratricopeptide repeats (TPR), which are essential for its function and have been implicated in protein-protein interaction. The same goes for the seven ~40-residue WD40 repeats identified in Tup1p. To exert its repressive function, Ssn6-Tup1 binds to a number of DNA-binding proteins specific for different sets of genes. For example, it represses a number of glucose-repressed genes through Mig1p, **a**-specific genes through the DNA-bound α2/Mcm1 complex and genes required for anaerobiosis through Rox1p (reviewed in Smith and Johnson, 2000). The modes of repression by Ssn6-Tup1 are multiple. Tup1 interacts with hypoacetylated histones H3 and H4 *in vitro* and mutation of H3 and H4 N-termini interferes with repression by Tup1p *in vivo* (Edmondson *et al*, 1996). Artificial recruitment of Ssn6-Tup1 leads to repression and histone-hypoacetylation (Bone and Roth, 2001). Furthermore Ssn6-Tup1 has been shown to interact with HDACs HDA1 (Wu *et al*, 2001) and Rpd3p (Watson *et al*, 2000) and with the RNA polymerase II holoenzyme through interaction with SRB/Mediator and CTD-associated components (Kuchin and Carlson, 1998; Lee *et al*, 2000b).

An indirect mechanism to downregulate transcription is antiactivation, in which the activator is prevented from performing its function. For example, activators may be targeted for destruction to discontinue their activity. The activator Gcn4, active under amino acid starvation, is phosphorylated and then ubiquitinated and targeted for destruction by the proteasome when starvation ends (Meimoun *et al*, 2000). It has been shown that the ubiquitination signal often resides in activation domains and that the potency of both functions correlates, stronger activators being less stable (Salghetti *et al*, 2000). Another way of targeting activators in repression is complex-formation of the activator with an inhibitor of activation function or DNA binding capability. The paradigm of this type of negative regulation, shielding of the Gal4p activation domain through association with the inhibitor Gal80p, is introduced in more detail below. In repression as well as in inactivation subcellular localization can be regulated. Snf1p phosphorylates the Mig1protein mentioned before, a DNA-binding mediator of repression, during the diauxic shift or when cells are shifted to poor carbon sources. This leads to its export from the nucleus (DeVit *et al*, 1997; Östling and Ronne, 1998; Treitel *et al*, 1998; Smith *et al*, 1999).

Regulation of transcription is a highly dynamic process and a clear distinction between activation and repression is not seldom difficult. Many of the regulatory factors mentioned, particularly the chromatin remodeling factors and other components of the general transcription machinery, have been shown to regulate some genes positively while at the same time regulating others negatively (Bernstein *et al*, 2000; Sudarsanam and Winston, 2000). These differences may be caused by chromosomal context, differential interactions with other DNA- and non-DNA-binding regulators or perhaps by yet different, so far unidentified mechanisms. By powerfull

methods such as comparative, microarray-based genome-wide screening of gene-expression (DeRisi and Iyer, 1999) new data are quickly being gathered that shed light on coregulation of classes of genes by individual regulators in response to environmental changes.

1.2 Regulation of transcription by carbon source in yeast

Glucose, through the intermediate glucose-6-phosphate produced from it in the cell, is central both in biosynthesis and energy production. It gives the highest net energy yield of all carbohydrates. It is therefore not surprising that glucose and other (poly)saccharides, easily converted to it, are preferred carbon sources. This does not mean that other carbon sources as glycerol or lactate, ethanol or acetate, fatty acids or even amino acids could not be used. Depending on the distribution of carbon sources in the natural environment, however, organisms vary in their capability to use any of these alternative carbon sources, or even alternative sugars, and in their avidity to use them in the presence of glucose.
The work described in the next sections deals with the unicellular yeasts, more particular the dairy yeast *Kluyveromyces lactis* and in comparison the baker's yeast *Saccharomyces cerevisiae*. These two species are closely related; yet show some striking differences in physiology. Those concerning the use of alternative carbon sources will be discussed here. *S. cerevisiae* is highly specialized in using glucose, due to the plentifull availability of it in its natural environment (wine grapes). *K. lactis* shows a broader carbon source spectrum since the dairy products that are its habitat are less abundant in glucose and contain different carbon sources in greater amounts (Wésolowski-Louvel *et al*, 1996). In all cases the choice which carbon source to use when several are available at one time is a regulated event. In general sugars repress the use of other carbon sources and the presence of glucose even represses the use of other sugars. This effect is known as carbon catabolite repression or glucose repression.
An additional important difference between the two yeasts is their use of oxygen. *S. cerevisiae* is one of few Crabtree-positive yeasts. In the presence of sugars fermentation is prevalent and respiration minimal in these yeasts, despite oxygen saturation. *K. lactis* on the other hand is Crabtree-negative. It will not ferment sugars unless starved for oxygen (reviewed in Breunig *et al*, 2000). Both yeasts can use galactose as an alternative carbon source. The regulon required for this consists of the *GAL* genes. *K. lactis* also grows very well on the disaccharide lactose, consisting of β-1,4-linked glucose and galactose moieties. Uptake and metabolism require two additional gene products, lactose permease (also required for galactose uptake) and β-galactosidase, encoded by the *LAC12* and *LAC4* genes, respectively. *S. cerevisiae*

cannot utilize lactose, but can grow on melibiose (α-1,6-linked glucose and galactose moieties) through *MEL1* gene product α-galactosidase that is secreted and extracellularly hydrolyses melibiose. Galactose is taken up by the Gal2p galactose permease. Regulon-organization and -regulation is conserved between the yeasts (Figure 1-3).

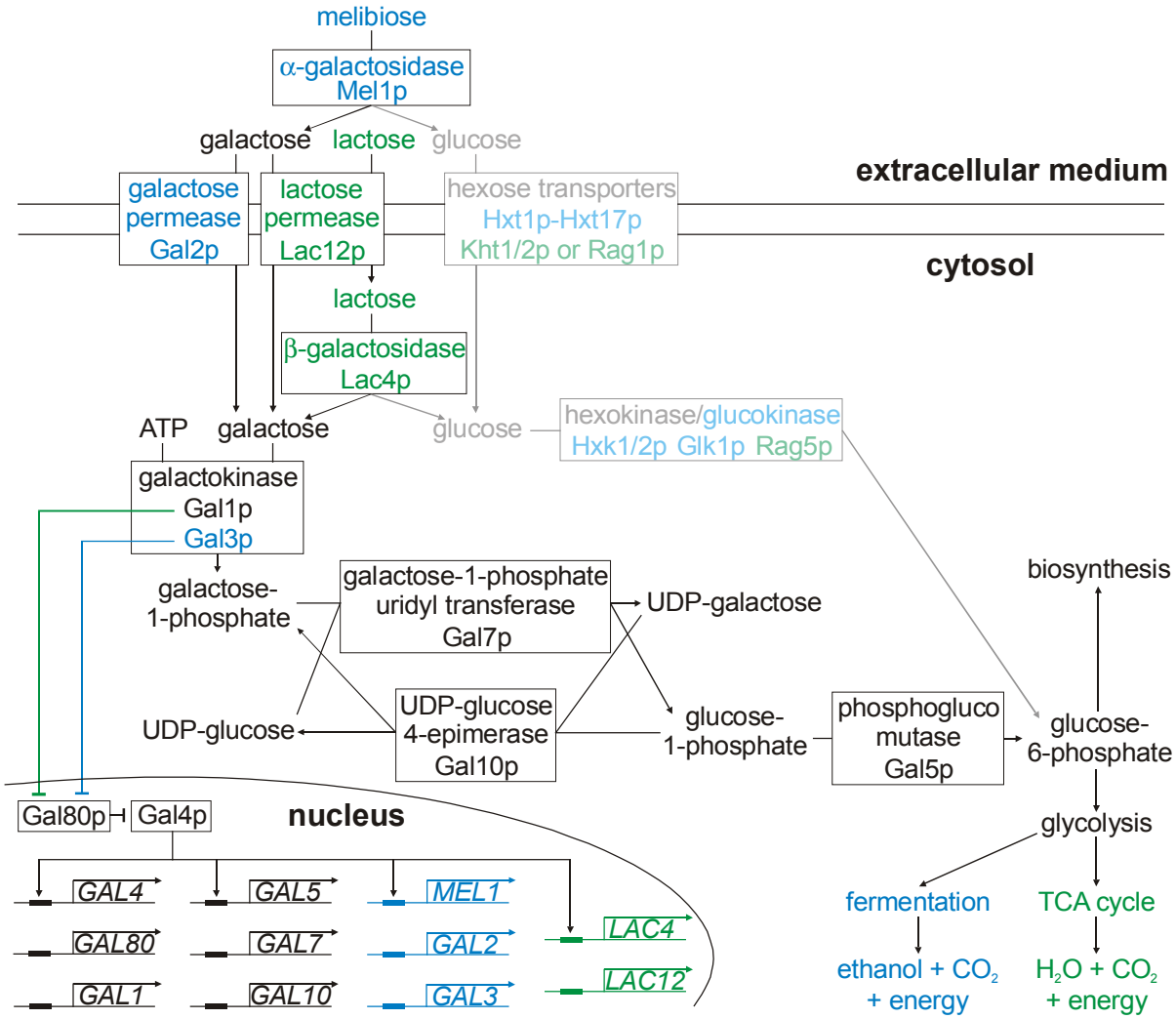


Figure 1-3: Metabolism of galactose/lactose in *Kluyveromyces lactis* and galactose/melibiose in *Saccharomyces cerevisiae*.
Common genes (nucleus), proteins (boxed) and intermediates are in black; those specific to *K. lactis* are in green and those specific to *S. cerevisiae* in blue. The uptake and phosphorylation of glucose is represented in grey.

1.2.1 Glucose repression

K. lactis cells metabolize glucose to CO_2 and H_2O through the tricarboxylic acid (TCA) cycle and respiration. Under those circumstances the genes encoding gluconeogenic and glyoxylate cycle enzymes are repressed. *S. cerevisiae* ferments

the glucose and respiratory genes are repressed as well, different from *K. lactis*. Exactly how glucose repression is mediated in *K. lactis* is unknown. In *S. cerevisiae* it is mediated at least partly by Mig1p, which recruits the Ssn6p-Tup1p corepressor (see above) to the promoters of the glucose-repressed genes. Through this direct repression of a number of gluconeogenic and glyoxylate cycle genes and indirect, through repression of transcription of the *ScCAT8* gene (see below) that encodes an activator of these genes, the pathways are rendered inactive. In *K. lactis*, unlike in *S. cerevisiae*, the invertase (converts sucrose to glucose) gene *INV1* is not dependent on KlMig1p for repression (Georis *et al*, 1999) and the promoter of the gluconeogenic fructose-1,6-bisphosphatase gene *KlFBP1* contains no potential Mig1p binding site. They may be repressed Mig1p-independently and/or require activation. In *S. cerevisiae* many components required for glucose repression have been identified but the system as a whole is not completely understood. Glucose uptake is required and accordingly multiple mutations of hexose transporter (*HXT*) genes interfere with growth on and repression by glucose, but a sensor mechanism at this level seems not to be important. Two genes that encode non-functional hexose transporter homologues, *SNF3* and *RGT2*, have signaling functions. Apart from enhancing the initial response to newly available sugar they are not required for glucose repression *per se*, however (reviewed in Gancedo, 1998; Carlson, 1999). At least one of the three enzymes hexokinase 1 (*HXK1*), hexokinase 2 (*HXK2*) or glucokinase (*GLK1*) has to be present (Sanz *et al*, 1996) and phosphorylation of glucose is required for repression (Ma *et al*, 1989). *HXK2* appears strongest in repression and recent evidence implies a nuclear function of Hxk2p in repression of *HXK1* and *GLK1* and glucose-autoinduction of *HXK2* (Rodriguez *et al*, 2001). As far as energy charge of the cell is concerned there are indications for both changes in AMP:ATP ratio (Wilson *et al*, 1996b) and cAMP level to play a role in regulation of glucose repression, but again both seem to be important for responding to changes in extracellular glucose rather than for steady state repression (reviewed in Gancedo, 1998). Absolutely required for relief from glucose repression is the serine/threonine protein kinase Snf1p (Celenza and Carlson, 1986). This kinase exists in a complex with the Snf4p activating subunit and one of three scaffold subunits, Sip1p, Sip2p or Gal83p, which are essential and determine the downstream target cascade of Snf1p (Schmidt and McCartney, 2000). Under glucose repressing conditions Snf1p is autoinhibited through interaction of its catalytic and regulatory domains. In absence of glucose Snf4p interacts with the regulatory domain of Snf1p and the catalytic domain is free to phosphorylate its targets (reviewed in Gancedo, 1998; Carlson, 1999). Strains deleted for the *SNF1* gene are unable to exit from the glucose-repressed state altogether. *Scsnf1* mutants therefore can only grow on glucose or fructose. Targets of Snf1p in glucose repression/derepression are Mig1p, which is phosphorylated and

exported from the nucleus (DeVit *et al*, 1997; Östling and Ronne, 1998; Treitel *et al*, 1998), and the transcriptional activators Cat8p and Sip4p, which require phosphorylation for activity (reviewed in Carlson, 1999). In *K. lactis* the homologues of the Snf1 and Gal83 proteins are encoded by the *FOG2* and the *FOG1* gene, respectively (Goffrini *et al*, 1996). Both the *S. cerevisiae GAL*/*MEL* and *K. lactis GAL*/*LAC* regulons are under glucose repression. Repression of the *GAL*/*MEL* regulon is achieved through regulation of expression of the transcriptional activator *GAL4* and galactokinase *GAL1* genes (see below). *ScGAL4* is regulated by binding of ScMig1p to its promoter on glucose, which leads to a 5-fold reduction of expression (Griggs and Johnston, 1991). Regulation of *ScGAL1* expression is more complex and the difference between repressed and induced expression is about 1000-fold. The gene contains a UAS with four ScGal4p binding sites in its promoter and a URS that contains two ScMig1p binding sites (Flick and Johnston, 1990; Nehlin *et al*, 1991; Flick and Johnston, 1992). Deletion of URS_{GAL1}, URS_{GAL4} or *ScGAL80* alone reduced glucose repression of *ScGAL1* by a factor of 10. The slight reduction by glucose in ScGal4p level alone, in a ΔURS_{GAL1} Δ*Scgal80* background, repressed *ScGAL1* expression 30-fold. ScGal80p alone, in a ΔURS_{GAL1} ΔURS_{GAL4} background, mediated 13-fold repression. URS_{GAL1} alone mediated only 3-fold repression of *ScGAL1* (Flick and Johnston, 1992). These three mechanisms together explain glucose repression of *ScGAL1* and the combined regulation of *ScGAL1* and *ScGAL4* explains regulation of the complete regulon.

In *K. lactis*, as in *S. cerevisiae*, glucose repression of the *GAL*/*LAC* regulon involves control of the *GAL4* and *GAL1* genes but the mechanism is different. KlMig1p does not repress expression of KlGAL4. It is activated through an autoregulatory loop that involves a weak KlGal4p binding site in the *KlGAL4* promoter (Zachariae and Breunig, 1993). Repression is achieved by inhibition of KlGal4p activity by KlGal80p. Increasing the level of KlGal4p by as little as 2-fold, through introduction of a second copy of *KlGAL4* or a naturally occurring promoter dimorphism that increases *KlGAL4* expression by an undefined mechanism, is sufficient to convert a glucose-repressible strain to a glucose-non-repressible one (Zachariae *et al*, 1993). So is deletion of *KlGAL80* (Zenke *et al*, 1993) in contrast to *S. cerevisiae* where deletion of *GAL80* does not relieve glucose repression (Nehlin *et al*, 1991). Both *KlGAL4* and *KlGAL80* are stronger expressed on glucose than their *S. cerevisiae* counterparts and the system at all times appears to be closer to the threshold between repression and induction. Attempts to over-express *KlGAL4* from multicopy plasmids were unsuccesfull, indicating that increased intracellular KlGal4p concentrations quickly become toxic (Breunig, 1989). KlMig1p represses *KlGAL1* during growth on glucose, as deletion of *KlMIG1* or mutation of the KlMig1p binding site in the *KlGAL1* promoter substantially relieves expression of most *GAL*/*LAC* genes from repression (Dong and

Dickson, 1997). However, since expression of these genes is still further induced by galactose, a KlMig1p-independent mechanism of glucose repression must exist.

1.2.2 Galactose induction

Gal4p, the acidic $Zn(II)_2Cys_6$ zinc-cluster transcriptional activator mentioned before, mediates induction of the *GAL*/*LAC* and *GAL*/*MEL* genes in response to galactose. The activity of Gal4p in turn is inhibited by Gal80p, which binds to the Gal4p activation domain under non-inducing conditions. Induction by galactose leads to release of Gal4p from inhibition by Gal80p (reviewed in Breunig, 2000; Bhat and Murthy, 2001) and this anti-inhibition requires the unique galactokinase KlGal1p in *K. lactis*. It has been show that the mode of action is a galactose- and ATP-dependent physical interaction of KlGal1p with KlGal80p (Zenke *et al*, 1996). KlGal1p contains both a catalytic and a regulatory activity that can be separated by mutation. This allows mutants that can no longer phosphorylate galactose but still bind KlGal80p and *vice versa*. KlGal1p-KlGal80p interaction may lead to dissociation of the complex from KlGal4p (dissociation model) or a shift to a different position on KlGal4p in a ternary complex (allosteric model). In *S. cerevisiae* the mechanism of induction is very similar, with the exception that in this yeast the catalytic and regulatory functions of galactokinase are separated. The *ScGAL1* and *ScGAL3* genes encode highly homologous proteins but only ScGal1p is a functional galactokinase whereas ScGal3p is the regulator and catalytically inactive due to a mutation in the catalytic site (Platt *et al*, 2000). *Scgal3* mutants take 3 to 5 days longer for induction to proceed. This "long adaptation" phenotype is caused by the exceedingly low expression of *ScGAL1* under repression. *Scgal1* mutants are unable to grow on galactose. Overexpression of either gene leads to constitutive expression of the *GAL* genes (Bhat and Hopper, 1992). In *S. cerevisiae* evidence for an ScGal4p-ScGal80p-Gal3p complex has been obtained (Platt and Reece, 1998).
Induction also requires *SNF1* in both yeasts. In *S. cerevisiae* Snf1p-dependent phosphorylation and nuclear export of Mig1p abolishes glucose repression of URS_{ScGAL4} and URS_{ScGAL1}, leading to full induction of *ScGAL4* and partial induction of *ScGAL1* (Nehlin *et al*, 1991; Flick and Johnston, 1992). Full induction of *ScGAL1* is then achieved through occupation of the UAS_{ScGAL1} by ScGal4p, which ends the "indirect", UAS_{ScGAL1}-mediated glucose repression of *ScGAL1* (Flick and Johnston, 1990). In *K. lactis* full induction of the *GAL*/*LAC* genes requires KlSnf1p, as deletion of *KlSNF1* strongly reduces induction of the regulon. There must however also be a KlSnf1p-independent component in induction, as the *Klsnf1* deletion mutant still shows induction of expression. The mechanism responsible for this residual induction is unknown (Dong and Dickson, 1997).

1.2.3 Derepression on poor carbon sources

Much less preferred than sugars are the poor C3 carbon sources glycerol or lactate or the C2 compounds ethanol or acetate. Both *K. lactis* and *S. cerevisiae* can metabolize these carbon sources and *K. lactis* even grows quite well on all of them. In contrast to glucose, however, catabolism of these carbon sources requires respiration and activity of the glyoxylate cycle to replenish the TCA cycle with oxaloacetate. The production of hexose phosphates for biosynthetic pathways additionally requires gluconeogenesis. In *K. lactis*, different from *S. cerevisiae*, respiratory genes need not be induced for they are expressed under all conditions. Expression of gluconeogenic genes *FBP1* (fructose-1,6-bisphosphatase) and *PCK1* (phosphoenol carboxykinase) and glyoxylate cycle genes *ICL1* (isocitrate lyase) and *MLS1* (malate synthase) must be activated for catabolism of lactate, ethanol or acetate (reviewed in Flores *et al*, 2000). Metabolism of glycerol only requires expression of *FBP1*. In *S. cerevisiae* derepression requires the Snf1p protein kinase (Celenza and Carlson, 1986) and transcriptional activator Cat8p. Strains deleted for the *SNF1* gene are unable to exit from the glucose-repressed state altogether, due in part to the failure to release glucose-repressed genes, amongst others *ScFBP1*, from repression by Mig1p. *Scsnf1* mutants therefore can only grow on glucose or fructose. But release from Mig1p-repression is not sufficient for expression of gluconeogenic and glyoxylate cycle genes. Transcription must be activated by Cat8p (Hedges *et al*, 1995), a protein identified as being required for activation of *FBP1* through $UAS1_{FBP1}$ (Niederacher *et al*, 1992). Deletion was shown to prevent expression of *FBP1*, *PCK1* and *ICL1*. ScCat8p is a 160 kDa protein that belongs to the Gal4p family of $Zn(II)_2Cys_6$ binuclear cluster transcriptional activators. It was shown to bind to a sequence called Carbon Source-Responsive Element (CSRE, consensus 5'-$CGGN^{T}/_{C}NAA^{T}/_{C}GG$ -3') (Rahner *et al*, 1999), first identified in the *ICL1* promoter (Schöler and Schüller, 1994). At least one CSRE has been identified in the promoters of *FBP1* (Vincent and Gancedo, 1995), *PCK1* (Proft *et al*, 1995), *ICL1* and *MLS1* (Caspary *et al*, 1997) but also in the promoters of several other genes: *ACS1* (acetyl-CoA synthetase) (Kratzer and Schüller, 1997), *SFC1* (*ACR1*, mitochondrial succinate-fumarate carrier) (Bojunga *et al*, 1998), *IDP2* (cytosolic NADP-dependent isocitrate dehydrogenase) and *JEN1* (lactate permease) (Bojunga and Entian, 1999), *MDH2* (cytosolic malate dehydrogenase) (Roth and Schüller, 2001) and *ADH2* (Walther and Schüller, 2001). Expression of the *ScCAT8* gene itself is dependent on Snf1p, for it is Mig1p-repressed as well. Also an Snf1p-dependent phosphorylation of ScCat8p is necessary for activity of the activator (Randez-Gil *et al*, 1997). Deletion mutants in *ScCAT8* do not express the afore-mentioned genes and cannot grow on poor carbon sources. In a two-hybrid screen for Snf1p-interacting proteins (SIPs) a

putative transcriptional activator was identified (Yang *et al*. 1992) that interacts with the Snf1 protein kinase through its Gal83p subunit (Lesage *et al*, 1996; Vincent and Carlson, 1999). The *SIP4* gene encodes a 96 kDa protein that, like ScCat8p, contains a $Zn(II)_2Cys_6$ binuclear cluster, a putative coiled-coil region and a putative C-terminal acidic activation domain (Lesage *et al*, 1996). Additionally a putative leucine zipper was identified, giving Sip4p two putative dimerization domains. Again as ScCat8p, Sip4p shows Snf1p-dependent phosphorylation on poor carbon sources. The *SIP4* promoter contains a CSRE sequence and its expression is glucose-dependent (Lesage *et al*, 1996; DeRisi *et al*, 1997; http://cmgm.stanford.edu/pbrown/) and ScCat8p-dependent (Vincent and Carlson, 1998; Haurie *et al*, 2001). Sip4p was itself shown to be a CSRE-binding protein and to function as a transcriptional activator through this sequence (Vincent and Carlson, 1998). Despite this fact deletion of *SIP4* gave no phenotype (Lesage *et al*, 1996) but when overexpressed *SIP4* could complement an *Sccat8* deletion (Vincent and Carlson, 1998).

In *K. lactis* it was found that *LAC4* gene expression and β-galactosidase activity were around 10-fold increased in cells grown on glycerol compared to the glucose-repressed level and as much as 50-fold increased when ethanol was the sole carbon source. More important, this expression was fully independent of *KlGAL4*. This derepressed "basal" activity was due to the -1066 to -1522 region of the *LAC4* promoter (Gödecke, PhD thesis 1990). Deletion of this promoter region had no influence on KlGal4p-mediated induction of *LAC4*. A sequence was identified in this Basal Control Region (BCR) that had UAS activity and showed strong homology to the CSRE (Schmidt, PhD thesis 1996). In electrophoretic mobility-shift assays a factor, *Kluyveromyces* derepression factor 1 (Kdf1), was identified that bound to this CSRE under derepressing growth conditions (Schmidt, PhD thesis 1996). Subsequently the *K. lactis* homologue of *ScCAT8*, *KlCAT8*, was cloned as a multicopy suppressor of the growth deficiency of a *Klsnf1* (*fog2*) mutant on glycerol (Georis *et al*, 2000). A *Klcat8* mutant grows poorly on ethanol and acetate but grows on glycerol like the wild-type. Induction of *KlFBP1* and *KlPCK1* is normal in this strain, whereas expression of *KlICL1* and *KlMLS1* is lost and reduced respectively.

1.3 Aim of the PhD thesis

The initial goal of the project in which the work reported here was embedded, was identification of factors involved in glucose-repression in *K. lactis*. The *GAL*/*LAC* regulon, more particularly the *LAC4*-*LAC12* locus in glucose-repressible strain JA6 was chosen as a model for a glucose-repressed system. Galactose induction and basal expression were both glucose-repressed and independent of each other. However, analysis of the Basal Control Region revealed elements responsible for activation by poor carbon sources rather than for repression by glucose.
Aim of this PhD thesis was to elucidate the mechanism of transcriptional regulation at the Basal Control Region. The $CSRE_{LAC4}$ was to be characterized more precisely in the context of the *LAC4* Basal Control Region. Isolation of upstream regulators of the $CSRE_{LAC4}$ and analysis of their functions and modes of action was to be performed.

2 Materials and Methods

2.1 Yeast strains and growth media

Kluyveromyces lactis

Strain	Genotype	Author
JA6	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2*	Breunig and Kuger, 1987
JA6/LR2	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 LAC4Δ2* (Δ -1066 / -1527)	Gödecke, PhD thesis 1990
JA6/DL4	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 lac4::ScURA3*	Gödecke, PhD thesis 1990
JA6/D1	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 gal1::ScURA3*	Zachariae, PhD thesis 1994
JA6/D802	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 gal80Δ2::ScURA3*	Zenke *et al*, 1993
JA6/831	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 gal80-31*	Zenke *et al*, 1999
JA6/209/831	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 gal1-209 gal80-31*	Zenke, PhD thesis 1995
JA6/DL9	α *ade1-600 adeT-600 trp1-11 ura3-12 lac9::ScURA3*	Breunig and Kuger, 1987
yIG8	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 Klcat8*	Georis *et al*, 2000
yIG10	α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2* (HA)-*KlCAT8*	Georis, personal communication

Strains constructed in this work

JA6/LR2K α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 LAC4Δ2*::$CSRE_{LAC4}$
Plasmid pLR2K was digested with *Xba*I and the fragments were transformed in strain JA6/DL4. Integrants were selected for reversion of the lac^- ura^+ penotype of strain JA6/DL4 to lac^+ ura^-. Replacement of *ScURA3* by the *LAC12*-promoter-*LAC4 Xba*I fragment by homologous recombination led to reconstitution of a functional *LAC4* gene. Correct integration of the $CSRE_{LAC4}$ in the LR2 deletion was confirmed by colony PCR with the primers mentioned and sequencing of the PCR product.

JA6/DS4 α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 Klsip4* (Δ -18 / +2182)
The *KlSIP4* Open Reading Frame was deleted by two-step gene disruption. Strain JA6 was transformed with *Xho*I-linearized plasmid pDS4 and selected for uracil prototrophy. Integrants were then selected for uracil auxotrophy on 5-FOA plates.

JA6/DS49 α *ade1-600 adeT-600 trp1-11 ura3-12 lac9::ScURA3 Klsip4 KlGAL4* (*LAC9*) was disrupted in strain JA6/DS4 by integration of *ScURA3*. Cells were transformed with *Eco*RI/*Ksp*AI (*Hpa*I)-linearized plasmid pDL9 and selected on glucose/lactose X-gal ura$^-$ plates for uracil prototrophy and white color due to lack of lactose-induction.

yIG8/DS4 α *ade1-600 adeT-600 trp1-11 ura3-12 LAC9-2 Klcat8 Klsip4 KlSIP4* was deleted in strain yIG8 as described for strain JA6/DS4.

yIG8/DL9 α *ade1-600 adeT-600 trp1-11 ura3-12 lac9::ScURA3 Klcat8 KlGAL4* was disrupted in strain yIG8 as described for strain JA6/DS49.

yIG8/DS49 α *ade1-600 adeT-600 trp1-11 ura3-12 lac9::ScURA3 Klcat8 Klsip4 KlGAL4* was disrupted in strain yIG8/DS4 as described for strain JA6/DS49.

Strains were grown in rich medium (YEP: 10 g/l yeast extract, 20 g/l bacto-peptone) or synthetic complete medium (SC: 6.7 g/l yeast nitrogen base with $(NH_4)_2SO_4$, supplemented with 14.4 mg/l Tyr, 38.4 mg/l Arg, His, Met and Trp, 48 mg/l Phe, 57.6 mg/l Ile, Leu, Lys, Thr and Val, 11.2 mg/l Ade and 38.4 mg/l Ura from a 20x stock solution) at 30°C. For selection of plasmid-containing cells the transformants were grown in synthetic complete medium lacking uracil. Carbon sources were added in the following concentrations: glucose 2%(w/v), galactose 2%(w/v), glycerol 3%(w/v), ethanol 2%(v/v) and sodium acetate 2%(w/v). For solid media the above liquid media were supplemented with 18 g/l agar and in the case of SC medium the pH was adjusted to 6.5 - 6.8 with NaOH.
Selection for reversion from uracil prototrophy to auxotrophy was performed on SC plates containing uracil, 2% glucose and 0.5 µM 5-fluoroortic acid (FOA) as described by Boeke *et al* (1984). For monitoring of β-galactosidase activity, plates were supplemented with 40 µg/ml X-gal from a 20 mg/ml stock solution in DMF.

2.2 *Escherichia coli* strains and growth media

DH5αF' F'(Φ80d*lacZ*ΔM15), Δ(*lacZYA-argF'*)U169, *recA1*, *endA1*, *hsdR17* r_k^- m_k^+, *supE44*, *thi-1*, *gyrA*, *relA1* (Gibco BRL, Gaitersburg MD, USA)

Bacteria were grown in LB medium (10 g/l bactotryptone, 5 g/l yeast extract and 5 g/l NaCl) at 37°C. For selection of plasmids the medium was supplemented with ampecillin at a final concentration of 100 mg/l from a 50 g/l stock solution in ethanol. Solid LB medium contained 18 g/l agar. For monitoring of β-galactosidase activity, plates were supplemented with 40 µg/ml X-gal from a 20 mg/ml stock solution in DMF and 0.1 mM IPTG from a 100 mM stock solution.

2.3 Plasmids

yeast	description	author
KEp6	*E coli K. lactis* shuttle vector; pKD1 S11 fragment in YIp5 *Bam*HI site; Amp^r Tc^r *ScURA3.*	Bianchi *et al*, 1987
pDL9	*LAC9* disruption plasmid, *LAC9* region -151 to + 682 replaced by *ScURA3.*	Breunig and Kuger, 1987
pDS4	*KlSIP4* disruption plasmid containing *KlSIP4* promoter-terminator fusion fragment in pBRURA.	this work
pE1	*E coli K. lactis* shuttle vector; pKD1 in YIp5 *Eco*RI site; Amp Tc *ScURA3.*	Bianchi *et al*, 1987
pEAGK	*E. coli galK* under *ScADH1* promoter in pTS32X.	Kuger and Breunig
pGID1	chromosomal *Sau*3A fragment carrying the 4338 bp *KlCAT8* gene in *Bam*HI in KEp6.	Georis *et al*, 2000
$pGID1_{1-1275}$	pGID1 was cut with *Nhe*I and the 10.9 kb fragment recircularized, truncating *Klcat8* at position +3819.	this work
$pGID1_{1-716}$	pGID1 was cut with *Spe*I and the 10.0 kb fragment recircularized, truncating *Klcat8* at position +2142.	this work
$pGID1_{1-305}$	pGID1 was cut with *Hin*dIII and the 7.8 kb fragment recircularized, truncating *Klcat8* at position +909.	this work
pGP3	chromosomal *Sau*3A fragment carrying *KlSIP4* in *Bam*HI in KEp6.	this work
pLR2	Multicopy *E. coli*-yeast plasmid carrying 827 bp of *LAC12*, the *LAC12-LAC4* promoter and *LAC4*. The -1066 to -1527 (ATG *LAC4* = +1) *Eco*RI-*Hpa*I promoter fragment was replaced by a *Sal*I linker.	Gödecke, PhD thesis 1990

pLR2K	The 3'- recessed ends of double stranded oligonucleotide $CSRE_{LAC4}$ and *Sma*I-linearized plasmid pLR2 were filled-in using Klenow polymerase. Blunt-ended plasmid and oligonucleotide were ligated and products transformed in *E. coli*. Insertion of the $CSRE_{LAC4}$ in pLR2 was tested by PCR with primers LR2 5' (-1634 / -1618; `5'- CCTAAATTTCCGCGGGG -3'`) and LR2 3' (-984 / -1000; `5'- TAGCCTAGATGGCTCCG -3'`).	this work

E. coli

pBluescript SK+	highcopy vector, Amp^r, *lacZ'*-gene for blue-white screening, f1-replication origin.	Stratagene, Heidelberg
pBR322	high copy vector, Amp^r, Tc^r.	Bolivar *et al*, 1977
pBRURA	*ScURA3* on 1.2 kb *Hin*dIII fragment in pBR322.	Kuger and Breunig
pCR2.1-topo	highcopy vector for T-A cloning of PCR fragments using topoisomerase I. Amp^r, Kan^r, *lacZ'*-gene for blue-white screening, f1-replication origin.	invitrogen, Karlsruhe
pCR-ΔS4	1144 bp *KlSIP4* promoter-terminator fusion PCR fragment (see 2.6.4) inserted in pCR2.1-topo	this work
pUC18m	highcopy vector, Amp^r, *lacZ'*-gene for blue-white screening, *Eco*RI-linker in filled-up *Hin*dIII site.	Yanish-Perron *et al*, 1985
pV2-1	139 bp *Acc*I fragment from pS2 (*LAC4* -1066 to -1190) in *Sma*I in pUC18m	Schmidt, PhD thesis 1996

2.4 Oligonucleotides

2.4.1 Double-stranded oligonucleotide probes for electrophoretic mobility shift assay DNA binding studies

All complementary oligonucleotides for use as probes in the elctrophoretic mobility shift assay were designed with *Eco*RI-compatible 5' overhanging ends for labelling purposes. Wild-type sequences are in capitals, non-wild-type bases in small case.

CSRE$_{LAC4}$: *LAC4* positions -1127 to -1114.

```
5'- aattcTCGGATGAAAGGGGg -3'
    3'- gAGCCTACTTTCCCCcttaa -5'
```

CSRE$_{LAC4}$-Adr1: *LAC4* positions -1127 to -1092.

```
5'- aattcTCGGATGAAAGGGGAATCGTATGAGATTGGAGAGGG -3'
    3'- gAGCCTACTTTCCCCTTAGCATACTCTAACCTCTCCCttaa -5'
```

CSRE$_{LAC4}$-mAdr1: *LAC4* positions -1127 to -1092, position -1102 mutated A > G, position -1101 mutated T > C and position -1099 mutated G > C.

```
5'- aattcTCGGATGAAAGGGGAATCGTATGAGGCTCGAGAGGg -3'
    3'- gAGCCTACTTTCCCCTTAGCATACTCCGAGCTCTCCcttaa -5'
```

CSRE2$_{KlSIP4}$: *KlSIP4* positions -469 to -456.

```
5'- aattcCCGGCTGAAAGGGAg -3'
    3'- gGGCCGACTTTCCCTcttaa -5'
```

CSRE3$_{KlSIP4}$: *KlSIP4* positions -466 to -479.

```
5'- aattcCCGGATCTGGGGAAg -3'
    3'- gGGCCTAGACCCCTTcttaa -5'
```

CSRE4$_{KlSIP4}$: *KlSIP4* positions -598 to -611.

```
5'- aattcCCGGATGAAAGGCTg -3'
    3'- gGGCCTACTTTCCGActtaa -5'
```

CSRE$_{KlCAT8}$: *KlCAT8* positions -100 to -113.

```
5'- aattcCCGGTACAAAGGGAg -3'
    3'- gGGCCATGTTTCCCTcttaa -5'
```

CSRE1$_{KlICL1}$: *KlICL1* positions -284 to -300.

```
5'- aatTCATGGTTAAATGGATG -3'
    3'- GTACCAATTTACCTACttaa -5'
```

CSRE2$_{KlICL1}$: *KlICL1* positions -573 to -557.

```
5'- aatTCAGGGATCAATGGACG -3'
    3'- GTCCCTAGTTACCTGCttaa -5'
```

CSRE3$_{KlICL1}$: *KlICL1* positions -798 to -812.

```
5'- aattcTCGGTTTAATGGCGG -3'
    3'- gAGCCAAATTACCGCCttaa -5'
```

CSRE$_{KlACS1}$: *KlACS1* positions -765 to -778.

```
5'- aattcGCGGTTGTGAGGGGg -3'
    3'- gCGCCAACACTCCCCcttaa -5'
```

2.4.2 Primers for construction of the Δ*Klsip4* construct

Wild-type sequences are in capitals; bases added for cloning purposes in small case.

DSIP4 Pr 5': *KlSIP4* positions -325 to -308.

5'- cgcgcgaattccGGATCCATCAATTAAGGG -3'

DSIP4 Pr-Ter: *KlSIP4* positions -19 to -33 (3' half) fused to 2200 to 2183 (5' half).

5'- CGGATGTTGATTCTTCTG|TTGGCGCTACTACAGG -3'

DSIP4 PTrev: complementary to DSIP4 Pr-Ter; *KlSIP4* positions 2183 to 2200 (3' half) fused to -33 to -19 (5' half).

5'- CCTGTAGTAGCGCCAA|CAGAAGAATCAACATCCG -3'

DSIP4 Ter 3': *KlSIP4* positions 2994 to 2979.

5'- agcggaattcgagCTCCCAATGCTAACCG -3'

2.4.3 RT-PCR primer pairs

KlHHT1, encoding histone H3:
KlHHT1 20 down (*KlHHT1* pos 20 to 38): 5'- CAGCAAGAAAGTCTACTGG -3'
KlHHT1 287 up (*KlHHT1* pos 287 to 270): 5'- GATTCTTGCAAAGCACCG -3'

KlSIP4:
182 down (*KlSIP4* pos 592 to 610): 5'- CCAAACTATATCCATGACC -3'
290 up (*KlSIP4* pos 1328 to 1311): 5'- ATCTTCAAATGAGGTGGG -3'

KlMLS1:
KlMLS1 26 down (*KlMLS1* pos 26 to 46): 5'- TCCAACTTTTAGTTGATGTCG -3'
KlMLS1 626 up (*KlMLS1* pos 626 to 606): 5'- TTATGATAGAAGTACAAACCG -3'

2.5 Transformation procedures

2.5.1 Transformation of *E. coli*

Chemocompetent cells were produced and transformed according to the rubidiumchloride / calciumchloride protocoll of Hanahan (1985).

2.5.2 Transformation of *K. lactis*

Frozen chemocompetent *K. lactis* cells were produced and transformed according to the protocoll of Klebe *et al* (1983) as modified by Dohmen *et al* (1991). Transformations in chemocompetent cells were typically performed with ~1 µg of circular plasmid DNA or, for chromosomal integration, with ~5 µg of linearized plasmid DNA. Electrocompetent cells were produced and transformed according to the protocoll of Sanchez *et al* (1993) as modified according to Wésolowski-Louvel (1996). Electrocompetent cells were transformed with ~ 200 ng of circular plasmid DNA.

2.6 Preparation and manipulation of DNA

2.6.1 Plasmid isolation from *E. coli*

Small and medium scale isolation of plasmid DNA for general purposes from *E. coli* was performed by the alkaline lysis method (Birnboim, 1983). High purity plasmid DNA (when required) was isolated using Qiagen column technology.

2.6.2 Plasmid rescue from *K. lactis*

For retrieval of plasmids from *K. lactis*, cells from 1.5 ml overnight cultures were resuspended in 200 µl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH8.0, 1 mM EDTA pH8.0). After addition of 200 µl of 0.4 mm glass beads and 200 µl of phenol/chloroform/isoamylalcohol (25:24:1) the suspensions were vortexed for at least 2 minutes at maximum speed. After centrifugation at 20,000 x g for 5 minutes the inorganic phase was recovered and 1-2 µl were used for transformation of *E. coli*.

2.6.3 Isolation of chromosomal DNA from *K. lactis*

Large or small scala preparation of chromosomal DNA from *K. lactis* was performed according to the yeast minilysate method (Fink *et al*, 1983). Cells from 1.5 ml / 40 ml overnight cultures in rich medium were collected and resuspended in 3 ml / 150 µl YML-buffer 1 (0.9 M Sorbitol, 0.1 M EDTA pH8.0, 14 mM β-mercaptoethanol). After addition of 20 µl / 5 µl zymolyase 100T (2.5 mg/ml) the suspensions were incubated for 30 minutes at 30°C and spheroplasts then collected by centrifugation. Spheroplasts were then resuspended in 5 ml / 250 µl YML-buffer 2 (50 mM Tris-HCl, 20 mM EDTA, pH7.0) and after addition of 500 µl / 25 µl 10% SDS incubated at 65°C

for 30 minutes. Then 1.5 ml / 75 µl 1.5 M KAc was added and the solutions were incubated on ice for at least 30 minutes. After 10 minute centrifugation at 10,000 x g and 4°C the supernatants were decanted through folded filters and the DNA was ethanol-precipitated, taking care not to sheare the DNA in mixing. After 10 minute centrifugation at 10,000 x g and 4°C the pellets were dried and dissolved in 1 ml / 50 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH7.5).

2.6.4 Two-step gene disruption of *KlSIP4*

Two-step gene disruption of *KlSIP4* was performed with the aim of creating a strain carrying a defined deletion of *KlSIP4* and no marker. For this purpose two primer sets were designed. PCR with the DSIP4 Pr 5' - DSIP4 Pr Ter primer set on plasmid pGP3 resulted in a fragment spanning *KlSIP4* promoter positions -325 to -19 fused to terminator positions 2183 to 2200. The DSIP4 PTrev - DSIP4 Ter 3' primer set yielded a fragment of promoter positions -33 to -19 fused to a terminator fragment spanning positions 2183 to 2994. The fragments were then used as megaprimers against each other and the resulting 1144 basepair full-length product was cloned in the pCR2.1-topo vector for T-A cloning of PCR products. The resulting plasmid pCR-∆S4 was digested with *Eco*RI in the *Eco*RI restriction sites introduced by primers DSIP4 Pr 5' and DSIP4 Ter 3'. The 1129 basepair *KlSIP4* promoter-terminator fragment was isolated from 0.8% agarose and inserted in the unique *Eco*RI restriction site of the yeast integrative plasmid pBRURA. The resulting *KlSIP4* disruption plasmid pDS4 was linearized at the unique *Xho*I restriction site at position -202 in the *KlSIP4* promoter and transformed into the target yeast strains. Integrants were selected for uracil prototrophy on SC ura^- 2% glucose plates. Homologous recombination of the linear pDS4 in the chromosomal *KlSIP4* promoter was checked for by colony-PCR on the uracil prototrophic transformants. Cells from positive clones were plated on SC ura^+ 2% glucose plates containing 0.5 µM of 5-fluoro-orotic acid (FOA) for selection of uracil auxotrophic revertants. Reversion can occur through mutations in the *ScURA3* gene, homologous recombination between the *KlSIP4* promoter regions or homologous recombination between the terminator regions. The latter, desired event results in removal of the entire pBRURA portion of pDS4, including the *ScURA3* marker gene, plus the *KlSIP4* gene from the chromosome. Uracil auxotrophic colonies were again tested by colony-PCR. The procedure is schematically depicted in Figure 2-1.

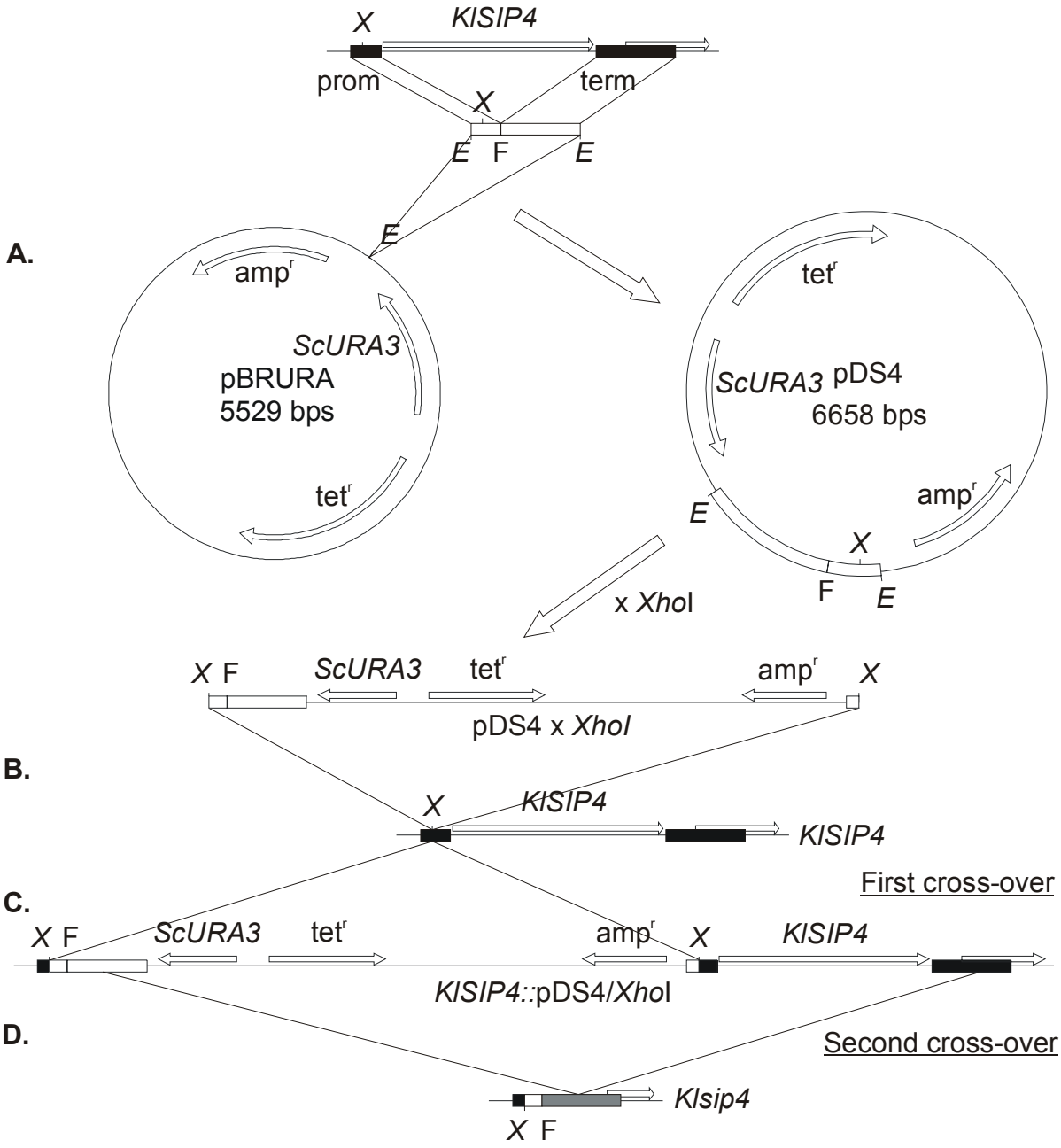


Figure 2-1: Strategy for the two-step disruption of *KlSIP4*.
A. *KlSIP4* promoter and terminator sequences were amplified. Using the fragments as megaprimers against eachother a promoter-terminator fusion fragment was generated and inserted in the single *Eco*RI site in disruption vector pBLURA. **B.** *KlSIP4* disruption plasmid pDS4 was cut with *Xho*I in the *KlSIP4* promoter region and cells were transformed with linearized plasmid and selected for uracil prototrophy on SC + 2% glucose plates without uracil. **C.** Uracil prototrophs were tested by PCR for correct integration of linear pDS4 in the *Xho*I site of the chromosomal *KlSIP4* promoter through homologous recombination. **D.** Positive clones were selected for uracil auxotrophy on SC + 2% glucose plates with uracil, containing 0.5 µM of FOA. Uracil auxotrophs were tested by PCR for loss of integrated pDS4 along with the *KlSIP4* gene through homologous recombination in the *KlSIP4* terminator region. prom: 313 bp *KlSIP4* promoter region. term: 816 bp *KlSIP4 terminator region.* F: point of fusion of *KlSIP4* promoter and terminator regions. *X*: *Xho*I restriction site in the *KlSIP4* promoter. *E*: *Eco*RI restriction sites. amp^r: *bla* gene for ampecillin resistance. tet^r: *tet* gene for tetracyclin resistance.

2.6.5 DNA sequencing

Cycle sequencing reactions were performed on high purity plasmid DNA using the ABI dRhodamine Termination Cycle Sequencing kit according to the manufacturers protocoll. Products were analysed on an ABI Abiprism 377 DNA Sequencer.

2.6.6 General enzymatic manipulation of DNA

General recombinant DNA techniques were performed according to the protocols of the suppliers of enzymes and reagents and as described in "Current Protocols in Molecular Biology" (Ausubel *et al* (Eds.), 1994-present).

2.7 Preparation and manipulation of RNA

2.7.1 Extraction of RNA from *K. lactis*

Total RNA for transcript analysis by RT-PCR was isolated using Qiagen RNeasy Mini spin column technology according to the suppliers protocol. For *K. lactis*, cell wall degradation by zymolyase followed by lysis of the spheroplasts was shown to be the procedure of choise. The alternative protocoll that employs glass bead disruption yielded negligible RNA yields under the recommended conditions. To minimize DNA contamination in the preparations, on-column DNaseI treatment was performed using Qiagen RNase-free DNaseI according to the suppliers protocol.

2.7.2 Transcript analysis by RT-PCR

In this method complementary DNA (cDNA) is synthesized on template RNA by reverse transcriptase (RT), a RNA-dependent DNA polymerase. This cDNA is then analysed by the polymerase chain reaction (PCR). First strand cDNA was synthesized on total RNA, employing the RevertAid first strand cDNA synthesis kit (MBI Fermentas) as indicated by the supplier, by reverse transcription using oligo$(dT)_{18}$ as primer. Aliquots of untranscribed RNA and cDNA were subjected to PCR amplification of *HHT1* (histone H3), *KlSIP4* or *KlMLS1* target sequences. All reactions contained identical amounts of RNA to allow comparison. The untranscribed RNA samples served as negative controls for contaminating chromosomal DNA and the *HHT1* product as internal control. The pilot experiment depicted in Figure 2-2 shows convincingly that DNase I digestion of RNA samples is a prime requirement in RT-PCR, as it removes contaminating chromosomal DNA to a degree that no background product is visible whereas in the untreated sample the

false-positive signal is as strong as that produced from cDNA under the conditions indicated. For each individual message the appropriate conditions for amplification (amount of RNA/cDNA, number of cycles *etc*.) were determined experimentally to find a range that allows signal detection without the reactions going into saturation.

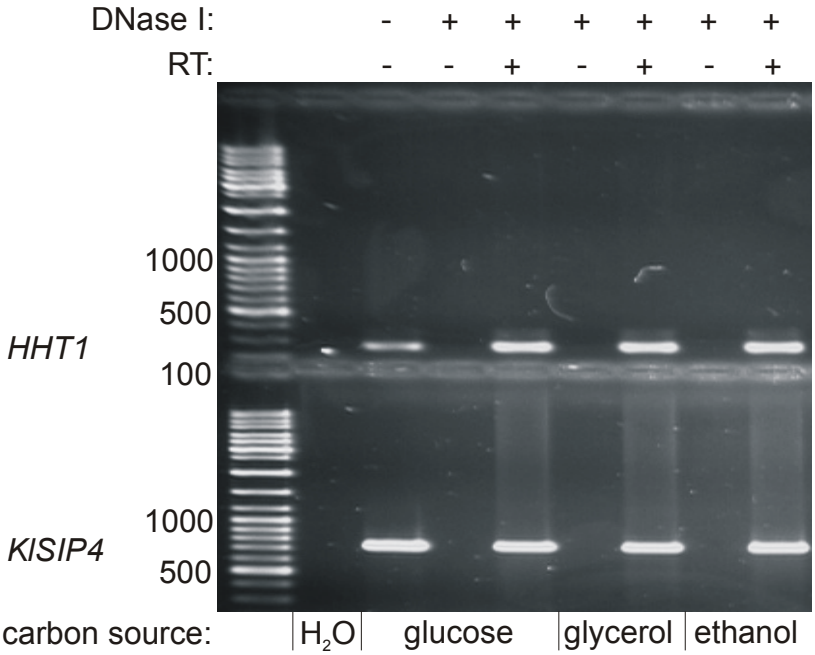


Figure 2-2: DNase I treatment of total RNA for RT-PCR is essential. Total RNA was isolated from wild-type cells grown in SC medium containing 2% glucose, 3% glycerol or 2% ethanol as carbon source. One charge of RNA from glucose-grown cells was not DNase I treated during isolation. First strand cDNA was synthesized using $(dT)_{18}$ as primer on 600 ng of total RNA. 30 ng of non-DNase I-treated, DNase I -treated untranscribed or DNase I-treated, reverse transcribed total RNA was amplified through 30 cycles of PCR using *HHT1*- and *KlSIP4*-specific primer sets yielding 268 and 737 basepair products, respectively. Half of each reaction was run on a 1.5% TAE agarose gel at 100 V for 1 hour. The molecular size marker shows 100 bp increments from 100 to 1000 bp.

2.8 Preparation and manipulation of proteins

2.8.1 Extraction of proteins from *K. lactis*

Whole cell extracts were prepared by glass-bead disruption. Typically 40 - 60 OD units of cells (3.0 - 4.5 x 10^9 cells; OD units = OD_{600} x volume (ml); using a Beckman DU 640 spectrophotometer for *K. lactis* OD_{600} = 1 ≅ 7.5 x 10^7 cells/ml) from 50 ml or 100 ml cultures grown to mid- to late-logarithmic phase were washed and resuspended in 400 µl of the appropriate icecold buffer and added to 500 µl of 0.4 mm glass beads in glass test tubes cooled on ice. Cells were disrupted (3 x 3 min., 4°C) in a Braun homogenizer at maximum speed. After transfer to 1.5 ml tubes debris was removed from the extracts by 10 minute centrifugation (20.000 x g, 4°C) in an Eppendorf cooled tabletop centrifuge. The supernatants were kept on ice until further processing.

2.8.2 Determination of protein concentration

Concentrations of soluble protein in extracts were determined using either the method developed by Lowry *et al*. (1951) or the method of Bradford (1976) in the "Bio-Rad Protein Assay Dye Reagent" ready-to-use format. Bovine serum albumin (BSA) was used as standard in concentrations from 1 to 15 µg/ml for the Lowry assay and from 1 to 20 µg/ml for the Bradford assay.

2.8.3 Electrophoretic mobility shift assay

Cells were glass bead-disrupted in TMEGA buffer (0.2 M Tris-HCl pH7.8, 0.3 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1.0 mM EDTA, 7.0 mM β-mercapto-ethanol, 10 % glycerol). To each sample 1.0 mM proteinase inhibitor PMSF was added immediately before disruption. Supernatants were transferred to centrifuge cups for a Beckmann TLA 100.2 rotor and spun at 100.000 x g for 1 hour at 4°C in a Beckmann Ultima Max tabletop ultracentrifuge. The resulting S100 supernatants were retrieved and stored at -70°C in 20 aliquots. Protein concentrations were determined according to Bradford. Radioactive probes were prepared by filling in the *Eco*RI 3' recessed ends of a restriction fragment or duoble-stranded oligonucleotides with α-^{32}P-dATP using klenow DNA polymerase. The labelled fragments were purified by size exclusion chromatography on Sephadex G50 (Ausubel *et al* (Eds.), 1994-present). For assessment of protein-DNA interaction reactions (≤ 20 µl if possible) were set up in binding buffer (20 mM Hepes-NaOH pH7.8, 0.1 M NaCl, 10 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM DTT, 10 % glycerol, 0.2 mg/ml BSA) containing 10 fmoles of ^{32}P-labelled probe (≥ 2.0 x 10^4 cpm) and 5.0 µg of sonicated calf thymus DNA to quench unspecific binding. Aliquots of the S100 extracts were thawed on ice and volumes corresponding to 10 to 40 µg of protein were immediately added to the reaction. Reactions were incubated 20 min. at room temperature and analyzed on a 4% polyacrylamide gel (acrylamide/bisacrylamide: 30/0.8) in TBE buffer (90 mM Tris, 90 mM H_3BO_3, 2.0 mM EDTA) that was run at 150 V for 2 to $2^1/_2$ hours. The gel was transferred to Whatman paper and dried under vacuum at 80°C for 2 hours. Bands were visualized through autoradiography on X-ray film.

2.8.4 Determination of enzyme activity

2.8.4.1 β-Galactosidase activity measurement

The principle of the assay used in this work is the detection of O-nitrophenol set free through cleavage of the lactose-analogon O-nitrophenyl-β-D-galactopyranoside

(ONPG) by the *LAC4* gene product β-galactosidase. The increase in absorbance at 420 nm due to accumulation of the O-nitrophenol over time is recorded photospectrometrically.
Cells were glass bead-disrupted in β-galactosidase buffer (5.0 mM Tris-HCl pH7.8; 5 % glycerol; 10 mM KCl). For each reaction 10 - 100 µl of extract, appropriately diluted in cold β-galactosidase buffer if required, was added to 1.0 ml of β-galactosidase buffer containing 4 mg/ml ONPG in quartz cuvettes, both prewarmed to 30°C. The increases in A_{420} was recorded for 5 minutes. For calculation of activities protein concentrations in the extracts were determined using the Lowry or Bradford methods. Extinction coefficient ε_{420} for O-nitrophenol = 4.5×10^6 M^{-1} cm^{-1}.

2.8.4.2 Isocitrate lyase activity measurement

Isocitrate lyase activity was determined by the method of Dixon and Kornberg (1959), modified according to Georis and Heinisch (Georis *et al*, 2000; personal communication). The principle of the assay is indirect detection of the glyoxylate produced, along with succinate, from isocitrate by isocitrate lyase. In the presence of phenylhydrazine, glyoxylate reacts with it to form glyoxylic acid phenylhydrazone. The increase in absorbance at 324 nm due to accumulation of the glyoxylic acid phenylhydrazone over time is recorded photospectrometrically.

Solutions:
- A: 33.8 mM sodiumphosphate buffer pH7.0
  5.0 mM $MgCl_2$
  (premade, store at 4°C, warm to 30°C before use)
- B: 103.1 mM L-cysteine-HCl·H_2O
  (freshly prepared, kept on ice)
- C: 112.6 mM phenylhydrazine-HCl
  (freshly prepared, kept on ice)
- D: 200 mM DL-isocitrate pH6.5
  (freshly prepared, kept on ice)
- E: 1000 volumes A + 1 volume B + 30 volumes C

Cells were glass bead-disrupted in solution A. For each reaction 20 - 50 µl of extract was added to solution E to a total volume of 950 µl in quartz cuvettes prewarmed to 30°C. After 2 - 3 minutes for the spontaneous reaction to subside, the reactions were started by addition of 50 µl of 200 mM DL-isocitrate and the increases in A_{324} recorded for 5 minutes. For calculation of activities protein concentrations in the extracts were determined using the Bradford method. Extinction coefficient ε_{324} for glyoxylic acid phenylhydrazone = 1.7×10^4 M^{-1} cm^{-1}.

3 Results

3.1 Analysis of the CSRE$_{LAC4}$

The promoter of the *LAC4* gene, encoding β-galactosidase, contains a region that is required for the moderate activation of transcription of the gene in cells grown on poor carbon sources like glycerol or ethanol but has no function in induction by galactose (Gödecke, PhD thesis 1990). This sequence, called Basal Control Region (BCR), can be divided into two regions, A and B, that are each responsible for about half of the activation mediated by the BCR. In electrophoretic mobility shift assays a complex with Region A was observed in extracts from cells grown on poor carbon sources. The protein forming this complex was named *Kluyveromyces* derepression factor 1 (Kdf1). DNAse I protection experiments had shown that Kdf1 protects the sequence 5' - TCGGATGAAAGGGG - 3' from digestion (Schmidt, PhD thesis 1996).

3.1.1 Competition of the CSRE$_{LAC4}$ for Kdf1-binding

To address the question whether this sequence was sufficient for complex formation, two complementary oligonucleotides were designed carrying just this sequence and a 5'-*Eco*RI halfsite for labelling purposes (see Materials and Methods 2.4.1). The double stranded oligonucleotide CSRE$_{LAC4}$ was tested in a competition gel shift experiment using the 198-basepair-fragment f198 as probe (Figure 3-1).

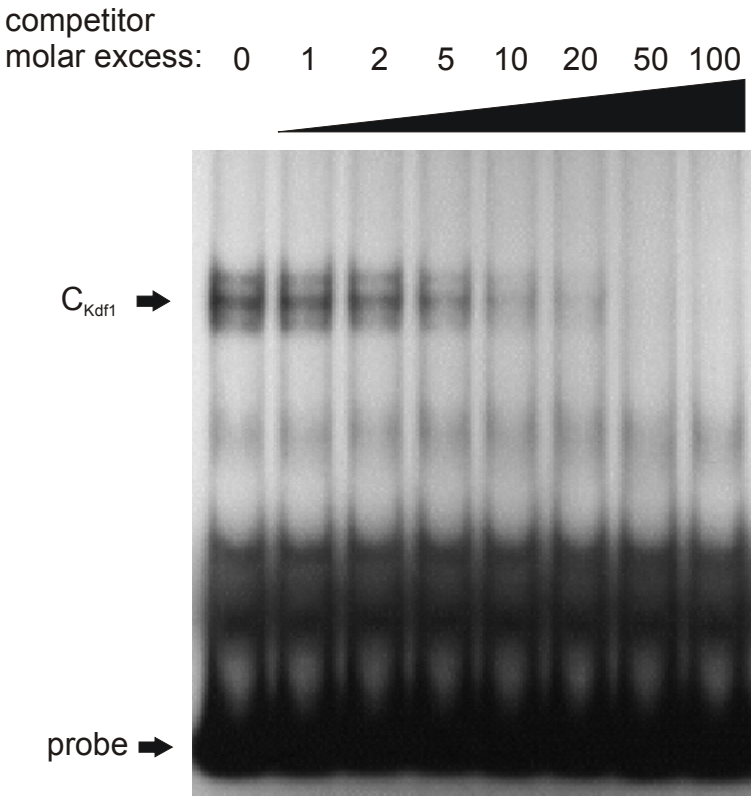
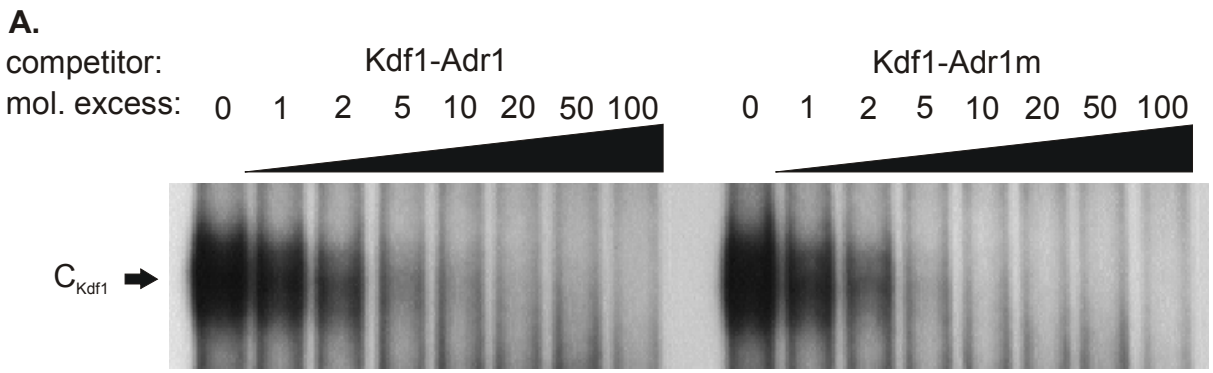


Figure 3-1: The CSRE$_{LAC4}$ competes with the Basal Control Region A for Kdf1-binding.
Each sample contained 10 fmoles of ^{32}P-labelled f198 fragment (2.5 x 10^4 cpm), 20 µg of protein (S100 extrakt from SC + 3% glycerol-grown wild-type cells) and no or 1-, 2-, 5-, 10-, 20-, 50- or 100-fold molar excess of unlabelled CSRE$_{LAC4}$ oligonucleotide.

This fragment carries the 124-basepair Region A from the Basal Control Region of the *LAC4* promoter. The autoradiograph shows that the oligonucleotide $CSRE_{LAC4}$ competes with f198 for binding of Kdf1. A 20- to 50-fold molar excess of unlabelled oligonucleotide sequestered all signals from the diffuse Kdf1-complex formed with f198 showing that the $CSRE_{LAC4}$ sequence per se is sufficient for sequence specific binding of Kdf1.

3.1.2 Binding of Kdf1 to the $CSRE_{LAC4}$ is not influenced by the adjacent putative Adr1p binding site but depends on the carbon source

In Region A the $CSRE_{LAC4}$ (-1114 to -1126) is flanked by a putative Adr1p binding site (-1094 to -1100). This consensus site has been shown to be protected from DNAse I digestion by a GST-ScAdr1p fusion protein produced in *E. coli*. Moreover, three point mutations within the putative Adr1 site led to decreased β-galactosidase activity on glycerol (Schmidt, PhD thesis 1996). To determine whether this Adr1p binding site affected the affinity of Kdf1 for the $CSRE_{LAC4}$, two double stranded oligonucleotides were compared in competition gel shift assays (Figure 3-2).



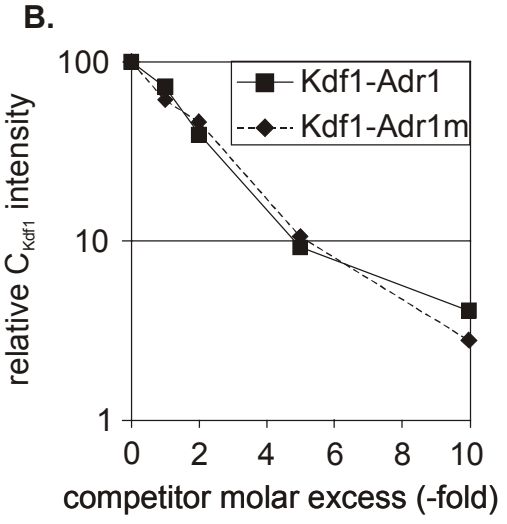


Figure 3-2: The putative Adr1p binding site flanking the $CSRE_{LAC4}$ has no influence on Kdf1-binding.
A. Samples contained 10 fmoles of ^{32}P-labelled f198 fragment (2.5 x 10^4 cpm), 20 µg of protein (S100 extract from SC + 3% glycerol-grown cells) and no or 1-, 2-, 5-, 10-, 20-, 50- or 100-fold molar excess of unlabelled competitor oligonucleotide Kdf1-Adr1 carrying the wild-type putative Adr1p binding site flanking the $CSRE_{LAC4}$ or Kdf1-Adr1m containing three point mutations in the Adr1p binding site.
B. Quantification of C_{Kdf1} complex-intensities in **A.**, from no competitor to 10-fold excess.

Oligonucleotide Kdf1-Adr1 represents the wild-type sequence whereas oligonucleotide Kdf1-Adr1m carries the mutation mentioned above. No difference in competition for Kdf1-binding is evident between wild-type and mutated Adr1 binding site. This supports the conclusion from Figure 3-1 that no other sequence apart from the $CSRE_{LAC4}$ is involved in Kdf1-binding.
To confirm the carbon source dependence of binding fo Kdf1and to test whether a complex was formed with the putative Adr1p binding site on carbon sources other than glycerol, oligonucleotides Kdf1-Adr1 and Kdf1-Adr1m were labeled and used as probes in EMSA. Extracts from cells grown in synthetic complete medium containing glucose, glycerol or sodium acetate were used in this experiment.

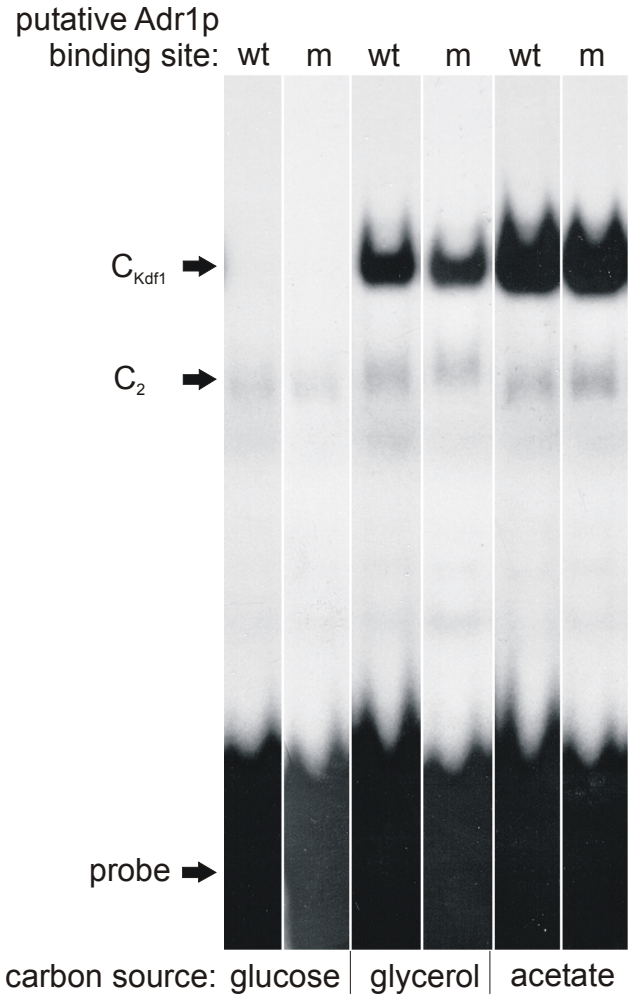


Figure 3-3: Kdf1-binding to the $CSRE_{LAC4}$ is carbon source-dependent and independent of the putative Adr1p binding site.
Samples contained 25 fmoles of ^{32}P-labelled oligonucleotide Kdf1-Adr1 (wt, 1.0 x 10^5 cpm) or Kdf1-Adr1m (m, 1.1 x 10^5 cpm) and 20 µg of S100 protein from wild-type cells grown in SC medium with 2% glucose, 3% glycerol or 2% acetate as sole carbon source.

Apart from the Kdf1-complex a weak complex, C_2, forms independent both of the Adr1p binding site and the carbon source (Figure 3-3). Binding of Kdf1 is absent in extracts from glucose-grown cells. In extracts from cells grown on the poor carbon sources, binding of Kdf1 occurs independent of the Adr1p binding site. On acetate the Kdf1-complex is stronger than on glycerol. Since the Adr1p binding site had no

effect on Kdf1-binding and oligonucleotide CSRE$_{LAC4}$ had been shown to compete with f198 for Kdf1-binding this oligonucleotide was subsequently used directly as a probe in gel shift experiments.

3.1.3 The CSRE$_{LAC4}$ alone is sufficient to replace the Basal Control Region

Now that it had been shown that the Basal Control Region contains a Carbon Source-Responsive Element that is the sole sequence bound by a protein / complex (at least in this Region A), the question arose whether this sequence alone could replace the BCR. To test this the CSRE$_{LAC4}$ oligonucleotide was inserted in place of the deleted BCR. β-Galactosidase activities were compared between wild-type, BCR deletion strain JA6/LR2 and CSRE$_{LAC4}$ insertion strain JA6/LR2K, grown on glucose, glycerol, ethanol and acetate.

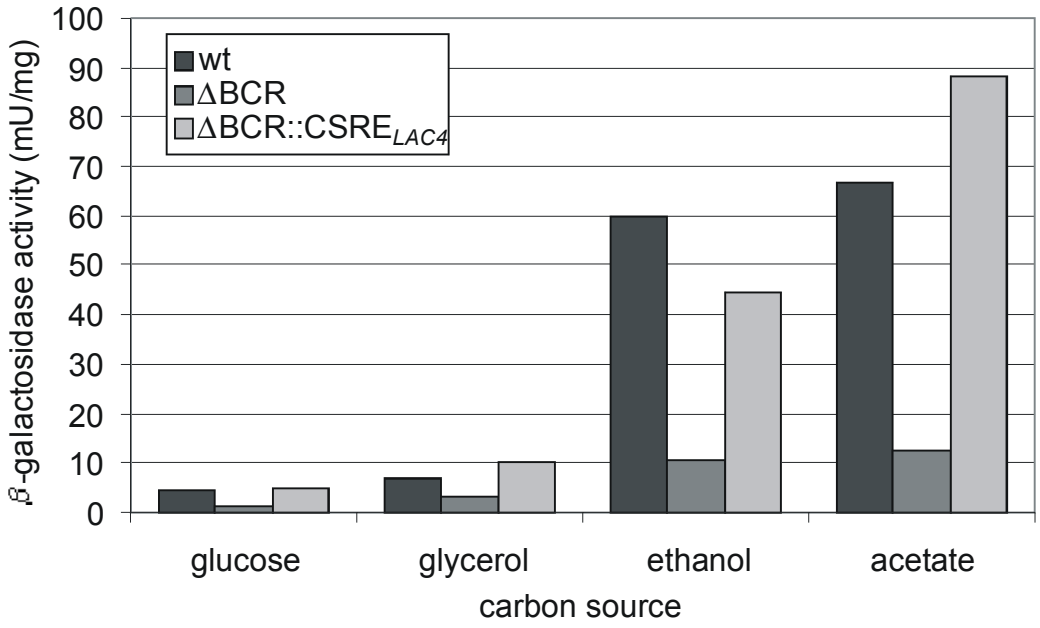


Figure 3-4: The loss of β-galactosidase activity due to deletion of the BCR is restored by reinsertion of the CSRE$_{LAC4}$.
β-galactosidase activity was determined in glassbead extracts from strains JA6 (wild-type), JA6/LR2 (ΔBCR) and JA6/LR2K (ΔBCR::CSRE$_{LAC4}$) grown in synthetic complete medium containing 2% glucose, 3% glycerol, 2% ethanol or 2% acetate as sole carbon source.

Deletion of the BCR led to a marked decrease in *LAC4* expression on all carbon sources tested (Figure 3-4), confirming and expanding the data obtained by Gödecke (PhD thesis 1990) and Schmidt (PhD thesis 1996). Reinsertion of the CSRE$_{LAC4}$ restored expression to near wild-type levels, indicating that this sequence alone is sufficient to mediate the activation of expression seen in the wild-type.

3.1.4 The quantity of Kdf1-CSRE$_{LAC4}$ complex formed depends not only on carbon source but also on growth medium

In extracts from cells grown in synthetic complete medium + 2% ethanol we observed Kdf1-CSRE$_{LAC4}$ complex-formation that varied, in comparison to the complex formed in glycerol extracts, from clearly weaker to virtually none at all. Previously Kdf1 binding to the Region A had been shown to be stronger in ethanol- than in glycerol-grown cells (Schmidt, PhD thesis 1996). These cells however were grown in rich medium.
We therefore tested the influence of the medium *per se* on Kdf1-CSRE$_{LAC4}$ binding.

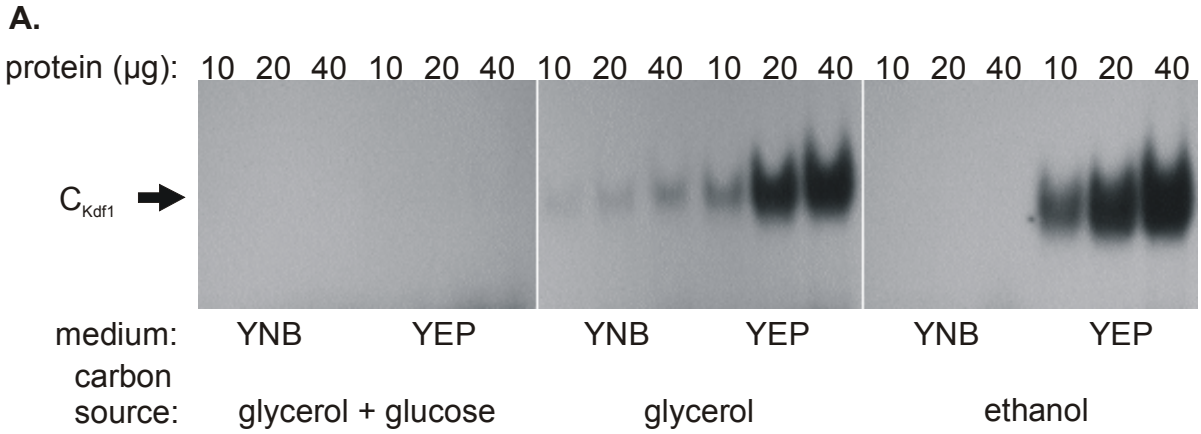


Figure 3-5: Growth medium composition, apart from the carbon source, influences *in vitro* Kdf1-complex formation.
A. Samples contained 10 fmoles of ^{32}P-labelled CSRE$_{LAC4}$ oligonucleotide (7.4 x10^4 cpm) and 10, 20 or 40 µg of S100 protein extracted from cells grown either in synthetic complete (YNB) or rich (YEP) medium supplemented with 3% glycerol + 2% glucose, 3% glycerol or 2% ethanol as sole carbon source.
B. Samples contained 25 fmoles of ^{32}P-labelled Kdf1-Adr1 oligonucleotide (1.0 x 10^5 cpm) and 20 µg of S100 protein extracted from cells grown in synthetic complete medium supplemented with 2% glucose, 3% glycerol or 2% ethanol as sole carbon source.

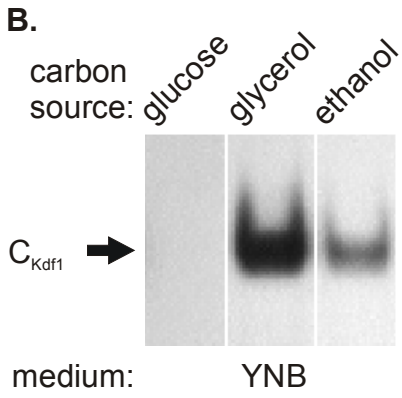


Figure 3-5 shows that this was the case; Kdf1 complex-formation is generally much stronger in extracts from rich medium-grown cells in comparison to synthetic complete medium-grown cells (Figure 3-5A). We asumed the batch to batch variation in quantity of Kdf1-CSRE$_{LAC4}$ complex formed in extracts from SC + 2% ethanol-grown cells (compare Figures 3-5 A and B) to be a result of reduced protein stability in these extracts.

3.1.5 Glucose leads to loss of Kdf1 complex-formation in the presence of glycerol

Formation of the Kdf1-CSRE$_{LAC4}$ complex is not observed when cells are grown on any poor carbon source plus glucose, showing that glucose repression is dominant in this respect (Schmidt, PhD thesis 1996). A preliminary experiment had shown that addition of glucose to a glycerol-grown culture led to loss of Kdf1 complex-formation and that this process took some hours. A more precise time course experiment was then performed to determine the kinetics of glucose-repression of Kdf1-binding (Figure 1-6).

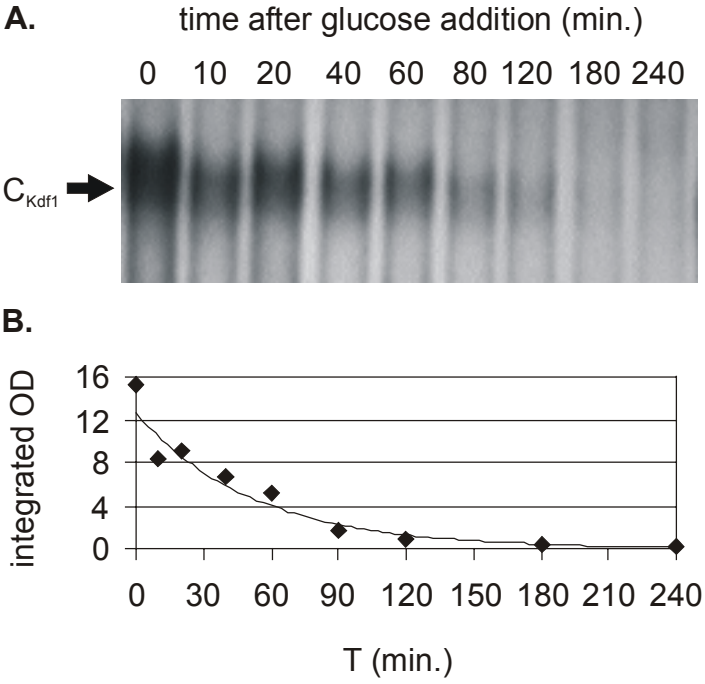


Figure 3-6: Glucose-repression of Kdf1 complex-formation is a slow process.
A. wild-type cells were grown to OD_{600} = 0.8 in SC + 3% glycerol. A sample was taken (t=0) and 2% glucose was added. At the timepoints indicated cells were collected and S100 extracts from all samples were prepared. 20 µg of each extract was incubated with 10 fmoles of ^{32}P-labelled f198 fragment (5.5 x 10^4 cpm).
B. Kdf1-complex band intensities from **A.** were quantified using the One-D Scan program.

Indeed a slow and steady decrease of the Kdf1-complex over time is observed until no complex is discernible anymore three to four hours after glucose addition (Figure 3-6A). Quantification of band-intensities showed a logarithmic decline with a half-life of 36 minutes (Figure 3-6B). This indicated a passive loss of Kdf1 through normal protein degradation combined with a lack of *de novo* synthesis rather than an active downregulation through mechanisms as (de)phosphorylation or targeted degradation.

3.2 Analysis of regulatory gene *KlCAT8*

The experiments described so far showed that $CSRE_{LAC4}$-binding of Kdf1 is repressed by fermentable sugars in general. In *Saccharomyces cerevisiae* a gene had been found, *CAT8*, which encodes a zinc-cluster protein which might be a transcriptional activator (Hedges *et al*, 1995). A strain deleted for *cat8* no longer grew on fermentable carbon sources and induction of expression of gluconeogenic and glyoxylate pathway genes on these carbon sources was lost. Overexpression of *CAT8* suppressed the growth defect on ethanol of a *snf1* strain. Based on this effect we tried to isolate a *K. lactis* homologue of *CAT8*.

3.2.1 Identification of *KlCAT8*

A multicopy suppression of the growth defect on ethanol of a *K. lactis fog2* (*Klsnf1*) (Goffrini *et al*, 1996) strain was attempted. As in *S. cerevisiae*, in *K. lactis* the Snf1p protein kinase function is required for growth on poor carbon sources. Among the clones mediating growth we identified an ORF 4335 basepairs in length, encoding a protein of 1445 amino acids that showed 40% identity to ScCat8p (Georis *et al*, 2000). Regions identified were a $Zn(II)_2Cys_6$ zinc-cluster in the N-terminus, a central sequence fitting the Major Homology Region consensus (Poch, 1997) and an asparagine-rich region in the C-terminus of the protein. The ORF was disrupted by deleting an internal 3357 basepair fragment by two-step gene replacement.

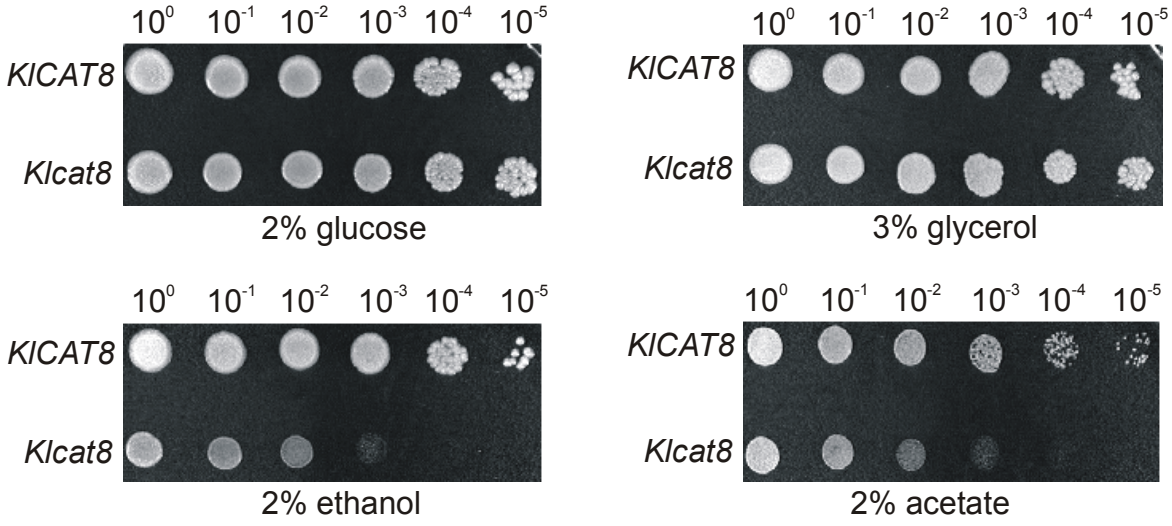


Figure 3-7: Deletion of *KlCAT8* impairs growth on ethanol and acetate, but not on glycerol.
Wild-type and *Klcat8* cells pregrown in rich medium + 2% glucose were spotted in serial 10-fold dilutions on SC agar plates with the indicated carbon sources and incubated at 30°C for 4 days.

The resulting *Klcat8* deletion strain shows a strong reduction in growth on ethanol and acetate but, in contrast to *S. cerevisiae*, grows like the wild-type on glycerol (Figure 3-7). This showed that Cat8p is involved in carbon source regulation of growth in *K. lactis* as in *S. cerevisiae* but the systems are not identical.

3.2.2 The influence of *KlCAT8* on Kdf1-binding to the $CSRE_{LAC4}$

The first question to answer was whether Kdf1-binding to the $CSRE_{LAC4}$ could be observed in the *Klcat8* strain, which would give an indication about the nature of Kdf1. Many zinc cluster transcriptional activators bind as homodimers to sequences characterized by two CGG triplets, oriented as direct, inverted or everted repeats, separated by a stretch of basepairs the length of which, rather than the sequence, is specific for the activators. The $CSRE_{LAC4}$ sequence, 5'- TCGGATGAAAGGGG -3', strongly resembles this pattern. Moreover KlCat8p contains the characteristic zinc cluster domain and deletion of the gene affects growth on C2 carbon sources, on which the Kdf1-$CSRE_{LAC4}$ complex forms most strongly. KlCat8p might therefore be itself Kdf1 or the DNA-binding component of a complex Kdf1.
We analysed Kdf1-$CSRE_{LAC4}$ complex formation in extracts from wild-type and *Klcat8* cells grown on SC medium with 3% glycerol. The result of this analysis is shown in Figure 3-8A. Deletion of *KlCAT8* led to a major decrease of Kdf1-binding but a residual complex of the same size was still present in the mutant. This indicated that KlCat8p is required for binding of Kdf1 to the $CSRE_{LAC4}$ but is not itself Kdf1.

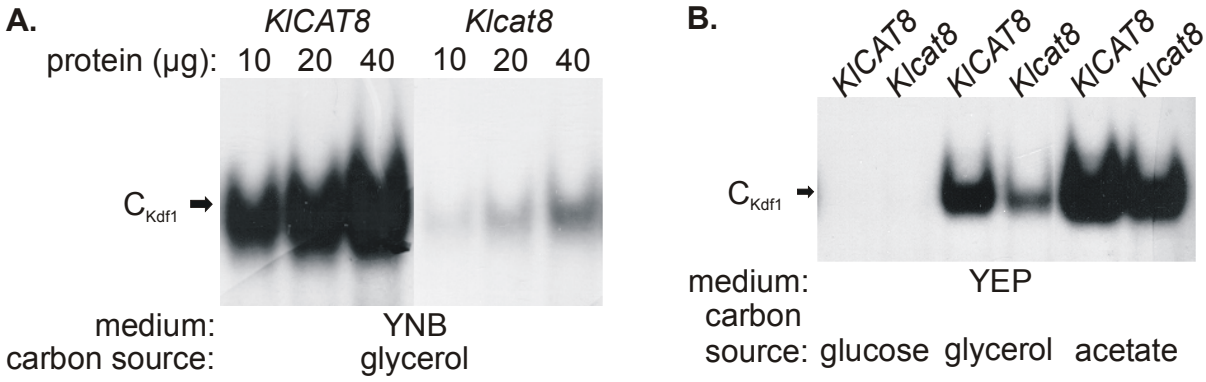


Figure 3-8: Kdf1-binding to the $CSRE_{LAC4}$ is reduced in a *Klcat8* mutant.
A. Samples contained 10 fmoles of ^{32}P-labelled $CSRE_{LAC4}$ oligonucleotide (7.0 x 10^4 cpm) and 10, 20 or 40 µg of S100 protein from *KlCAT8* wild-type or *Klcat8* disruption strains grown in SC medium + 3% glycerol.
B. Samples contained 25 fmoles of ^{32}P-labelled oligonucleotide KDF1-Adr1 (1.0 x 10^5 cpm) and 20 µg of S100 protein from wild-type or *Klcat8* cells grown in rich medium + 2% glucose, 3% glycerol or 2% potassium acetate.

Figure 3-8B shows that the reduction of Kdf1-binding observed on glycerol occurred on actate as well although the complexes formed in the extracts from both wild-type and *Klcat8* mutant grown on acetate were much stronger than on glycerol.

3.2.3 KlCat8p is not the factor binding to the CSRE$_{LAC4}$

The most likely cause for the residual complex-formation in the *Klcat8* mutant (Figure 3-8A) is decreased abundance or binding activity of Kdf. The possibility of another protein than Kdf1 binding to the CSRE$_{LAC4}$, forming a complex with the same mobility as the Kdf1-CSRE$_{LAC4}$ complex and hidden by it in the wild-type, cannot be excluded. In a further approach to address this question, a heamagglutinin (HA) epitope tag was fused to the N-terminus of KlCat8p. The HA-*KlCAT8* construct on an integrative plasmid was then inserted at the site of the disrupted gene in the wild-type chromosomal context. The growth phenotype of the integrant was indistinguishable from the wild-type and the same is valid for Kdf1-binding to the CSRE$_{LAC4}$ as tested by mobility shift experiment (results not shown). Using this strain we now tried whether the Kdf1-CSRE$_{LAC4}$ complex could be supershifted by addition of an α-HA antibody. As negative control an α-(HIS)$_5$ antibody, targeted against a 5 x histidine stretch, was used to exclude non-specific antibody-binding. Figure 3-9 shows that no band of lower mobility than the Kdf1-CSRE$_{LAC4}$ complex, which would result from the added mass of the bound antibody, occurs.

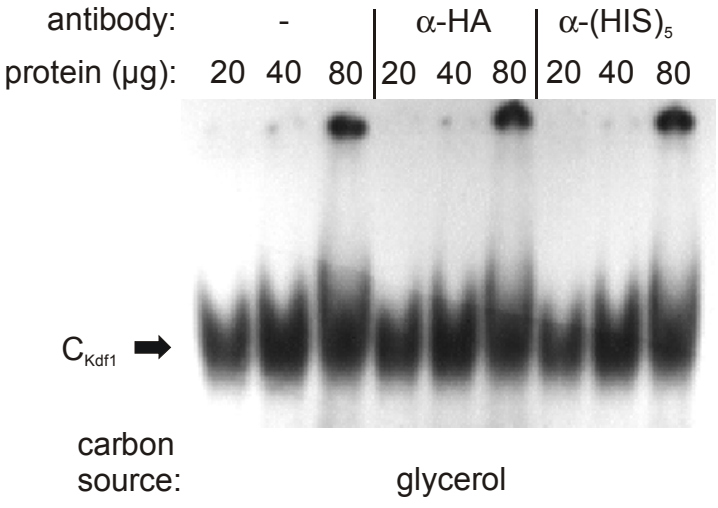


Figure 3-9: Kdf1 is not supershifted in extracts from cells expressing HA-KlCat8p. Samples contained 50 fmoles of ^{32}P-labelled oligonucleotide CSRE$_{LAC4}$ (2.0 x 10^4 cpm) and 20, 40 or 80 µg of S100 protein from a SC + 3% glycerol-grown strain carrying a chromosomal HA-*KlCAT8* fusion. After 20-minute incubation for Kdf1-binding 30 ng α-HA or 30 ng α-(His)$_5$ antibody were added and samples incubated for another 10 minutes.

In a second approach to try and discern between the residual complex being Kdf1 itself or a different complex with identical mobility we analysed the effect of expression of progessively shortenend KlCat8p variants on Kdf1-binding. By

removing restriction fragments we created C-terminal deletions in KlCat8p of 170, 729 and 1140 amino acids in size, resulting in clones pGID1$_{1-1275}$, pGID1$_{1-716}$ and pGID1$_{1-305}$ that encode *Klcat8* 1-1275, *Klcat8* 1-716 and *Klcat8* 1-305, respectively (Figure 3-10B). All three clones still contain the N-terminal zinc-cluster DNA binding domain and the coiled-coil dimerization domain C-terminally adjacent to it. We considered that expression of those truncated KlCat8p versions in the deletion strain would result in smaller complexes in addition to the residual complex if KlCat8p did bind to the CSRE$_{LAC4}$. Extracts were prepared from glycerol-grown cultures of the wild-type strain and the *Klcat8* deletion strain carrying the the empty vector, full length *KlCAT8* or either of the three truncated alleles and tested for Kdf1-binding to the CSRE$_{LAC4}$ in gel shift assay.

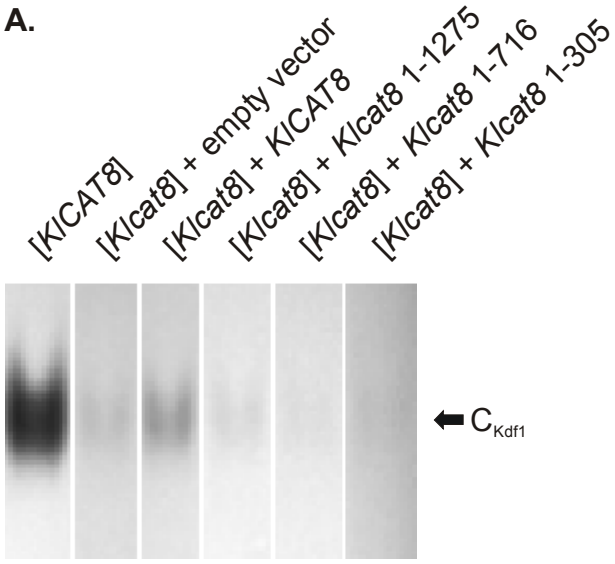


Figure 3-10: C-terminal deletion of up to 79% of KlCat8p does not influence the size of the residual Kdf1-CSRE$_{LAC4}$ complex.
A. Samples contained 10 fmoles of ^{32}P-labelled CSRE$_{LAC4}$ oligonucleotide (7.5 x 10^4 cpm) and 40 µg of S100 protein extract from wild-type cells grown in SC + 3% glycerol or *Klcat8* cells carrying empty vector KEp6 or the *KlCAT8* alleles indicated, grown in SC + 3% glycerol w/o uracil.
B. Graphical representation of the wild-type and truncated alleles. Zn = $Zn(II)_2Cys_6$ binuclear cluster, CC = coiled-coil, MHR = Major Homology Region, ASNn = poly-asparagine stretch.

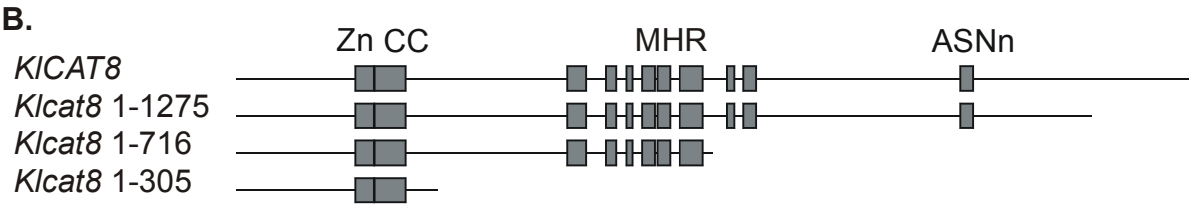


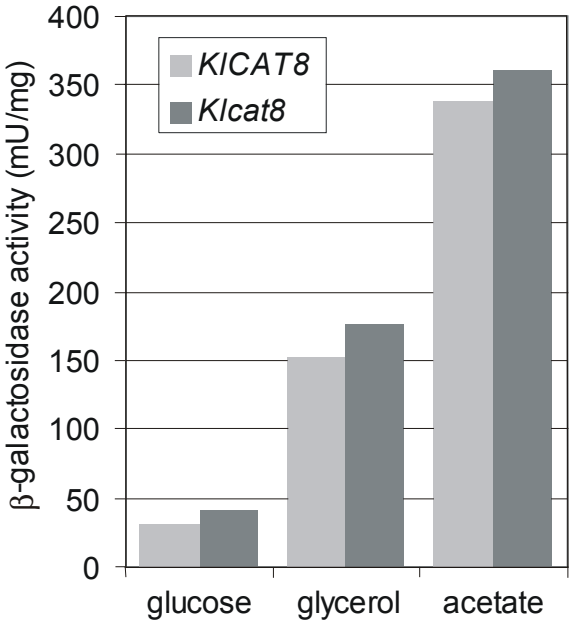
Figure 3-10A shows that only Kdf1-CSRE$_{LAC4}$ complexes of the same size as in the wild-type form with the extracts from any of the transformants. This is another indication that KlCat8p is not Kdf1. Overexpression of *KlCAT8* suppressed the growth defects on ethanol of both the *fog2* (*Klsnf1*) (Georis *et al*, 2000) and *Klcat8* strains (Figure 3-12). The suppression of the *Klcat8* mutation by plasmid-borne *KlCAT8* on a multicopy vector however is only partial, showing that overproduction of KlCat8p had a toxic effect on the cell (Figure 3-10 shows that this

condition did not fully restore Kdf1-binding. The slight restoration of Kdf1-binding caused by the wild-type gene is lost again already with the smallest, 170 amino acid C-terminal deletion. This indicates that already this deletion removes a region of KlCat8p that is required for normal regulation of Kdf1-binding.

3.2.4 The influence of deletion of *KlCAT8* on *LAC4* expression

Deletion of *KlCAT8* led to a considerable decrease of Kdf1-binding to the $CSRE_{LAC4}$. This sequence had been shown to be responsible for approximately half of the basal expression level of *LAC4* (Schmidt, PhD thesis 1996). Moreover the $CSRE_{LAC4}$ could substitute for the BCR in activation of *LAC4* transcription on poor carbon sources (compare strains JA6/LR2 and JA6/LR2K in figure 3-4). If Kdf1 functioned as an activator of transcription, irrespective of whether KlCat8p were Kdf1 or an upstream regulator of it, reduction of Kdf1-$CSRE_{LAC4}$ binding in *Klcat8* cells on poor carbon sources should lead to reduced *LAC4* expression and lower β- galactosidase activity.

Figure 3-11: Deletion of *KlCAT8* does not influence β-galactosidase activity on poor carbon sources. β-galactosidase activities were determined by the standard assay in glassbead extracts from wild-type and *Klcat8* cultures grown in rich medium + 2% glucose, 3% glycerol or 2% sodium acetate to an OD_{600} of 0.8 to 1.8.



Kdf1-binding and β-galactosidase activities in wild-type and *Klcat8* cells grown on glucose, glycerol or acetate were compared. Figure 3-11 shows that β-galactosidase activity was not affected by the *Klcat8* deletion. Derepressed activity was about five-fold higher on glycerol and ten-fold higher on acetate than the glucose-repressed activity. This corresponded with the increased Kdf1-binding to the $CSRE_{LAC4}$. The reduction in Kdf1-binding in the *Klcat8* mutant compared to the wild-type was not reflected in the β-galactosidase activities, which were unaffected by the deletion. This means that Kdf1-binding to the $CSRE_{LAC4}$ and activation of *LAC4* are not as tightly linked as previously thought.

3.3 Analysis of the regulatory gene *KlSIP4*

Since the decreased binding to the $CSRE_{LAC4}$ in the *Klcat8* deletion mutant suggested that KlCat8p was an upstream regulator of Kdf1, we set out to identify target genes of *KlCAT8*. The approach we chose was to search for multicopy suppressors of the growth defect of *Klcat8* on ethanol.

3.3.1 Identification of *KlSIP4*

Electrocompetent cells of strain yIG8 (*Klcat8*) were transformed with the KEp6-based genomic library “Curie pool” (Wesolowski-Louvel *et al*, 1992) and plated on synthetic complete medium plates lacking uracil for selection of transformants. With 2% glucose as carbon source approximately 9000 colonies were obtained whereas on 2% ethanol 45 colonies formed. After plasmid rescue from these latter transformants and retransformation in yIG8 growth on ethanol was verified and six of the suppressing plasmids were further analysed. Restriction pattern comparison and Southern hybridisation using a digoxygenin-labelled *KlCAT8* probe showed five out of six plasmids to carry *KlCAT8* on the inserts. One plasmid, pGP3, showed no signal in the Southern hybridisation and was further analysed. The 6.5 kilobase insert in pGP3 was sequenced and two Open Reading Frames were found. A 2151 base pair Open Reading Frame encoded a 717 amino acid putative protein. A scan for homologies (PROSCAN on the Network Protein Sequence @nalysis page at the Pôle Bio-Informatique Lyonnais: http://npsa-pbil.ibcp.fr/) against the PROSITE domain database (http://www.expasy.ch/prosite/) using this sequence showed identity to the fungal type $Zn(II)_2Cys_6$ binuclear cluster from position 62 to 90. A homology search against the Swissprot and translated EMBL databases was performed using the BlastP algorithm (Altschul, 1997) on the same server. The search yielded *Saccharomyces cerevisiae* Sip4p (ScSip4p: 829 AA) as the closest match, followed at some distance by *Neurospora crassa* Acu-15 (NcAcu15: 865 AA), the FacB proteins from *Emericella nidulans* (EnuFacB: 867 AA), *Aspergillus oryzae* (AouFacB: 859 AA) and *Aspergillus niger* (AnuFacB: 862 AA), all involved in regulation of acetate metabolism and the Cat8 proteins from *Kluyveromyces lactis* (KlCat8p: 1445 AA), *Saccharomyces cerevisiae* (ScCat8p: 1433 AA) and *Candida albicans* (CaCat8p: 1056 AA). Based on these data we named the ORF *KlSIP4* and concluded that KlSip4p is capable of suppressing the deficiency to utilize ethanol as a carbon source of a *Klcat8* deletion (Figure 3-12).

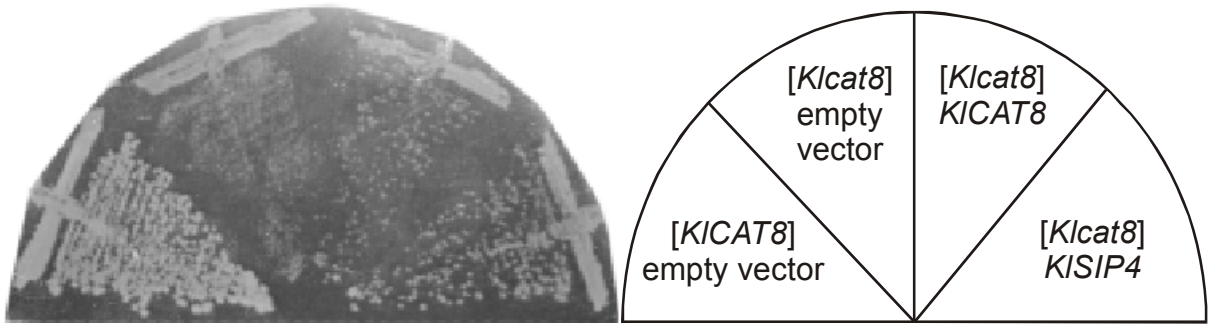


Figure 3-12: *KlSIP4* suppresses the *Klcat8* growth phenotype on ethanol.
Strains were streaked on a SC-agar plate + 2% ethanol w.o. uracil as indicated and incubated for 4 days at 30°C.

The set of *SIP4* / *CAT8*-related sequences was aligned on the NPS@ server using Clustal W Protein (Thompson *et al*, 1994) with default parameters:

Pairwise alignment type: FAST;

Fast pairwise alignment parameters:

K-tuple (word) size: 1; Number of top diagonals: 5; Window size: 5; Gap penalty: 3; Scoring method : percentage;

Multiple Alignment Parameters:

Weight matrix: GONNET; Gap opening penalty: 10.0; Gap extension penalty: 0.2; Residue-specific gap penalties OFF: no; Hydrophilic gaps OFF: no; Hydrophilic residues: GPSNDQERK; Percent of identity for delay: 30; Gap separation distance: 8; No end gap separation penalty: yes.

The alignment showed that primary sequence conservation is restricted to the N-terminal zinc cluster and the C-terminally adjacent 27 amino acid region. In the Acu-15, FacB and Cat8p proteins this region constitutes a 12 amino acid linker and the C-terminal half of a conserved 33 animo acid coiled-coil dimerization domain (Figure 3-13). This domain (primary consensus +sYTEs**L**EERVRx**L**EsEx+x**L**xxLxDx+--xh) is highly conserved in all of the aligned sequences except the Sip4p proteins. In both, ScSip4p and KlSip4p, sequence similarity breaks off after the second leucine residue. The high degree of conservation of the coiled-coil domain in the Acu-15, FacB and Cat8p proteins characterizes a subfamily of Cat8p-related proteins within the Gal4p family of zinc-cluster DNA-binding proteins. The truncated coiled-coil domain in the Sip4p proteins indicated that these do not belong to this subfamily.

A.

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                          10        20        30        40        50        60        70        80
                          |         |         |         |         |         |         |         |
KlSip4p       52  ASTKVKRFSQACDRCRLKKIKCDGIKPSCSNCKKIGYHCSTSDKLTRRGFPRGYTEMLENEVIKLQRLCGMVDENGETVIDGAVAAVA
ScSip4p       35  ITDFSVRKAHACDRCRLKKIKCDGLKPNCSNCAKIDFPCKTSDKLSRRGLPKGYTELLEKEVVRLTNMNASSSANANSNLPFINDTFY
NcAcu15       13  GNSSQSRIAQACDRCRSKKIRCDGIRPCCSQCANVGFECKTSDKLSRRAFPRGYTESLEERVRALEAEIRELKDLLDEKDEKLDMLSK
AnuFacB       13  GNGSQSRIVLACDRCRSKKIRCDGVRPCCTQCANVGFECKTSDKLSRRALPRGYTESLEERVRALESEVRDLKNLLDEKDEKIDVLSR
AouFacB       13  GNGAQSRIAQACDRCRSKKIRCDGIRPCCTQCANVGFECKTSDKLSRRAFPRGYTESLEERVRALEAEVRDLKNLLDEKDEKIDVLSR
EnuFacB       13  GNNTQSRIAQACDRCRSKKIRCDGIRPCCTQCANVGFECKTSDKLSRRAFPRGYTESLEERVRTLEAEVRELKSLLDEKDEKIDVLSR
KlCat8p      172  TTTPSYRVAQACDRCRAKKIRCDGKRPQCTQCAAVGFECKISDKLSRRAFPRGYTETLEERVRELEAENRRLVALCDLKEEQLHLVSK
ScCat8p       59  LSTPIYRIAQACDRCRSKKTRCDGKRPQCSQCAAVGFECRISDKLLRKAYPKGYTESLEERVRELEAENKRLLALCDIKEQQISLVSQ
CaCat8p       43  PGSKVERVAQACDRCRAKKTKCDGQNP-CSTCQSVGLECIVSDRLTRKSYPKAYTETLEERVRQLEAENKKLAGLLDMRDEQLELLNG
                      *   ****** ** :*** .* *: *  :.  *  **:* *:. *:.*** **:.*  **:* : *  * * ::: : ::
Prim.cons.            R   ACDRCR KK +CDG  P Cs C  h h C  SD+L R+shP+sYTE LE  V  LEsE + L  L D +-- h hh
```

B.

	Kl Sip4p	Sc Sip4p	Nc Acu15	Anu FacB	Aou FacB	Enu FacB	Kl Cat8p	Sc Cat8p	Ca Cat8p
Kl Sip4p	-	23	19	18	18	17	17	17	18
Sc Sip4p	23	-	19	18	18	18	18	18	17
Nc Acu15	19	19	-	28	28	27	24	25	19
Anu FacB	18	18	28	-	29	28	25	24	18
Aou FacB	18	18	28	29	-	28	25	24	18
Enu FacB	17	18	27	28	28	-	25	24	18
Kl Cat8p	17	18	24	25	25	25	-	26	19
Sc Cat8p	17	18	25	24	24	24	26	-	20
Ca Cat8p	18	17	19	18	18	18	19	20	-

Figure 3-13: The zinc-cluster is conserved between transcription factors involved in growth on poor carbon sources in different genera.

A. Multiple alignment of zinc-cluster, linker and coiled-coil regions of the Sip4p / Cat8p-like proteins. For nomenclature see text. Numbers on the left indicate the position of the first residue shown. Underlined: fungal $Zn(II)_2Cys_6$ binuclear cluster, cysteines in bold face; italics: coiled-coil region, leucines in bold italics. '*' indicates identity; ':' and '.' indicate strong and weak similarity, respectively. In the consensus '+' indicates positively charged residues (R, K, H), '-' negatively charged residues (D, E, Q, N), 'h' bulky aliphatic or aromatic residues (I, L, M, V, F, Y, W) and 's' small residues (P, A, G, S, T).

B. Pairwise comparison of the number of identical residues in the 29 residue $Zn(II)_2Cys_6$ zinc-clusters aligned in **A**. Dark, middle and light grey indicate 100% - 85%, 85% - 70% and 70% - 55% identity, respectively.

The only sequence from the databases that shows similarity to the putative KlSip4p sequence over its entire length is ScSip4p. The alignment of the two sequences using Clustal W is shown in Figure 3-14A. Overall identity is 24.5% and strong and weak similarities combined comprise 26.7% of the KlSip4p sequence. Block-pairs in Figure 3-14A represent aligned sequences. Lines represent unaligned regions opposite gaps opened in the other sequence. In the alignment the region containing the zinc-cluster, linker and truncated coiled-coil, showing 60% identity between the two sequences, is indicated.

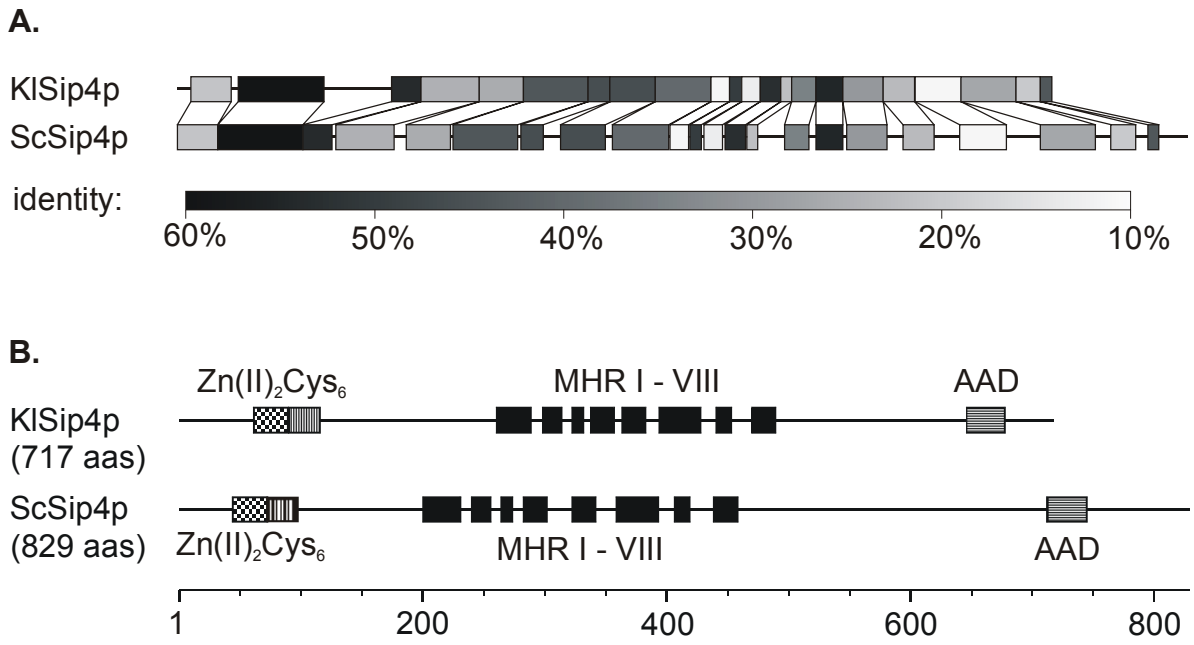

Figure 3-14: Overall sequence conservation between KlSip4p and ScSip4p is low but structural features are conserved.
A. Alignment of KlSip4p and ScSip4p (Clustal W, standard parameters) shows an overall identity of 24.5% and similarity of 26.7%. Identity is highest at 60.0% in the zinc-cluster region. Shading of regions corresponds to identity.
B. The locations of the $Zn(II)_2Cys_6$ binuclear cluster flanked by linker and truncated coiled-coil, the Middle Homology Region (MHR I - VIII) and putative acidic activation domains are indicated.

A number of short stretches found in ScSip4p is missing in KlSip4p, which in contrast contains, adjacent to the truncated coiled-coil, a long "insertion" that is missing in the ScSip4p sequence. Although the block structure is artificial it is evident that a number of regions exist that show an identity of 40% to 50%, separated by regions of considerably lower conservation.
Closer scrutiny of the central part of KlSip4p showed that it contains the Middle Homology Region. This region was originally identified by Poch (1997) from multiple alignment of a large set of zinc-cluster protein sequences. It consists of eight motifs

separated by 'spacers' of highly variable length. The consensus for the Middle Homology Region is one of amino acid characteristics combined with predicted secondary structure rather than a residue consensus *per se*, and therefore rather loose at first sight. The Middle Homology Region is conserved among the nine homologous proteins (Figures 3-14B and 3-15). An alignment of the KlSip4p and ScSip4p MHRs is shown in Figure 3-16.

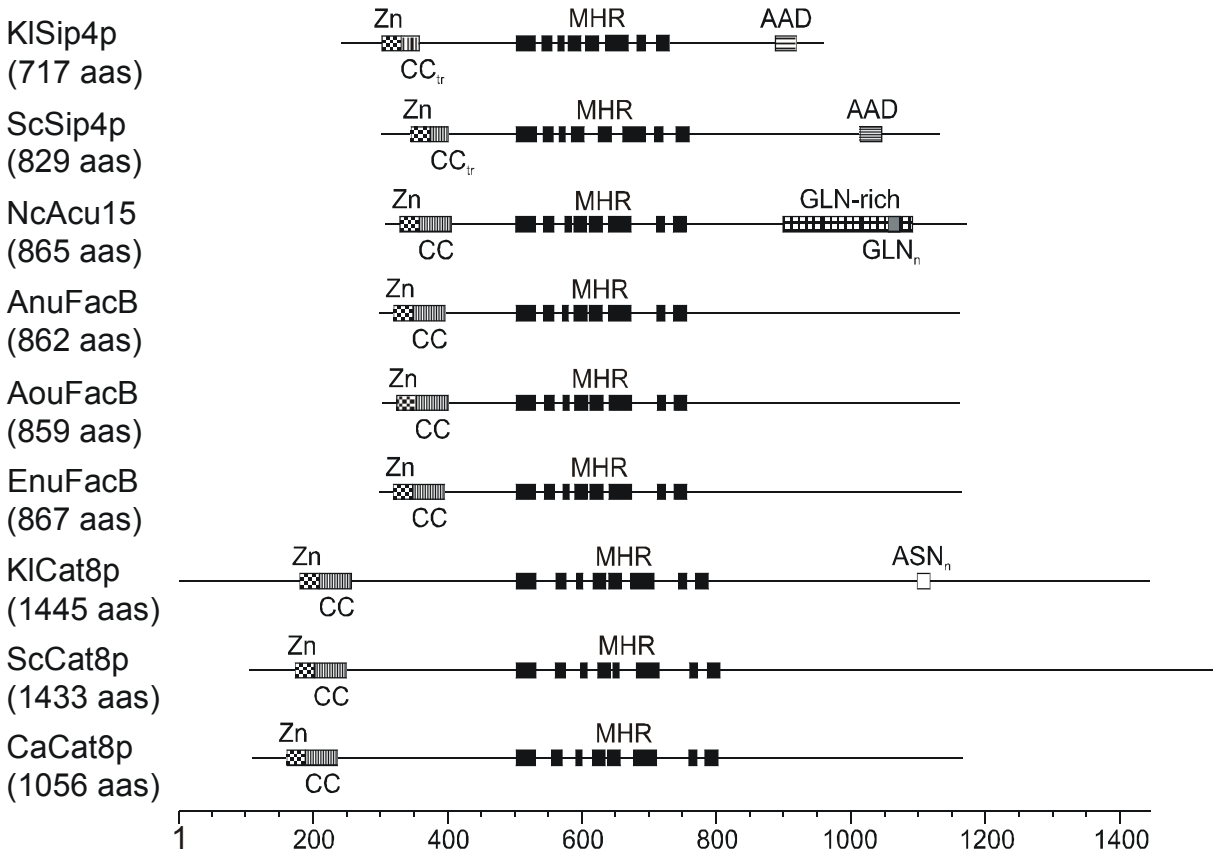

Figure 3-15: The proteins most closely related to KlSip4p all share the Zn(II)$_2$Cys$_6$ binuclear cluster and the Middle Homology Region.
The schematic renditions of the eight proteins showing significant similarity to KlSip4p in a BLAST search and KlSip4p itself show the features identified in a search for known motifs. All share the fungal-type Zn(II)$_2$Cys$_6$ binuclear cluster (Zn) and the Middle Homology Region Motifs I to VIII (MHR). The 11aa linker and 34aa coiled-coil regions (CC) are highly conserved in all but the Sip4 proteins, where the coiled-coil is truncated after residue 15 (CC$_{tr}$). Some features identified are apparently unique or shared only within subgroups. The Sip4 proteins contain C-terminal regions of 24 to 30 aas rich in negatively charged and hydrophobic residues, typical of acidic activation domains (AAD). A glutamine-rich region (GLN-rich) containing a poly-glutamine stretch (GLN$_n$) is unique to *N. crassa* Acu-15 whereas a poly-asparagine stretch (ASN$_n$) is unique to *K. lactis* Cat8p. The sequences are represented to scale and aligned on the first residue of MHR Motif I.

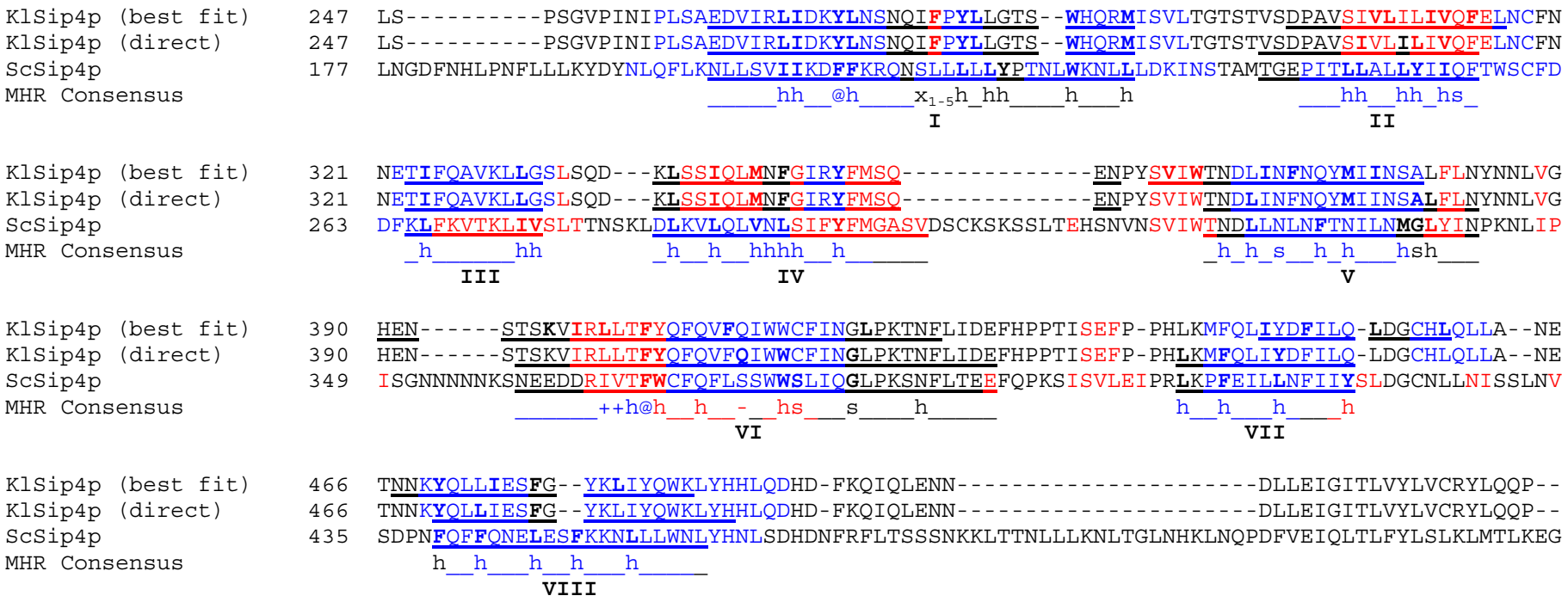

Fig 3-16: The Middle Homology Region is conserved in KlSip4p.
ClustalW alignment of KlSip4p residues 247 to 524 with ScSip4p residues 177 to 520. Below the alignment the consensus sequence for the Middle Homology Region (MHR) Motifs I to VIII is shown (Poch, 1997). These are characteristic for members of the Gal4 family of DNA-binding proteins. Consensus code is 'h' for bulky residues (I, L, M, V, F, Y, W), '@' for aromatic residues (F, Y, W), 's' for small residues (P, A, G, S, T), '-' for negatively charged residues (D, E, Q, N) and '+' for positively charged residues (H, K, R). MHR residues conserved in the Sip4 sequences are **bold**, the corresponding locations of the Motifs I to VIII are underlined. Predictions for the consensus secondary structure within the MHR motifs for the Sip4 proteins were obtained using the PHD program (Rost and Sander, 1993; 1994). Predicted α-helical regions are shown in blue, β-strand regions in red. The 'best fit' alignment for the KlSip4p MHR motifs (upper sequence) locates individual motifs to obtain the optimal alignment with the MHR consensus (conservation = 77%). The 'direct' alignment of the KlSip4p MHR (middle sequence) locates the MHR motifs in positions identical to the locations of the motifs in ScSip4p, based on primary sequence-alignment of KlSip4p and ScSip4p. Conservation for KlSip4p with the consensus in this case: 66%.

3.3.2 Deletion of *KlSIP4*

After identifying *KlSIP4* we tested whether deletion of the gene would have a phenotype. Deletion of *SIP4* in *Saccharomyces cerevisiae* had no discernible effect on growth on poor carbon sources, although overexpression suppressed the growth defect of a *Sccat8* deletion. Since one-step gene disruption through homologous recombination is inefficient in *K. lactis* we chose a two-step gene disruption scheme (see Materials and Methods) analogous to the *CAT8* deletion protocol (Georis *et al*, 2000). We created a fusion of the *KlSIP4* promoter and terminator regions, lacking the *KlSIP4* open reading frame, in a disruption plasmid. The *KlSIP4* deletion plasmid was linearized in the *KlSIP4* promoter and transformed into wild-type and *Klcat8* cells. Uracil prototrophic integrants were PCR-screened to obtain integrants in the *KlSIP4* locus. These were selected on 5-FOA to obtain uracil auxotroph derivatives that had lost the integrated plasmid along with the intact copy of the *KlSIP4* gene through a second cross-over event in the identical terminator regions. One colony each, which showed only the expected fragment in PCR analysis, was derived from wild-type strain JA6 and from *Klcat8* strain yIG8.

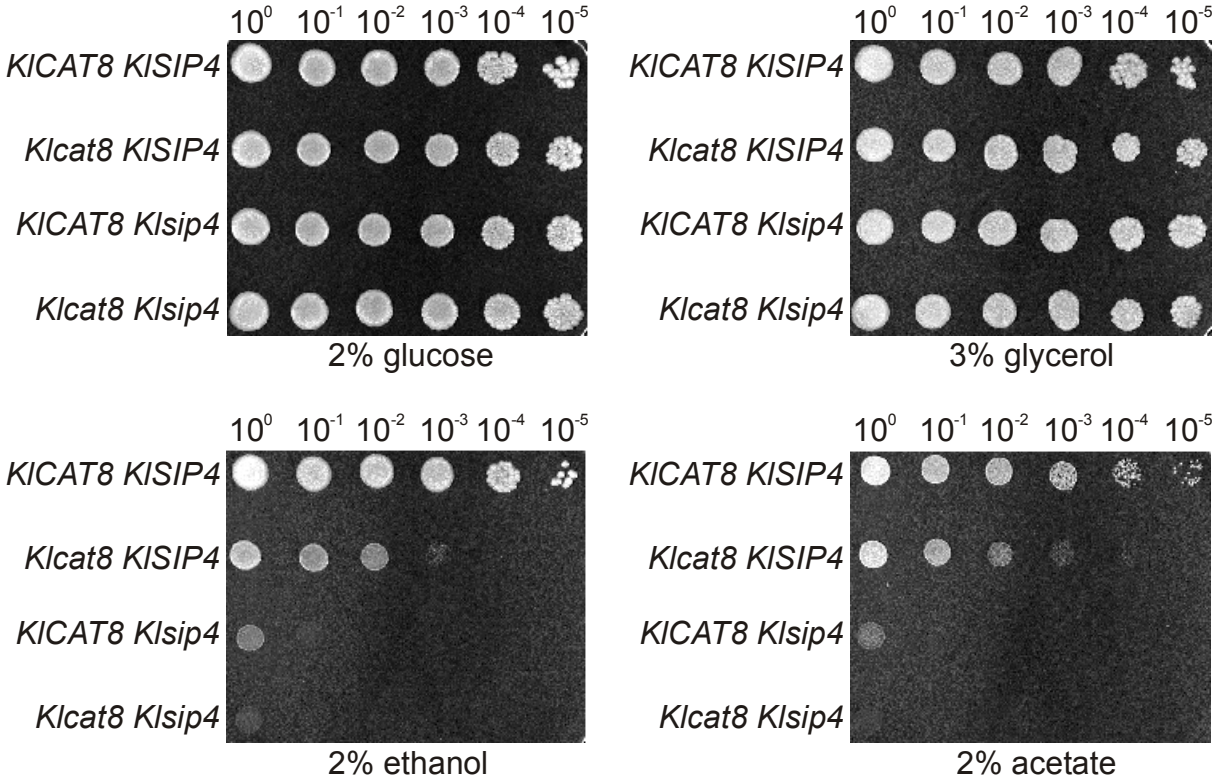

Figure 3-17: Deletion of *KlSIP4* alone or together with *KlCAT8* impairs growth on ethanol and acetate, but not on glycerol.
Wild-type, *Klcat8*, *Klsip4* and *Klcat8 Klsip4* cells pregrown in rich medium + 2% glucose were spotted in serial 10-fold dilutions on SC agar plates with the indicated carbon sources and incubated at 30°C for 4 days.

The *Klsip4* derivatives were named JA6/DS4 and yIG8/DS4 respectively. The spot test in Figure 3-17 shows that the *Klcat8*, *Klsip4* and *Klcat8 Klsip4* mutants share the same phenotype for growth on poor carbon sources. On glycerol neither strain showed any growth defect in comparison to the wild-type, whereas deletion of *KlCAT8* impaired growth on ethanol and acetate and deletion of *KlSIP4* did so even stronger. Deletion of both genes had a slight cumulative effect. This effect is a strong contrast with the situation in *S. cerevisiae*, were deletion of *SIP4* had no discernible effect on growth of the mutant on any poor carbon source.

3.3.3 In a *Klsip4* deletion strain Kdf1-binding is severely impaired

To address the question whether Kdf1-binding was altered in the *Klsip4* mutant strains a gel shift experiment was performed using extracts from the wild-type, the single mutants and the double mutant. As all strains grew equally well on glycerol, irrespective of the decreased binding of Kdf1 to the $CSRE_{LAC4}$ that was observed in the glycerol-grown *Klcat8* mutant, we grew the cultures for this experiments on this carbon source.

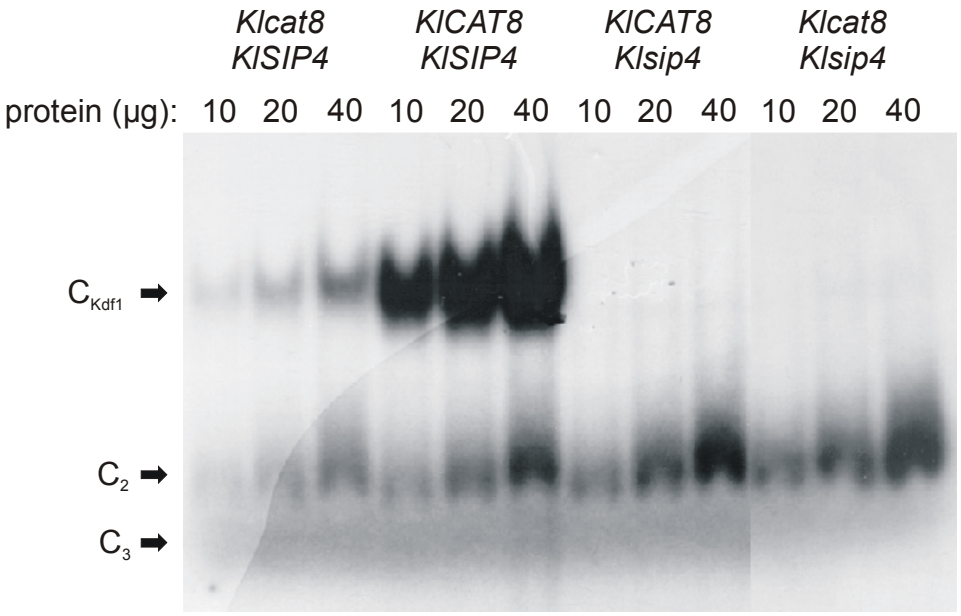

Figure 3-18: Deletion of *KlSIP4* does not abolish Kdf1-binding to the $CSRE_{LAC4}$.
Samples contained 10 fmoles (7.3 x 10^4 cpm) of ^{32}P-labelled $CSRE_{LAC4}$ oligonucleotide and 10, 20 or 40 µg of S100 protein from *Klcat8*, wild-type, *Klsip4* or *Klcat8 Klsip4* cells grown in SC medium + 3% glycerol.

Figure 3-18 shows that Kdf1-binding to the $CSRE_{LAC4}$ was extremely reduced by deletion of *KlSIP4*, but a residual complex still remained. The same was seen in the *Klcat8 Klsip4* double mutant. This indicates that KlSip4p is not Kdf1, in analogy to KlCat8p.

3.3.4 The effect of *Klcat8* and *Klsip4* deletions on β-galactosidase activity

To determine whether *Klsip4* deletion affected the expression of β-galactosidase, the single mutants and the double mutant grown in glycerol were analysed. The results represented in Figure 3-19 show that deletion of *KlCAT8* alone had no significant effect on β-galactosidase activity whereas deletion of *KlSIP4* led to a slight but significant increase of activity at about twice the level of the wild-type. More remarkable, deletion of both genes led to a β-galactosidase activity which is identical to the wild-type activity.

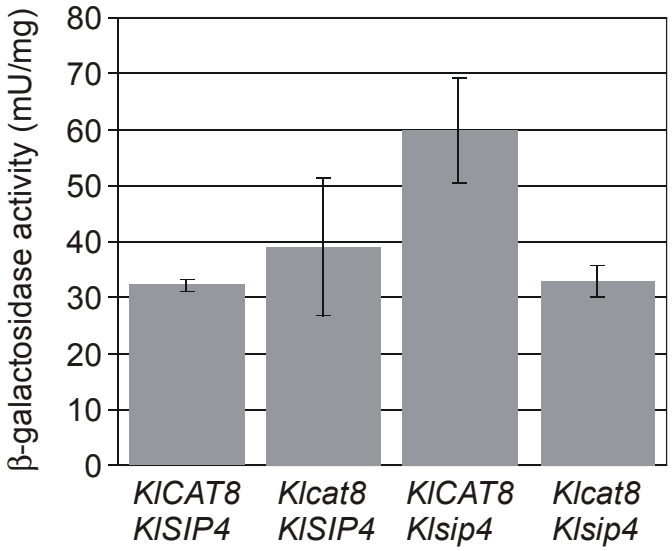

Figure 3-19: Deletion of *KlSIP4* but not of *KlCAT8* leads to an increase in β-galactosidase activity in glycerol-grown cells. β-galactosidase activity was measured in glassbead extracts from wild-type, *Klcat8*, *Klsip4* and *Klcat8 Klsip4* cells grown in synthetic complete medium + 3% glycerol to an OD_{600} of 0.8 to 1.6. Values are average with SD for three independent measurements of each strain.

For the wild-type strain it had been shown that β-galactosidase expression on glycerol is independent of KlGal4p. To test whether the absence of Kdf1 affected KlGal4p function we disrupted the gene in the *Klcat8*, *Klsip4* and *Klcat8 Klsip4* strains. For this purpose chemocompetent cells were transformed with the *KlGAL4* disruption plasmid pDL9 linearized with *Eco*RI and *Ksp*AI. Transformants were selected on synthetic complete medium lacking uracil, containing 2% glucose + 2% lactose and X-gal. Blue and white colonies arose in a 2:1 ratio. White colonies were streaked on plates as above as well as on plates lacking glucose to select for Lac^- growth phenotype. Integration in the *KlGAL4* locus in those strains unable to grow on lactose as sole carbon source was confirmed by PCR analysis. The *Klcat8 Klgal4*, *Klsip4 Klgal4* and *Klcat8 Klsip4 Klgal4* strains along with a preexisting *Klgal4* single mutant strain were grown in glycerol and β-galactosidase activity was determined. The data in Figure 3-20 show that expression of *LAC4* on glycerol was not affected by the *Klgal4* deletion in any of the four strains.

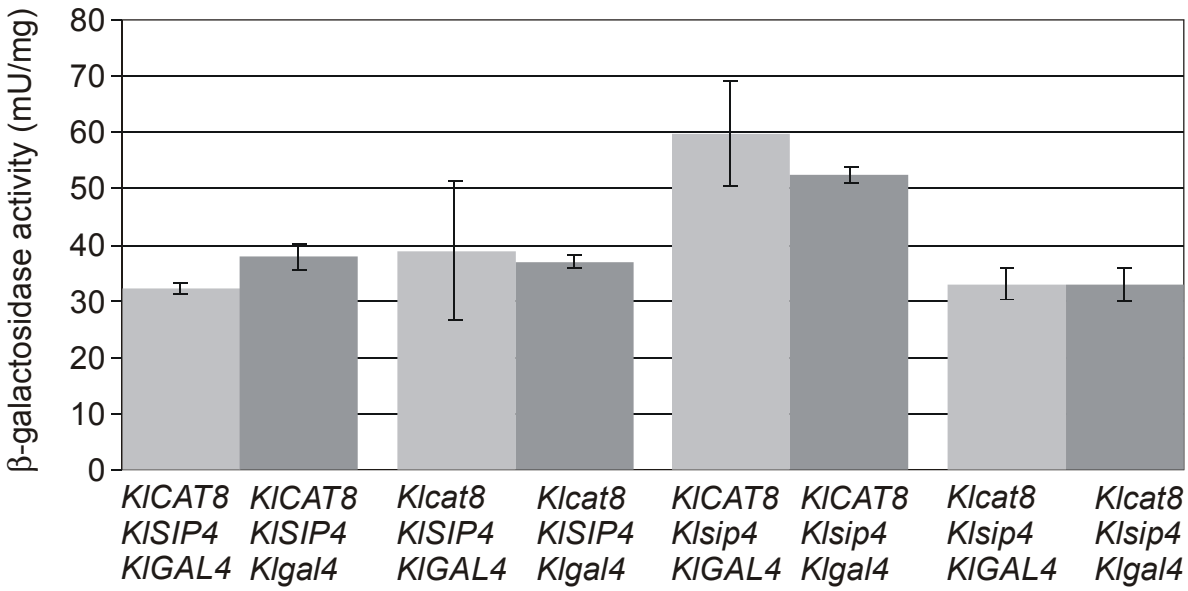

Figure 3-20: β-galactosidase expression on glycerol in wild-type, *Klcat8*, *Klsip4* and *Klcat8 Klsip4* strains is independent of *KlGAL4*.
β-galactosidase activity was measured in glassbead extracts of the strains indicated, grown in synthetic complete medium + 3% glycerol to an OD_{600} of 0.8 to 1.6. Values are average with SD for three independent measurements of each strain.

This result confirmed that KlGal4p function does not contribute to gene expression in non-induced cells. Specifically, the higher expression level observed in the *Klsip4* deletion mutant lacking Kdf1 is not due to elevated KlGal4p activity.
Taken together the data clearly show that, despite the apparent correlation of Kdf1 binding-activity and β-galactosidase levels in uninduced cells, Kdf1 is not responsible for derepression of *LAC4*. Rather the absence of KlSip4p seems to stimulate *LAC4* expression to some extent in a *KlCAT8*-dependent manner. However, KlCat8p is not responsible for most of the derepressed gene activity. This result is striking as the $CSRE_{LAC4}$ stimulated *LAC4* expression in the ΔBCR strain. It suggests that other $CSRE_{LAC4}$-binding factors exist.

3.4 Analysis of regulation mediated by *KlSIP4*

KlCAT8 is required for growth on the C2 carbon compounds ethanol and acetate, but not for growth on the C3 carbon compound glycerol. Gluconeogenesis from glycerol only requires fructose-1,6-bisphosphatase activity. In *K. lactis* the expression of gluconeogenic genes *KlFBP1* and *KlPCK1* has been compared between wild-type and *Klcat8* strains grown on glucose, glycerol or ethanol in Northern Blot experiments (Georis *et al*, 2000). Expression of *KlFBP1* was strongly induced both on glycerol and ethanol whereas *KlPCK1* was induced only on ethanol. Induction of both was KlCat8p-independent, however. In contrast, activity of glyoxylate cycle enzymes malate synthase and isocitrate lyase was mostly (malate synthase) or absolutely (isocitrate lyase) dependent on KlCat8p. These data fit the observed phenotype of the *Klcat8* mutant: normal growth on glycerol but no growth on ethanol or acetate.
In *S. cerevisiae* many genes that are induced during the diauxic shift (DeRisi *et al*, 1997; http://cmgm.stanford.edu/ pbrown/) or under growth on poor carbon sources were shown to be ScCat8p-dependent (Haurie *et al*, 2001) and to contain functional CSREs in their promoters. Among those are the gluconeogenic genes *FBP1* (Vincent and Gancedo, 1995) and *PCK1* (Proft *et al*, 1995) and the glyoxylate cycle genes *ICL1*, *MLS1* and *MDH2* (Schöler and Schüller, 1994; Caspary *et al*, 1997; Roth and Schüller, 2001). The transcriptional activator-encoding *ScSIP4* gene also belongs in this group (Lesage *et al*, 1996; Vincent and Carlson, 1998), but not *ScCAT8* itself (Randez-Gil *et al*, 1997).
Deletion of *KlSIP4* caused a phenotype even stronger than that of the *Klcat8* mutant. Therefore the effect of *Klsip4* deletion on expression of the *K. lactis* homologues of several of those genes or activity of their products was investigated and in parallel identification of CSRE sequences in the promoters of the genes was attempted.

3.4.1 Identification of *KlSIP4* target genes

The transcriptional activator genes *KlSIP4* and *KlCAT8*, glyoxylate cycle genes *KlICL1* and *KlMLS1* and acetyl-CoA synthetase genes *KlACS1* and *KlACS2* were analysed. As the *KlFBP1* promoter contains no obvious CSRE (J.J. Heinisch, personal communication), *KlFBP1* and *KlPCK1* expression was *KlCAT8*-independent and the *Klsip4* mutant grew as well on glycerol as the wild-type or the *Klcat8* mutant, these genes are most likely *KlSIP4*-independent as well. Expression of *KlFBP1* and *KlPCK1* was therefore not analysed.

3.4.2 The *KlSIP4* promoter contains two CSREs that bind Kdf1

The promoter of *KlSIP4* was searched for possible CSREs as *KlSIP4* might well be autoregulated as is *ScSIP4.* Four sequences were found (Table 3-1) that show similarity to the $CSRE_{LAC4}$ and the consensus CSRE-sequence from *S. cerevisiae*. $CSRE1_{KlSIP4}$ (-468 to -458) shows a mismatch to $CSRE_{LAC4}$ in position 4. $CSRE2_{KlSIP4}$ (-599 to -609) is identical to the $CSRE_{LAC4}$. Both sites were tested for Kdf1-binding in electrophoretic mobility shift experiments (Figure 3-21).

promoter site	position	sequence
$CSRE_{LAC4}$	-1129 / -1112	5′- AGT CGGATGAAAGG GGA -3′
KlSIP4$_{-137/-127}$	-140 / -124	5′- AAA CGGA**CCTGG**GG AAA -3′
$CSRE1_{KlSIP4}$	-471 / -455	5′- ATC CGG**C**TGAAAGG GAC -3′
KlSIP4$_{-467/-477}$	-464 / -480	5′- AGC CGGAT**CTGG**GG AAA -3′
$CSRE2_{KlSIP4}$	-596 / -612	5′- GTC CGGATGAAAGG CTA -3′
consensus *Sc*		5′- CGGNYNAAYGG -3′

Table 3-1: The *KlSIP4* promoter contains four sequences that show similarity to the $CSRE_{LAC4}$.
Shown are the $CSRE_{LAC4}$ and four *KlSIP4* promoter sequences and their positions (**A**TG = +1). The CSRE consensus sequence from *S. cerevisiae* (Roth and Schüller, 2001) is shown for comparison. Underlined: CSRE core, bold: bases differing from $CSRE_{LAC4}$. N = any base, Y =T/C.

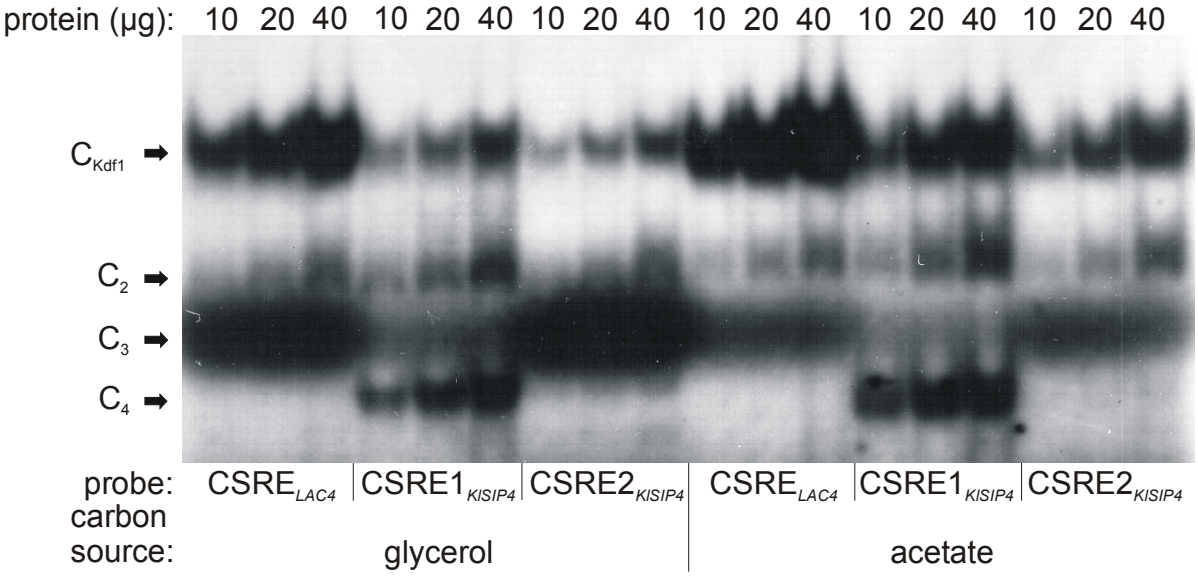

Figure 3-21: The *KlSIP4* promoter contains two Carbon Source-Responsive Elements.
Samples contained 30 fmoles of ^{32}P-labelled oligonucleotide ($CSRE_{LAC4}$, $CSRE1_{KlSIP4}$ or $CSRE2_{KlSIP4}$; 1.0 x 10^5 cpm each) and 10, 20 or 40 µg S100 protein extracted from wild-type cells grown in synthetic complete medium containing 3% glycerol or 2% acetate as sole carbon source.

Both oligonucleotides show binding of Kdf1 on glycerol and acetate, allbeit weaker than CSRE$_{LAC4}$. Complex C_2 formed with all probes in similar abundance and might represent a binding activity with low sequence specificity. Also, formation of this complex is not glucose-repressed (compare Figures 3-3). The nature of fuzzy complex C_3 is unclear. This band was not single stranded probe and its generation depended on the presence of protein extract in the sample (results not shown). It to some extent correlates with formation of complexes C_{Kdf1} and C_4 (compare Figures 3-26, 3-28 and 3-29) but not C_2 (compare Figure 3-23). Finally, complex C_4 is formed only with CSRE1$_{KlSIP4}$.
The sequences *KlSIP4*$_{-137/-127}$ and *KlSIP4*$_{-467/-477}$ diverge from the CSRE$_{LAC4}$ in containing the sequence TGG instead of AA$^{T}/_{C}$ in positions 7 to 9. Additionally *KlSIP4*$_{-137/-127}$ shows bases CC in positions 5 and 6 instead of TG and *KlSIP4*$_{-467/-477}$ a C in position 6. Among them however they show only one mismatch in the 11 basepairs corresponding to the CSRE. The three 3'-adjacent residues are identical as well. For this reason *KlSIP4*$_{-467/-477}$, most identical to CSRE$_{LAC4}$, was tested in the gel shift assay. First the oligonucleotide carrying this sequence was used in a competition experiment shown in Figure 3-22.

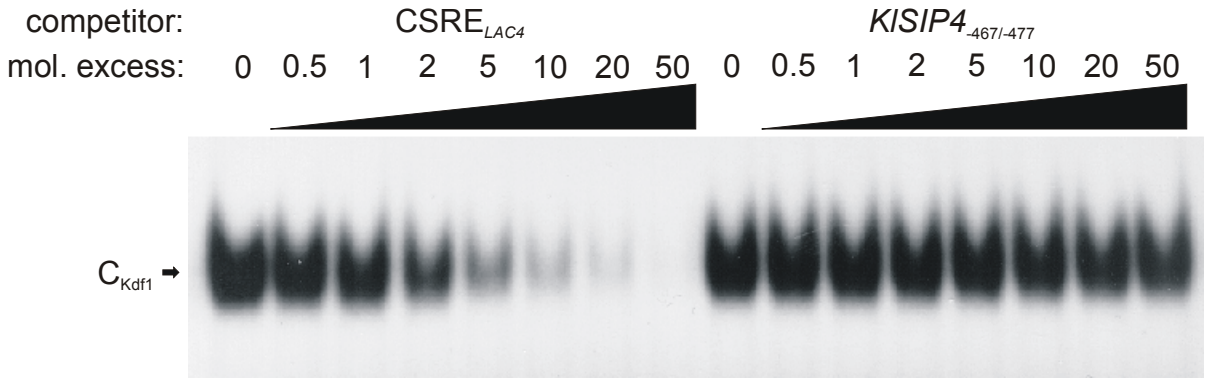

Figure 3-22: *KlSIP4*$_{-467/-477}$ does not compete for Kdf1-binding to CSRE$_{LAC4}$.
All samples contained 25 fmoles of ^{32}P-labelled CSRE$_{LAC4}$ oligonucleotide (3.0 x 10^4 cpm), 20 µg of S100 protein from wild-type cells grown in SC medium + 3% glycerol and no or 0.5-, 1-, 2-, 5-, 10-, 20- or 50-fold molar excess of unlabelled CSRE$_{LAC4}$ or *KlSIP4*$_{-467/-477}$ oligonucleotide.

Whereas cold CSRE$_{LAC4}$ added as competitor to labelled CSRE$_{LAC4}$ in a 1:1 molar ratio gave a reduction in band intensity of about 50% and 50-fold molar excess completely sequestered Kdf1 from the probe, 50-fold molar excess of *KlSIP4*$_{-467/-477}$ led only to a minor decrease in band intensity, indicating a very low affinity for Kdf1. This experiment indicated that despite similarity with CSRE$_{LAC4}$, this sequence may not represent a functional CSRE. When *KlSIP4*$_{-467/-477}$ was directly used as probe in a

mobility shift assay no Kdf1-complex was detected (Figure 3-23). Notably, complex C_2 does form with this sequence and is present in the glucose extract.

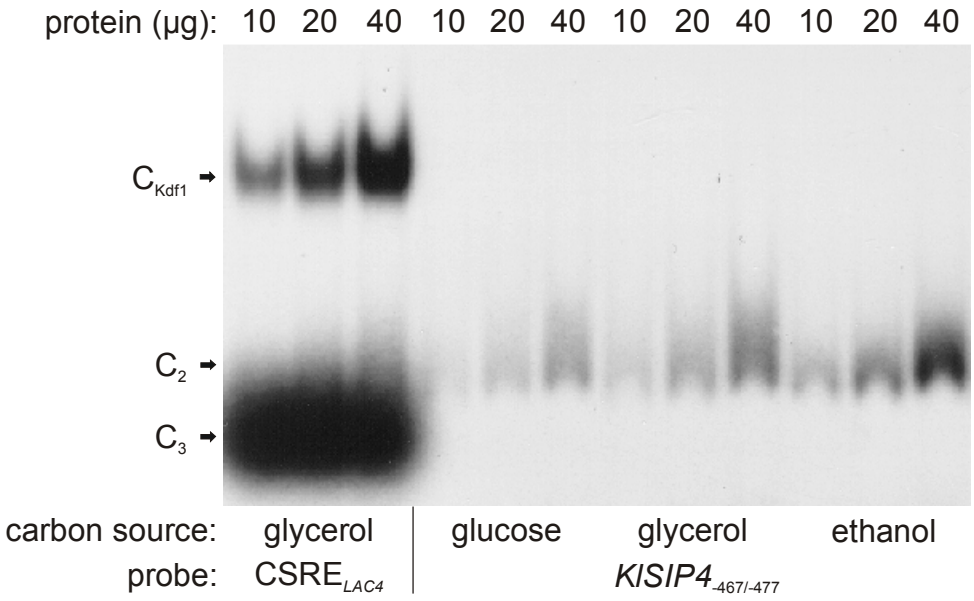

Figure 3-23: *KlSIP4*$_{-467/-477}$ is not bound by Kdf1.
Each sample contained 25 fmoles of ^{32}P-labelled probe (2.5 x 10^4 cpm for oligonucleotide CSRE$_{LAC4}$; 3.5 x 10^4 cpm for oligonucleotide *KlSIP4*$_{-467/-477}$) and 10, 20 or 40 µg of S100 protein from wild-type cells grown in synthetic complete medium with 2% glucose, 3% glycerol or 2% ethanol as sole carbon source.

The conclusion was that the conserved residues AA at positions 7 and 8 of CSRE$_{LAC4}$, as in the *S. cerevisiae* consensus, are essential to give high affinity Kdf1-binding. For this reason *KlSIP4*$_{-137/-127}$ was not further analysed.

3.4.3 Regulation of *KlSIP4* gene expression

The binding of Kdf1 to the *KlSIP4* promoter CSREs 1 and 2 in the gel shift experiment suggested carbon source-regulation (and the possibility of auto-regulation) of transcription of *KlSIP4*. Detection of this regulation was attempted by means of the reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA was isolated from wild-type cells grown in synthetic complete medium with 2% glucose, 3% glycerol or 2% ethanol as sole carbon source. A primer pair against bases +20 to +287 of the *HHT1* gene, encoding histone H3, was used to amplify a 268 basepair fragment as internal control. A *KlSIP4* primer pair was used that amplifies a 737 basepair fragment corresponding to bases +592 to +1328 of *KlSIP4*. The RT-PCR product-abundances (Figure 3-24) indicated an upregulation of *KlSIP4* RNA in ethanol-grown cells compared to glucose but no difference between glycerol- and glucose-grown cells.

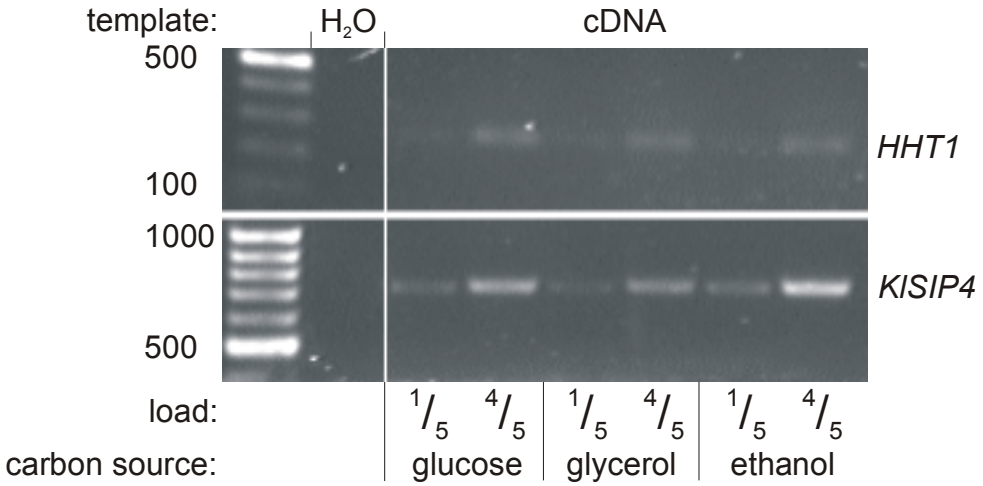

Figure 3-24: Carbon source regulation of *KlSIP4* transcription is weak. First strand cDNA was synthesized using $(dT)_{18}$ as primer on 600 ng of total RNA isolated from wild-type cells grown in synthetic complete medium containing 2% glucose, 3% glycerol or 2% ethanol as sole carbon source. cDNA corresponding to 12 ng of total RNA was subjected to 29 cycles of amplification with a *HHT1*-specific primer set to yield a 268 bp fragment or 26 cycles of amplification with a *KlSIP4*-specific primer set to yield a 737 bp fragment. Samples were separated on a 1.5% TAE agarose gel for 1 hour at 100 V. The gel was stained in TAE with 0.5 µg/ml ethidium bromide. The molecular size marker shows 100 bp increments in the range from 100 to 1000 bps.

3.4.4 Multicopy *KlCAT8* does not suppress the growth defect of the *Klsip4* deletion

Since the *KlSIP4* gene had been isolated by suppression of *Klcat8* a test whether the two known CSRE-binding factors could replace eachother was performed. It was tested whether the multicopy *KlCAT8* plasmid pGID1 complemented the *Klsip4* deletion for growth on ethanol. In parallel a possible influence of multicopy *KlSIP4* on growth, as observed for multicopy *KlCAT8*, was analysed.

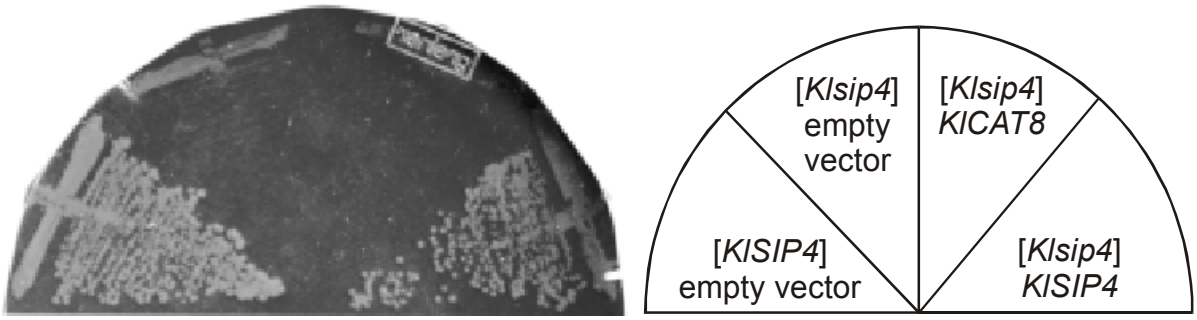

Figure 3-25: *KlCAT8* does not suppresses the *Klsip4* growth defect on ethanol. Strains were streaked on a SC-agar plate + 2% ethanol w.o. uracil as indicated and incubated for 4 days at 30°C.

KlCat8p expressed from multicopy plasmid pGID1 does not suppress the *Klsip4* growth defect on ethanol (Figure 3-25). This result supports the conclusion that *KlCAT8* is an upstream regulator of *KlSIP4*, possibly functioning through the CSREs in its promoter region. Overexpression of KlSip4p restores growth of the *Klsip4* deletion strain to wild-type and seems to have no adverse effect on growth, as was the case for KlCat8p (Georis *et al*, 2000). There is no evidence that *KlCAT8* can replace *KlSIP4*.

3.4.5 The *KlCAT8* promoter contains no carbon source-responsive element

The *KlCAT8* promoter also contained a CSRE-like sequence at positions -101 to -111 (Table 3-2). This potential CSRE contains three mismatches to the $CSRE_{LAC4}$. Two are in positions 4 and 6 where the *S. cerevisiae* consensus allows for any base. The third is an adenosine in position 5, where the *K. lactis* CSREs identified sofar have a thymidine and the *S. cerevisiae* consensus allows a cytidine as well. Kdf1-binding to $KlCAT8_{-101/-111}$ was tested in a gel shift assay (Figure 3-26).

promoter site	position	sequence
$CSRE_{LAC4}$	-1129 / -1112	5′- AGT CGGATGAAAGG GGA –3′
$KlCAT8_{-101/-111}$	-98 / -114	5′- CAC CGG**TAC**AAAGG GAT -3′
consensus *Sc*		5′- CGGNYNAAYGG –3′

Table 3-2: The *KlCAT8* promoter contains a CSRE-like sequence. Shown are the $CSRE_{LAC4}$ and the CSRE-like *KlCAT8* promoter sequence and their positions (**A**TG = +1). The CSRE consensus sequence from *S. cerevisiae* (Roth and Schüller, 2001) is shown for comparison. Underlined: CSRE core, bold: bases differing from $CSRE_{LAC4}$. N = any base, Y =T/C.

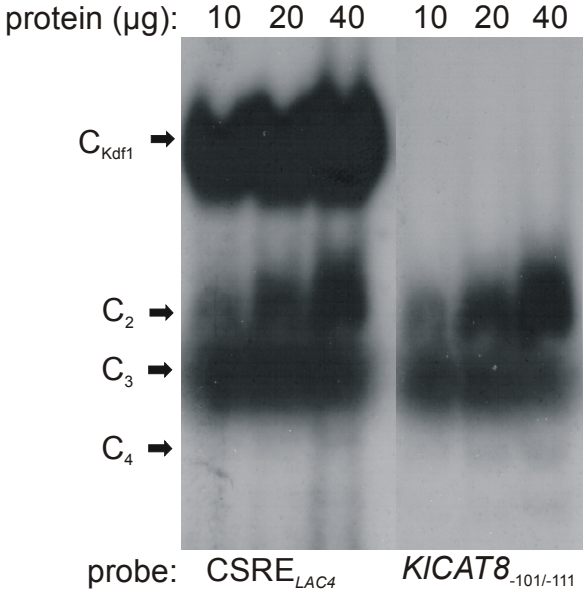

Figure 3-26: The single putative CSRE in the *KlCAT8* promoter shows no Kdf1-binding. Samples contained 10 fmoles of ^{32}P-labelled probe (1.0×10^5 cpm for $CSRE_{LAC4}$, 8.0×10^4 cpm for $KlCAT8_{-101/-111}$) and 10, 20 or 40 µg of S100 protein from wild-type cells grown in synthetic complete medium containing 3% glycerol as sole carbon source.

Despite the minor difference to $CSRE_{LAC4}$ no Kdf1-binding to *KlCAT8*$_{-101/-111}$ was observed. Thus, the conserved pyrimidine at position 5 seems to be crucial. The results indicate that *KlCAT8* like *ScCAT8* is not regulated through a CSRE; in contrast to *KlSIP4*.

3.4.6 The effect of *KlSIP4* on isocitrate lyase

It had already beenshown that in *K. lactis* expression of *ICL1* is induced by ethanol but not by glycerol. The induction on ethanol was *KlCAT8*-dependent as in *S. cerevisiae* (Georis *et al*, 2000). As the deletion of *KlSIP4* had an even stronger effect on carbon utilisation than the *KlCAT8* deletion, it was of interest to know whether isocitrate lyase induction required *KlSIP4*. Therefore isocitrate lyase enzyme activity assays were performed on the wild-type and *Klsip4* strains, grown in synthetic complete medium containing 2% glucose, 3% glycerol or 2% ethanol.

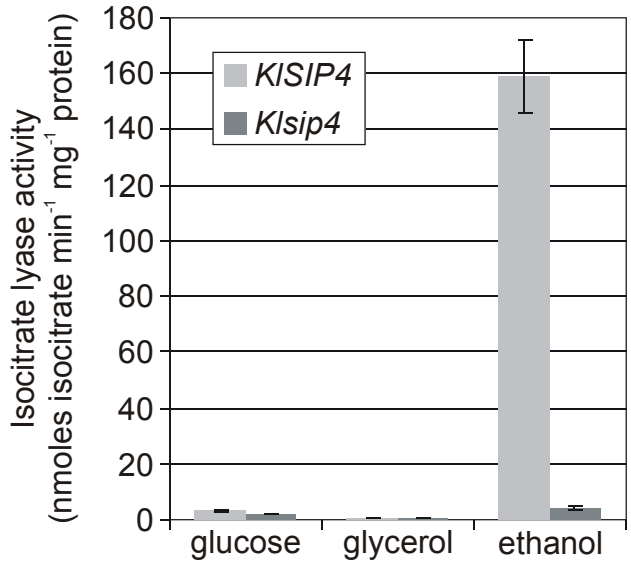

Figure 3-27: Induction of isocitrate lyase on ethanol is *KlSIP4*-dependent. Isocitrate lyase activity was determined in glassbead extracts from wild-type and *Klsip4* cells grown in SC medium with 2% glucose, 3% glycerol or 2% ethanol, to an $OD_{600}$of 0.6 to 1.4. Values are averages with SD from three independent cultures for each condition.

From the graph in Figure 3-27 it is clear that isocitrate lyase is *KlSIP4*-dependent as it is *KlCAT8*-dependent. In the wild-type the level of enzyme activity was at the limit of detection both on glucose and on glycerol and this activity was not influenced by deletion of *KlSIP4*. On ethanol a 50-fold induction compared to glucose was observed in the wild-type whereas the *Klsip4* deletion strain showed only 2-fold induction of isocitrate lyase activity. This was the same as was observed in the *Klcat8* deletion strain and suggests that KlCat8p functions through KlSip4p. The lack of derepression of *KlICL1* alone explains the severe growth defect on ethanol and acetate.

3.4.7 The *KlICL1* promoter contains a low-affinity CSRE

The previous experiment showed that KlSip4p plays an important role in regulation of at least *KlICL1* expression. We searched the known 1417 basepair *KlICL1* promoter region for a potential CSRE. Three sequences show a good fit to the $CSRE_{LAC4}$ (Table 3-3). Of the three promoter sequences only one, located around -800, contains a cytosine in position 1. This cytosine is conserved in all CSREs. *In vitro* this sequence showed binding to Kdf1 and was called $CSRE1_{KlICL1}$ (Figure 3-28).

site	position	sequence
$CSRE_{LAC4}$	-1129 / -1112	5′- AGT CGGATGAAAGG GGA –3′
KlICL1$_{-287/-297}$	-284 / -300	5′- TCA **T**GG**T**T**A**AA**T**GG ATG -3′
KlICL1$_{-570/-560}$	-573 / -557	5′- TCA **G**GGAT**C**AA**T**GG ACG -3′
$CSRE1_{KlICL1}$	-796 / -812	5′- ATT CGG**T**T**T**AA**T**GG CGG -3′
consensus *Sc*		5′- CGGNYNAAYGG –3′

Table 3-3: Three *KlICL1* promoter sequences show similarity to the $CSRE_{LAC4}$.
Shown are the $CSRE_{LAC4}$ and three *KlICL1* promoter sequences and their positions (**A**TG = +1). The CSRE consensus sequence from *S. cerevisiae* (Roth and Schüller, 2001) is shown for comparison. Underlined: CSRE core, bold: bases differing from $CSRE_{LAC4}$. N = any base, Y =T/C.

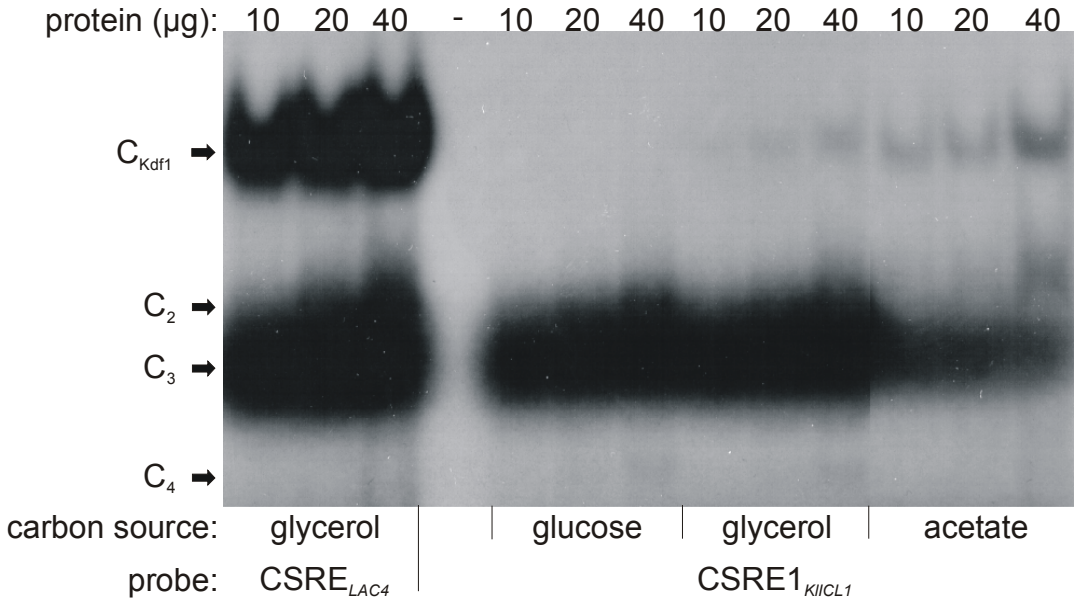

Figure 3-28: Kdf1 binds to the $CSRE1_{KlICL1}$.
Samples contained 10 fmoles of ^{32}P-labelled probe (1.0 x 10^5 cpm for $CSRE_{LAC4}$, 8.0 x 10^4 cpm for $CSRE1_{KlICL1}$) and 10, 20 or 40 µg of S100 protein from wild-type cells grown in synthetic complete medium containing 2% glucose, 3% glycerol or 2% sodium acetate as sole carbon source.

Kdf1 binding to the CSRE1$_{KlICL1}$ is very much weaker than the binding observed with the CSRE$_{LAC4}$. It shows the same carbon source-dependence. Complex C_3 is weaker on acetate than on glycerol as was the case for CSRE$_{LAC4}$, CSRE1$_{KlSIP4}$ and CSRE2$_{KlSIP4}$ (Figure 3-21). Complex C_4, is present in the extracts from glucose- and glycerol-grown cells, but not on acetate.

With sequences *KlICL1*$_{-287/-297}$ and *KlICL1*$_{-570/-560}$ no Kdf1-binding could be observed despite the strong overexposure of the autoradiogram, which even reveals an extremely weak binding of Kdf1 to the CSRE$_{LAC4}$ in extract from glucose-grown cells (Figure 3-29). Complexes C_2, C_3 and C_4 do appear however.

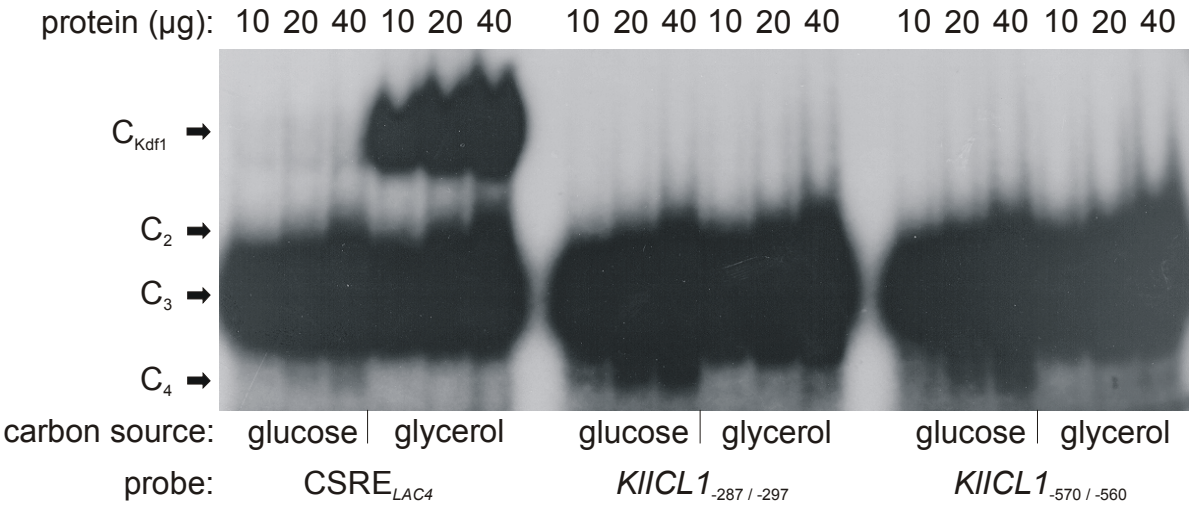

Figure 3-29: Kdf1 does not bind to the CSRE-like sequences -287 / -297 and -570 / -560 of *KlICL1*.
Samples contained 10 fmoles of ^{32}P-labelled oligonucleotide (7.5 x 10^4 cpm for CSRE$_{LAC4}$, *KlICL1*$_{-287 / -297}$ and *KlICL1*$_{-570 / -560}$ each) and 10, 20 or 40 µg of S100 extract from wild-type cells grown in synthetic complete medium containing 2% glucose or 3% glycerol as sole carbon source.

3.4.8 The effect of *KlSIP4* on malate synthase

K. lactis malate synthase enzyme activity was increased about 2-fold in glycerol-grown cells and 8-fold in ethanol-grown cells when compared to glucose-grown cells (Georis *et al*, 2000). The induction on ethanol is KlCat8p-dependent, for the *Klcat8* deletion mutant showed an only 3-fold increase in enzyme activity compared to glucose as sole carbon source. The activity in glycerol-grown cells was not affected in the mutant. The influence of deletion of *KlSIP4* on the expression of malate synthase was analysed. No *K. lactis* gene encoding malate synthase has been cloned to date but in the course of the french Génolevures project, the genomic

exploration of Hemiascomycetous yeasts (Feldmann, 2000), four expressed sequence tags from *K. lactis* were sequenced that show high degrees of identity to segments of the 1665 basepair *ScMLS1* and *ScDAL7* genes, both encoding malate synthase (Bolotin-Fukuhara *et al*, 2000). An 810 nucleotide segment from one EST, EMBL accession number AL427874, shows 72.0% identity to *ScMLS1* nucleotides 1-810 and 70.6% identity to *ScDAL7* nucleotides 1-810. The 270 residue translation of the sequence shows 79.3% identity to the corresponding sequence of ScMls1p and 76.7% identity to ScDal7p. A 486 nucleotide sequence shared by ESTs AL429931 and AL429748 shows 67.1% and 65.4% identity to *ScMLS1* and *ScDAL7* nucleotides 1209-1665 respectively. The 161 residue translation of the segment shows 73.3% and 68.3% identity to ScMls1p and ScDal7p residues 394-554 respectively. A 457 nucleotide sequence from the fourth EST, AL426607, is identical to the last 457 nucleotides from the two identical sequences mentioned above. From the sequence homologous to the 5'-end of *ScMLS1*/*ScDAL7* two oligonucleotides were designed for RT-PCR and regulation of *KlMLS1* mRNA was analysed. The preparation of total RNA, reverse transcription and subsequent PCR on cDNA was performed as described for *KlSIP4*.

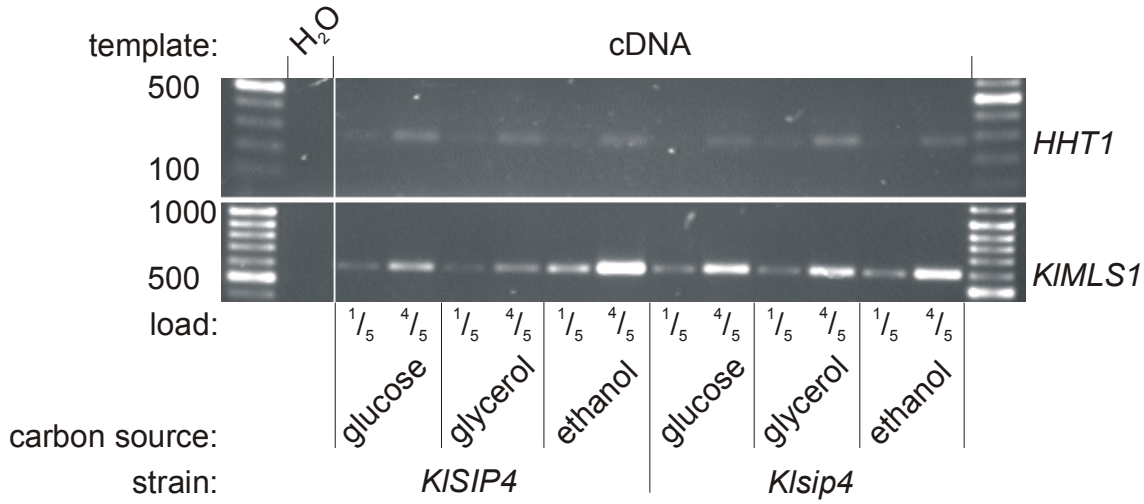

Figure 3-30: Induction of transcription of *KlMLS1* on ethanol is decreased in a *Klsip4* deletion strain.
First strand cDNA was synthesized using $(dT)_{18}$ as primer on 600 ng of total RNA isolated from wild-type or *Klsip4* cells grown in synthetic complete medium containing 2% glucose, 3% glycerol or 2% ethanol as sole carbon source. cDNA corresponding to 12 ng of total RNA was subjected to 29 cycles of amplification with a *HHT1*-specific primer set to yield a 268 bp fragment or 26 cycles of amplification with a *KlMLS1*-specific primer set to yield a 601 bp fragment. Samples were separated on a 1.5% TAE agarose gel for 1 hour at 100 V. The gel was stained in TAE with 0.5 µg/ml ethidium bromide. The molecular size marker shows 100 bp increments in the range from 100 to 1000 bps.

The *KlMLS1* primer set yielded a single band of the expected size (Figure 3-30). On RNA from wild-type cells grown on ethanol the abundance of product was clearly elevated over glucose or glycerol, indicating increased transcription on this carbon source. In the *Klsip4* deletion strain this increase does not occur, confirming that KlSip4p is required for activation of *KlMLS1* transcription on ethanol, as is KlCat8p. Quantification of the *KlMLS1* bands normalized against the corresponding *HHT1* signals showed no difference between glucose- and glycerol-grown wild-type cells and a 4.5-fold higher intensity in ethanol-grown cells. In the *Klsip4* mutant signals for glucose- and ethanol-grown cells were equally strong whereas the glycerol signal was only half as strong. Interestingly expression of *KlMLS1* on glucose appeared to be 4-fold stronger in the *Klsip4* mutant than in the wild-type. These quantitations should be regarded with great caution but overall they confirm what was expected from the analysis of the *Klcat8* mutant.

3.4.9 Acetyl-CoA synthetase is not regulated through a CSRE

In *K. lactis* two genes, *KlACS1* and *KlACS2*, encode acetyl-CoA synthetases (Zeeman *et al*, 2000). The enzymes catalyse the ATP-dependent linkage of coenzyme A to acetate to form acetyl-CoA. *S. cerevisiae* has three *ACS* genes. The transcription of only one of these genes, *ACS1* encoding the cytosolic isozyme, is activated by ScCat8p through a CSRE. The promoters of *KlACS1* and *KlACS2* were searched for putative CSRE sequences.

promoter site	position	sequence
$CSRE_{LAC4}$	-1129 / -1112	5′- AGT <u>CGGATGAAAGG</u> GGA –3′
$KlACS1_{-766/-776}$	-763 / -779	5′- GTG <u>CGG**T**TG**TG**AGG</u> GGA -3′
consensus *Sc*		5′- CGGNYNAAYGG –3′

Table 3-4: The *KlACS1* promoter contains one CSRE-like sequence. Shown are $CSRE_{LAC4}$, the putative $CSRE_{KlACS1}$ and their positions (**A**TG = +1). The *S. cerevisiae* CSRE consensus sequence (Roth and Schüller, 2001) is shown for comparison. Underlined: CSRE core, bold: bases differing from $CSRE_{LAC4}$. N = any base, Y =T/C.

In the *KlACS1* promoter one CSRE-like sequence was found (Table 4-4) whereas none could be identified in the *KlACS2* promoter. Positions 7 and 8 of the sequence differ from $CSRE_{LAC4}$, however, and more resemble the sequences $KlSIP4_{-137/-127}$ and $KlSIP4_{-467/-477}$. Kdf1 did not bind to $KlACS1_{-766/-776}$ in the gel shift assay, showing that it is no CSRE. Complex C_2 however did form as with $KlSIP4_{-467/-477}$.

3.5 Galactose repression of Kdf1-CSRE$_{LAC4}$ binding

Strong efforts have been made so far to elucidate the signalling cascades involved in glucose sensing and repression. Little is known however about repression by other sugars or hierarchy in carbon source quality.

3.5.1 The influence of galactose on Kdf1-binding to the CSRE$_{lac4}$

Since binding of Kdf1 to the CSRE$_{LAC4}$ is absent in glucose-repressed cells and present in cells grown on poor carbon sources like glycerol, Kdf1-binding to the CSRE$_{LAC4}$ in cells grown on galactose was addressed. Extracts from wild-type cells grown on glucose, galactose or glycerol were compared in the electrophoretic mobility shift assay.

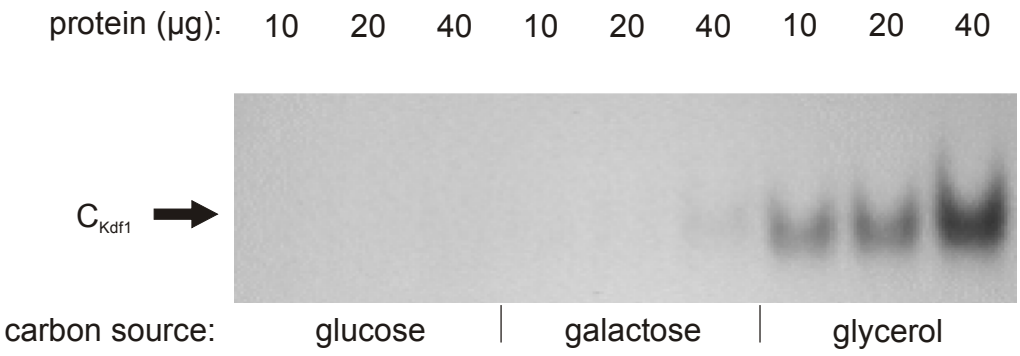

Figure 3-31: Galactose represses Kdf1-binding to the CSRE$_{LAC4}$. Samples contained 10 fmoles of ^{32}P-labelled CSRE$_{LAC4}$ oligonucleotide (7.4 x 10^4 cpm) and 10, 20 or 40 µg of S100 protein from wild-type cells grown in synthetic complete medium containing 2% glucose, 2% galactose or 3% glycerol as carbon source.

Figure 3-31 shows a faint band with 40 µg of extract from galactose-grown cells, in contrast to glucose-grown cells. Kdf1-binding in glycerol-grown cells is much stronger. This means that, although the repression by galactose is less tight than by glucose, both sugars prevent Kdf1 from binding to the CSRE$_{LAC4}$. Weak CSRE$_{LAC4}$-binding of Kdf1 similar to that in galactose is seen in cells grown on 2% fructose (results not shown).

3.5.2 The role of galactokinase KlGal1p in regulation of CSRE$_{LAC4}$-binding of Kdf1

The previous experiment indicated that Kdf1-binding is generally repressed by sugars. As the regulatory circuit that regulates galactose induction is established but no repression by this suger was known, the effect of galactose on Kdf1-CSRE$_{LAC4}$ binding was studied. In *K. lactis KlGAL1* encodes the single, bifunctional galactokinase (Meyer *et al*, 1991). Its first role is the enzymatic function catalyzing phosphorylation of galactose to galactose-1-phosphate. The second role of KlGal1p is to interact with inhibitor KlGal80p in a galactose- and ATP- dependent manner (Zenke *et al*, 1996), thereby releaving from inhibition the transcriptional activator KlGal4p which in turn then activates transcription of the *GAL*/*LAC* genes required for growth on lactose or galactose. Since Kdf1 did not bind to the CSRE$_{LAC4}$ in galactose-grown cells and KlGal1p plays a central role in galactose induction, the requirement for galactokinase for regulation of Kdf1-binding was analysed. Kdf1-binding in extracts from wild-type and *Klgal1* strains was compared in a gel shift assay (Figure 3-32). The 198 basepair promoter fragment was used as probe. The cultures were grown in 3% glycerol or 3% glycerol + 2% galactose since the *Klgal1* strain does not grow on galactose or lactose as sole carbon sources (Meyer *et al*, 1991).

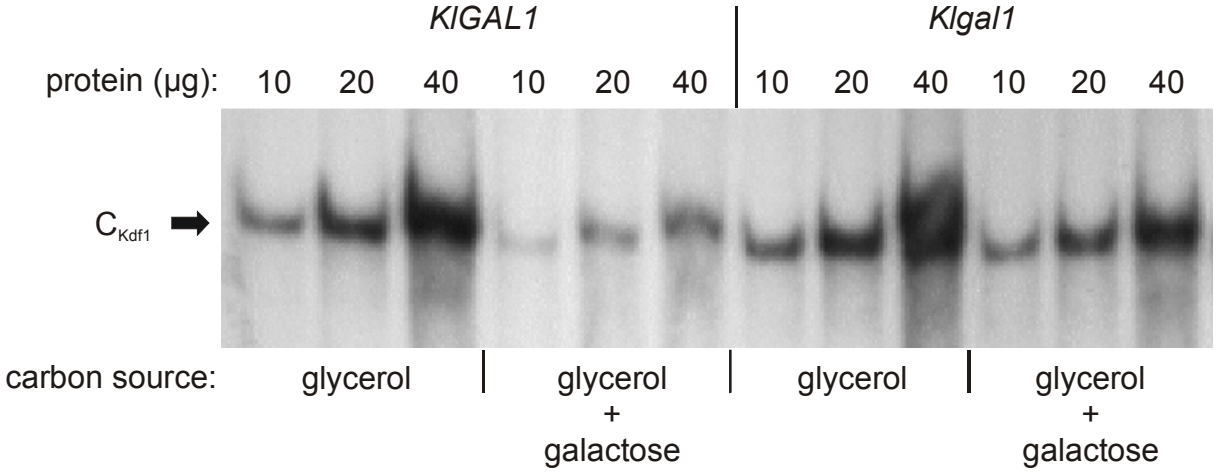

Figure 3-32: Influence of KlGal1p on Kdf1-binding to the CSRE$_{LAC4}$.
Wild-type and *Klgal1* strains were grown in synthetic complete medium containing 3% glycerol or 3% glycerol + 2% galactose. Samples contained 5 fmoles of ^{32}P-labelled f198 fragment (3.0 x 10^4 cpm) and 10, 20 or 40 µg of S100 extract.

The deletion of *Klgal1* had no influence on Kdf1-binding in glycerol-grown cells but in the presence of galactose binding was stronger in the deletion strain than in the wild-type, indicating that galactokinase is involved in the signaling that leads to repression of Kdf1-binding.

3.5.3 Influence of *Klgal1* on regulation of Kdf1-binding in a *Klgal80* background

A complication in the experiment descibed before is the fact that in absence of Gal1p activation of the *GAL*/*LAC* regulon does no longer take place. This problem can be overcome by inactivation through deletion or mutation of Gal80p, leading to constitutive transcription of the *GAL*/*LAC* genes. The effect of the *Klgal80-31* mutation on Kdf1-binding to the $CSRE_{LAC4}$ was tested. This particular mutation prohibits Gal80p-Gal4p interaction and thereby inhibition, but does not affect Gal80p-Gal1p interaction (Zenke *et al*, 1999). As shown in Figure 5-3 the mutation had no qualitative effect on Kdf1-$CSRE_{LAC4}$ complex formation on glycerol or glycerol + galactose. The effect of a *Klgal1* mutation on this regulation was again determined, now in the *Klgal80-31* background. For this experiment not the *Klgal1* deletion strain was used but a strain carrying, in addition to the *Klgal80-31* allele, the *Klgal1-209* allele. This allele encodes a galactokinase that has no kinase activity, due to a point mutation that only affects the kinase function of KlGal1p. The mutant protein is still capable of performing its regulatory function (Riley and Dickson, 1984). Since this requires interaction with KlGal80p it indicates that the mutation does not affect the structural integrity of the protein. Binding of Kdf1 to the $CSRE_{LAC4}$ was compared in a gel shift essay for wild-type, *Klgal80-31* and *Klgal80-31 Klgal1-209* strains, using the radiolabelled $CSRE_{LAC4}$ oligonucleotide as probe (Figure 3-33).

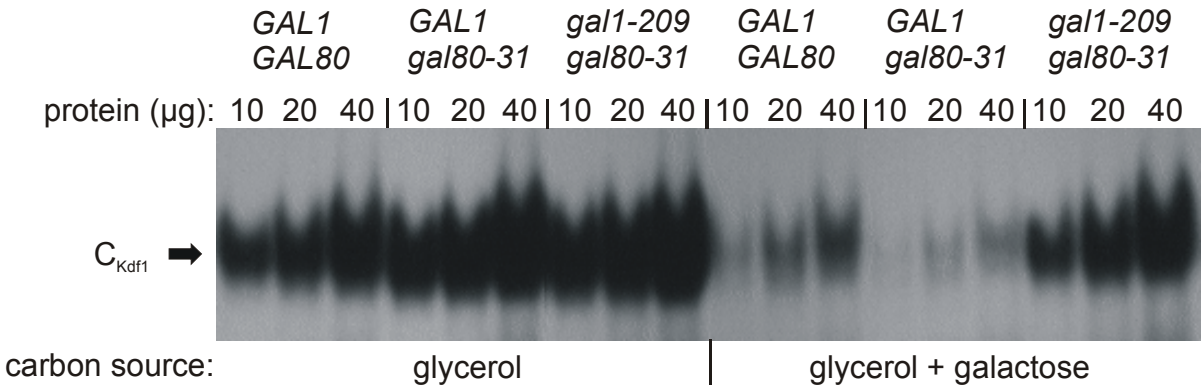

Figure 3-33: Loss of galactokinase activity abolishes galactose repression of Kdf1-binding to the $CSRE_{LAC4}$.
Samples contained 10 fmoles of ^{32}P-labelled $CSRE_{LAC4}$ oligonucleotide (7.4 x 10^4 cpm) and 10, 20 or 40 µg of S100 protein extracted from wild-type, *Klgal80-31* or *Klgal1-209 Klgal80-31* cells grown in synthetic complete medium with 3% glycerol or 3% glycerol + 2% galactose as carbon source.

In the double mutant grown on glycerol + galactose binding of Kdf1 to the $CSRE_{LAC4}$ was about as strong as in either strain grown on glycerol only. This result indicates

that galactokinase enzymatic activity is required for repression of Kdf1-binding. Its mutation leads to complete loss of regulation. No major differences in strength of the Kdf1-CSRE$_{LAC4}$ complex could be seen between strains grown on glycerol.

The previous experiment showed that galactokinase KlGal1p is required for down-regulation by galactose of Kdf1-binding to the CSRE$_{LAC4}$. The fact that the mutation in KlGal1-209p affects its galactokinase function suggests that galactose-phosphorylation is the signal for wild-type regulation, although a different regulatory function of KlGal1p cannot be excluded. To test the importance of galactose phosphorylation the *Klgal1-209 Klgal80-31* strain was transformed with pEAGK1; a multicopy vector expressing the *EcgalK* gene, encoding *E. coli* galactokinase, from the constitutive *ScADH1* promoter. GalK suppresses *gal1* deletions in *Saccharomyces cerevisiae* (Bhat *et al*, 1990) but not in *K. lactis* (Meyer *et al*, 1991). The transformant strain was first tested for growth phenotype and β-galactosidase activity in plate assays (Figure 3-34).

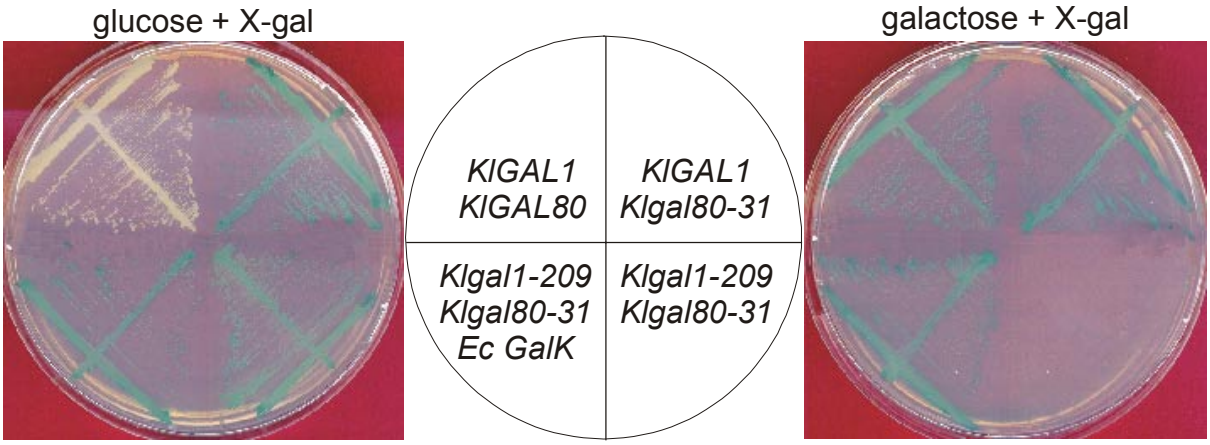

Figure 3-34: *E. coli* galactokinase complements a *Klgal1* mutation.
For testing growth phenotype and β-galactosidase activity, strains were streaked on SC medium containing 2% glucose or 2% galactose and 40 µg/ml X-gal.

All strains grew well on glucose (Figure 3-34 left) since growth on this carbon source is not affected by mutations in the *GAL*/*LAC* regulon. The *Klgal80-31* mutation leads to constitutive activation of the regulon since it cannot inhibit KlGal4p activity. This can be seen in the form of a blue coloration of the colonies due to degradation of X-gal by the *LAC4* gene product β-galactosidase (Figure 3-34 left). On galactose wild-type and *Klgal80-31* strains grew and β-galactosidase was expressed in the wild-type as well. The *Klgal1-209 Klgal80-31* double mutant did not grow since metabolism is blocked at the step of galactose phosphorylation (Figure 3-34 right). Expression of *E. coli* galactokinase in the double mutant allowed it to grow on galactose again. Thus

GalK functionally complements the inability of KlGal1-209p to phosphorylate galactose.

3.5.4 Influence of galactose phosphorylation on regulation of Kdf1-binding

The plate test showed that GalK restores galactose phosphorylation and thus growth in the *Klgal1-209* mutant strain. The next question was whether this also restores regulation of Kdf1-binding on galactose medium. Therefore cultures from wild-type and *Klgal1 Klgal80* strains and the *Klgal1 Klgal80 EcgalK* transformant were grown in synthetic complete medium containing 3% glycerol and 2% galactose to allow direct comparison. A wild-type culture grown on 3% glycerol served as a control.

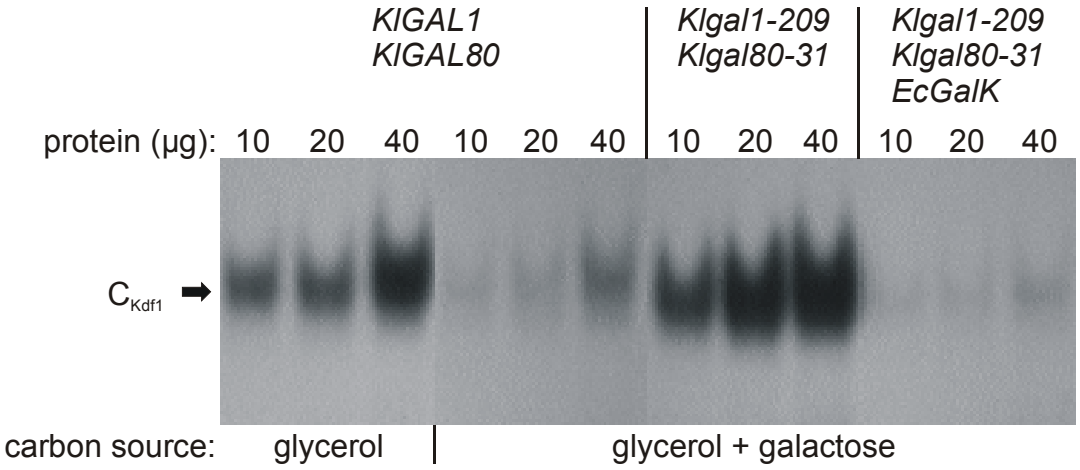

Figure 3-35: Galactose phosphorylation is required for galactose repression of Kdf1-binding to the CSRE$_{LAC4}$.
10, 20 or 40 µg of S100 extract from the wild-type strain grown on glycerol (lanes 1, 2 and 3) or wild-type (lanes 4 - 6), *Klgal1-209 Klgal80-31* (lanes 5 - 9) or *Klgal1-209 Klgal80-31* expressing *E. coli* galactokinase (lanes 10 - 12), grown in SC medium with 3% glycerol + 2% galactose were incubated with 10 fmoles of ^{32}P-labelled CSRE$_{LAC4}$ oligonucleotide (7.4 x 10^4 cpm).

As Figure 3-35 shows *E. coli* galactokinase not only suppressed the growth defect of the *Klgal1-209* mutant but also restored repression of Kdf1-binding. Binding of Kdf1 to the CSRE$_{LAC4}$ was still present but reduced to wild-type level or even below. This strongly suggests that phosphorylation of galactose is the function of KlGal1p that is required and sufficient for regulation of Kdf1.

4 Discussion

4.1 The *LAC4* Carbon Source-Responsive Element

For many years the *S. cerevisiae GAL*/*MEL* and *K. lactis GAL*/*LAC* regulons have been studied to clarify the regulatory mechanisms of glucose repression (reviewed in Gancedo, 1998; Carlson, 1999; Breunig *et al*, 2000) and galactose induction (reviewed in Breunig, 2000; Bhat and Murthy, 2001) that govern their expression. It was found that expression of the *K. lactis LAC4* gene, encoding β-galactosidase, was significantly increased by poor carbon sources. Moreover, this activation by poor carbon sources was KlGal4p-independent and a central region of the divergent *LAC12-LAC4* promoter was required that did not influence galactose induction. In this Basal Control Region two regions were assigned, A and B. A Carbon Source-Responsive Element, $CSRE_{LAC4}$, was identified in Region A that formed a complex *in vitro* with *Kluyveromyces* derepression factor 1. The complex only formed on derepressing carbon sources. The $CSRE_{LAC4}$ was protected from DNAse I-digestion by Kdf1. Binding of Kdf1 to promoter fragments or oligonucleotides was dependent on this sequence (Schmidt, PhD thesis 1996). Finally, a $CSRE_{LAC4}$ oligonucleotide efficiently competed with Region A for binding of Kdf1.

An important issue is the role of the $CSRE_{LAC4}$ *per se* and in the context of the BCR in basal expression of *LAC4*. Deletion of the complete BCR (-1066 to -1522) led to complete loss of *LAC4* basal expression (Figure 4-1, ΔBCR (Reg. A+B)). Deletion of Region B only showed no effect on basal expression, which implied that this region is dispensable for BCR function (Figure 4-1, ΔReg. B). Deletion of the Region A (-1066 to -1190), however, reduced basal expression by only 40% (Figure 4-1, ΔReg. A; Schmidt, PhD thesis 1996). This indicates that Region B does influence basal transcription in the absence of Region A. Similar complex effects are seen for changes within Region A itself. Substitution of the BCR by the $CSRE_{LAC4}$ alone completely reversed the loss of *LAC4* basal expression caused by the BCR deletion. It confirmed the importance of this sequence for basal expression. Deletion of the $CSRE_{LAC4}$ in the otherwise wild-type context, on the other hand, led to only 40% and 60% loss of basal activity on glycerol and ethanol, respectively (Figure 4-1, mut. CSRE). Thus, sequences in the BCR region or spacers can partially substitute for the absence of $CSRE_{LAC4}$.

The $CSRE_{LAC4}$ is flanked by a sequence identical to the sequence bound by transcriptional activator Adr1p in *S. cerevisiae*: 5'-ATTGGAGAG-3'. Additionally this sequence contains a CCAAT-box, which is the target of the Hap2/3/4/5 complex

(Ramil *et al*, 2000). A bacterially expressed GST-Adr1 fusion protein, consisting of glutathion-S-transferase fused to the DNA binding domain plus one activation domain of Adr1, bound to the BCR Region A *in vitro* (Schmidt, PhD thesis 1996). Binding was dependent on the Adr1 binding site and mutation of the Adr1 binding site, ATTGG to GCTCG (MC mutation), interfered with binding. *In vivo* the Adr1 binding site mutation led to ~45% and ~30% reduction in β-galactosidase activity on glycerol (Figure 4-1, mut. Adr1 cons.) and lactate, respectively. LacZ-activity in a promoter test construct on lactate was lost (Schmidt, PhD thesis 1996). On ethanol, on the other hand, this mutation had no effect on β-galactosidase activity (Figure 4-1) and did not reduce Region A-driven Lac*Z*-activity in the promoter test construct. Therefore it was carefully tested whether the Adr1 site had an influence on protein-complex formed with the $CSRE_{LAC4}$. In the comparison of Kdf1-binding to two oligonucleotide sequences, with and without Adr1 site, no differences in number, size or abundance of the complexes were detected.

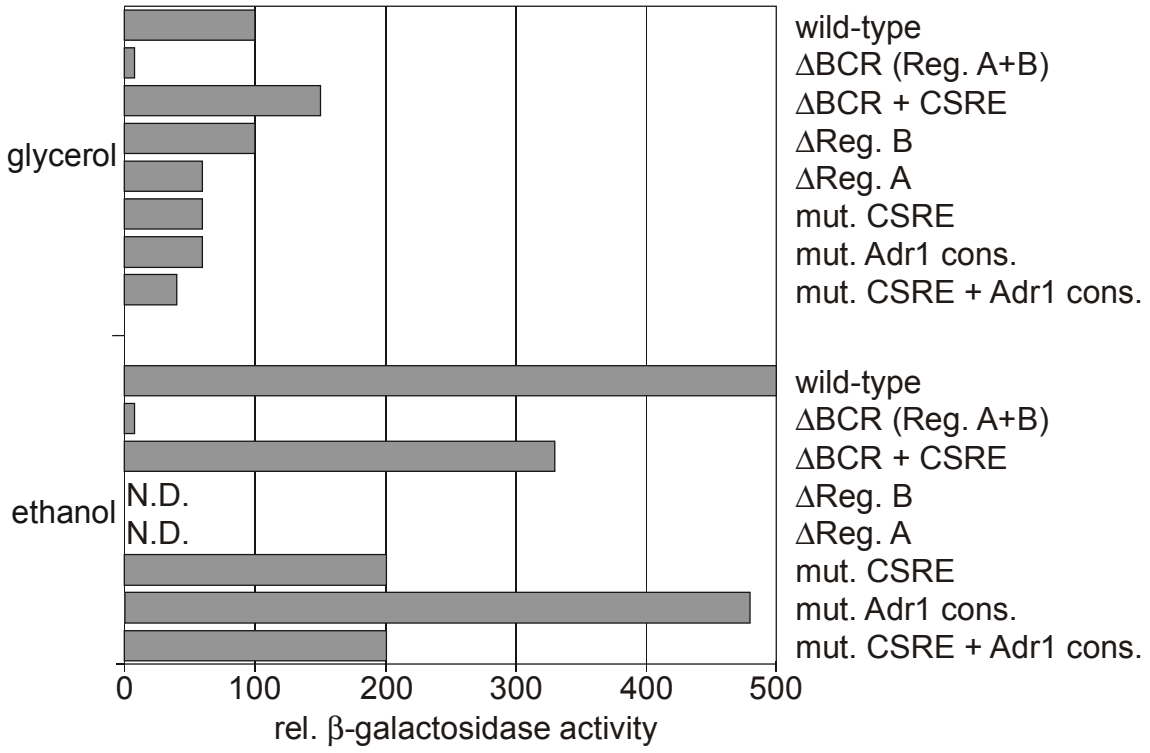

Figure 4-1: Qualitative comparison of the effects of different mutations in the *LAC4-LAC12* promoter Basal Control Region on β-galactosidase activity.
β-galactose activity data from Schmidt (thesis, 1996) and this work were combined and represented in comparison to the wild-type (JA6) on glycerol (strain names in parentheses). ΔBCR (Reg. A+B) = Δ -1066 to -1522 (JA6/LR2); ΔBCR + CSRE = $CSRE_{LAC4}$ integrated in BCR deletion (JA6/LR2K); ΔReg. B = Δ -1190 to -1522 (JA6/IC); ΔReg. A = Δ -1066 to -1190 (JA6/D123); mut. CSRE = -1113 to -1125 > GT (JA6/DO); mut. Adr1 cons. = -1098 C > G, -1100 A > G, -1101 T > C (JA6/MC); mut. CSRE + Adr1 cons. = combination of the $CSRE_{LAC4}$ and Adr1 mutations (JA6/MCDO).

Still, involvement of a *K. lactis* Adr1p homolog, Hap2/3/4/5 or a yet unknown factor (that would only play a role when cells are grown on C3 carbon sources) might explain the observed effects of the MC mutation. An additional complex may form *in vivo*, either independently or in concert with Kdf1, that is degraded during extract preparation or that does not bind under the experimental conditions. The fact that the triple basepair change in the potential Adr1p binding site led to 40% loss of basal *LAC4* expression on glycerol (Figure 4-1, mut. Adr1 cons.) indicates that a *K. lactis* Adr1 homologue may serve this function. Combination of the $CSRE_{LAC4}$ deletion and the Adr1p binding site mutation further decreased expression by 20% on glycerol (Figure 4-1, mut. CSRE + Adr1 cons.) but on ethanol had no stronger effect than $CSRE_{LAC4}$ deletion alone. However, combination did not lower β-galactosidase activity to the level of the BCR deletion strain, suggesting that the situation is still more complex. Factors involved in Adr1 site-mediated activation might be identified as genomic library clones that increase *LAC4* expression driven by the wild-type but not the mutated sequence.

The *S. cerevisiae ACS1* (Kratzer and Schüller, 1997) and *ADH2* (Walther and Schüller, 2001) genes were shown to be coregulated by Adr1p and Cat8p. In the case of *ACS1*, were the $UAS1_{Adr1}$ and the $CSRE_{ACS1}$ are 200 bp apart, *ADR1* and *ScCAT8* function additively. Deletion of either gene or binding site reduced activation by 40%. In the *ADH2* promoter on the other hand $UAS1_{Adr1}$ and $CSRE_{ADH2}$ are separated by only 3 bp and Adr1p and ScCat8p show a strong synergy in activation. Deletion of either *ADR1* or *ScCAT8* reduced activation of *ADH2* by 85%. In the *LAC4-LAC12* promoter of *K. lactis* the $CSRE_{LAC4}$ and putative UAS_{Adr1} are separated by 13 bp, similar to *ScADH2*, but the activation they mediate is additive, as for *ScACS1*. Furthermore, the *K. lactis* factor that may act through the putative UAS_{Adr1} appears to do so on glycerol but not ethanol, in contrast to *S. cerevisiae* Adr1p, which is active under all derepressing conditions. A difference in ethanol compared to the other derepressing carbon sources was also observed when the $CSRE_{LAC4}$ replaced the entire BCR.

The $CSRE_{LAC4}$ was the only sequence that showed protein-binding and this binding is carbon source dependent. The Kdf1-$CSRE_{LAC4}$ complex was detected in the presence of poor carbon sources glycerol, ethanol and acetate both in rich and synthetic complete medium. Generally complexes in extracts from cells grown in rich medium were stronger than in extracts from synthetic complete medium-grown cells, but there was a difference in complex abundance in the gel shift assay in extracts from ethanol-grown cells that depended on the medium used and the preparation tested. In extracts from cells grown in rich medium the Kdf1 complex was at least as

abundant on ethanol as on glycerol. In SC medium the Kdf1 complex was much weaker when cells were grown in ethanol in comparison to glycerol. This effect may have two explanations. One is that rich medium contains a compound that induces Kdf1-binding to the $CSRE_{LAC4}$. The second explanation is that in extracts from cells grown in synthetic complete medium with ethanol, Kdf1 or protein in general is less stable than in extracts from either rich medium with ethanol or synthetic complete medium with any other carbon source used in this work. Repeated experiments with different extract preparations strongly suggest that the latter is the most important reason for the observed difference.

In glucose-grown cells or upon addition of glucose to a glycerol-grown culture, Kdf1-binding to the $CSRE_{LAC4}$ is lost. This glucose repression effect could be exerted directly at the level of the Kdf1 protein through regulation of binding activity or indirectly through regulation of synthesis of Kdf1. Post-transcriptional regulation leads to functional changes in seconds to minutes while regulation of transcription rather takes in the range of minutes to hours to exert effects at the level of protein function. The time course of Kdf1-$CSRE_{LAC4}$ complex-loss upon addition of glucose, which showed a half-life of 36 minutes for the complex, argued for the second mechanism.

4.2 Role of *KlCAT8* in growth on poor carbon sources

In an attempt to identify the $CSRE_{LAC4}$-binding factor, two homologues of genes that encode CSRE-binding factors in *S. cerevisiae* were identified. The *K. lactis CAT8* gene was identified as a multicopy suppressor of the growth defect of a *fog2* (*Klsnf1*) strain on ethanol (Georis *et al*, 2000). The screen that was performed was based on the capability of *CAT8* to suppress the ethanol growth defect of a *snf1* mutation in *S. cerevisiae* (Hedges *et al*, 1995). Snf1p is a protein kinase that is required for derepression of glucose-repressed genes in *S. cerevisiae* (Carlson *et al*, 1981; Celenza and Carlson, 1986). The *K. lactis FOG2* gene was identified in a mutant screen for inability to grow on a number of fermentable and all poor carbon sources (Goffrini *et al*, 1996). Its homology to *SNF1* was shown by its sequence and its ability to complement a *snf1* mutation. The *KlCAT8* gene encodes a 1445 residue protein with 40% identity to ScCat8p and contains an N-terminal $Zn(II)_2Cys_6$ zinc cluster DNA binding domain and coiled-coil dimerization domain, a Middle Homology Region consensus (Poch, 1997) in the center and a C-terminal asparagine-rich sequence. Functional homology was shown by complementation of the growth phenotypes on glycerol and ethanol of a *Sccat8* deletion by *KlCAT8* (Georis *et al*, 2000).

A *Klcat8* disruption strain was created by deletion of a 3.3 kb internal fragment encompassing the zinc cluster, coiled-coil and Middle Homology Region. The *Klcat8* mutant grew only poorly on ethanol or acetate, but surprisingly growth on glycerol was unaffected, whereas in *S. cerevisiae* a *cat8* deletion affected growth on glycerol as well (Hedges *et al*, 1995). Gluconeogenesis from glycerol requires only expression of *FBP1*. A difference in regulation of gluconeogenesis through *FBP1* alone or both *FBP1* and *PCK1* could explain the differentiation between glycerol and other poor carbon sources by *K. lactis*. Indeed we could show that expression of *FBP1* and *PCK1* is *KlCAT8*-independent whereas isocitrate lyase and (to a lesser extent) malate synthase are *CAT8*-dependent as in *S. cerevisiae* (Georis *et al*, 2000).

Kdf1-CSRE$_{LAC4}$ complex formation was strongly reduced in the *Klcat8* mutant on glycerol in synthetic complete medium. In rich medium the reduction was slightly less, but clearly observed with both glycerol or acetate as sole carbon source. However, the residual complex observed in the mutant indicated that KlCat8p is most likely not itself the CSRE$_{LAC4}$-binding protein. The inability to produce a supershifted Kdf1 complex in a strain expressing a Haemagglutinin-tagged KlCat8p strengthened this notion. In a *Sccat8* mutant complete loss of complex formation with CSRE$_{ICL1}$ (Rahner *et al*, 1996) or CSRE UAS1$_{MLS1}$ (Caspary *et al*, 1997) was observed. Moreover, by use of deregulated ScCat8p variants ScCat8p-CSRE$_{ICL1}$ complex formation could be demonstrated *in vitro* (Rahner *et al*, 1999). A clear supershift of a FLAG-tagged deregulated ScCat8p variant (Rahner *et al*, 1999) was seen with CSRE3$_{MDH2}$ from the cytosolic malate dehydrogenase gene *MDH2* (Roth and Schüller, 2001). Thus, the DNA-binding proteins forming complexes on CSRE elements in *K. lactis* and *S. cerevisiae* are not necessarily the same.

Size variants of *KlCAT8* were created by deletion of 3' sequences resulting in carboxy-terminal truncations of 171, 772 and 1138 amino acids. KlCat8 1-1275p did not lack any identified features whereas KlCat8 1-716p lacked the asparagine-rich region located around position 1110 and the regions VII and VIII of the Middle Homology Region. KlCat8 1-305p finally retained only the zinc cluster and adjacent coiled-coil domains, resulting in a protein that should theoretically still be capable of dimerization and DNA-binding. A deregulated truncated ScCat8p variant of similar size as KlCat8 1-305p produced a complex with increased mobility with CSRE$_{ICL1}$ (Rahner *et al*, 1999). None of the KlCat8p size variants complemented the *Klcat8* mutation nor increased Kdf1-CSRE$_{LAC4}$ complex formation. The residual complex observed with the size variants was unaltered in size. This is another indication that KlCat8p is not Kdf1. It should be noted that full length KlCat8p expressed from the multicopy plasmid only poorly complemented *Klcat8* for Kdf1 binding. This may

correlate with a toxic effect observed when KlCat8p was overexpressed. These data in combination rule out the possibility that KlCat8p is Kdf1 or a subunit of a multiprotein Kdf-complex.

The data rather suggest that KlCat8p is an upstream regulator of the CSRE-binding factor in *K. lactis*. The results with the truncated KlCat8p variants suggest that the C-terminal 171 amino acids of KlCat8p are required for this regulatory activity. Although no known motifs of any kind could be identified in the C-terminus of KlCat8p or ScCat8p, a necessity of this region for activation has been shown for ScCat8p. Analysis of ScCat8p size variants showed that a variant C-terminally truncated by 170 amino acids retained activity whereas larger truncations abolished activity (Hedges *et al*, 1995; Rahner *et al*, 1996; Randez-Gil *et al*, 1997). The structural and functional conservation of Cat8p between *K. lactis* and *S. cerevisiae* suggest that this feature is conserved as well, despite a divergence in target UAS sequence.

Considering the strong requirement for *KlCAT8* in Kdf1-binding to the $CSRE_{LAC4}$ and the importance of the $CSRE_{LAC4}$ for *LAC4* transcription on poor carbon sources, a reduction in β-galactosidase activity on these carbon sources in the *Klcat8* mutant compared to the wild-type was expected. However, there was no difference in enzyme activity between the strains on any carbon source tested. One explanation is that the residual Kdf1-complex is still sufficient to mediate normal activation. A second possibility is that Kdf1 binds to the $CSRE_{LAC4}$ as part of a larger complex, perhaps involving the putative Adr1p, that can as a whole still efficiently form *in vivo* despite reduced $CSRE_{LAC4}$ occupancy by Kdf1. A third, and the most likely, possibility is that basal transcription of the *LAC4* gene involves multiple activities, Kdf1, Adr1p and other factors, that target the Basal Control Region. The sum of their interactions, if not physical then at least functional, determines β-galactosidase activity. If the activities of these factors were additive, similar to activation of *ScACS1* transcription by ScCat8p, Adr1p and pleiotropic activator Abf1p (Kratzer and Schüller, 1997), the effect of removing one factor could be quenched by the remaining factors. The data on regulation of the *LAC4* promoter mutants discussed before support this notion. This means, however, that Kdf1-$CSRE_{LAC4}$ binding does not correlate with *LAC4* transcriptional activation. The functions of other sequences in the BCR need clarification for better insight in regulation at this promoter.

4.3 The role of *KlSIP4* in growth on poor carbon sources

A screen for multicopy suppressors of the negative growth phenotype on ethanol of the *Klcat8* mutant was performed to identify downstream effectors. Most clones that

suppressed the growth defect carried *KlCAT8*. One clone did not and the sequence of the library insert showed an Open Reading Frame encoding a 717 residue putative protein. A $Zn(II)_2Cys_6$ binuclear cluster motif was identified in the aminoterminus of the translation. The *S. cerevisiae* Sip4 protein was the only database entry that aligned to the putative protein with regions of similarity and identity distributed throughout both sequences. Seven other transcriptional activators involved in growth on poor carbon sources, the Cat8 proteins from *K. lactis* (Georis *et al*, 2000), *S. cerevisiae* (Hedges *et al*, 1995) and *C. albicans* (unpublished), the FacB proteins from *E. nidulans*, *A. oryzae* and *A. niger* (Todd *et al*, 1997) and the Acu-15 protein from *N. crassa* (unpublished) only showed similarity in the zinc cluster and coiled-coil regions. The Cat8, FacB and Acu-15 proteins share a highly conserved coiled-coil region directly C-terminal to the zinc cluster DNA-binding domain that is not found in this form in any of the other Gal4p family members and defines a subfamily of Cat8p-related transactivators. Interestingly, the Sip4 proteins share this feature between residues 1 to 14 of this region but the remaining 19 residues following the second leucine are absent. This suggests that the Sip4 proteins do not homodimerize by means of this structure, as other zinc-cluster proteins do. Closer scrutiny revealed that the Middle Homology Domain of the Gal4 protein family (Poch, 1997), defined by amino acid properties and predicted secondary structure rather than primary sequence, is also well conserved in KlSip4p andScSip4p but the spacing of the eight individual, conserved motifs increases with protein length. The role of the Middle Homology Region is still unclear. It is only found in combination with the $Zn(II)_2Cys_6$ binuclear cluster DNA-binding domain and data suggest that it is involved in prevention of factor-binding to non-cognate $CGGN_xCCG$ sequences and regulation of activation function (Schjerling and Holmberg, 1997; Poch, 1997).
S. cerevisiae SIP4 (Snf1-interacting protein) was originally identified as a gene encoding a product that interacted with the Snf1 protein kinase in a two-hybrid screen (Yang *et al*, 1992). Subsequent analyses showed it to be a zinc cluster transcriptional activator under glucose control. Both its expression and activity are induced on poor carbon sources by a *SNF1*-dependent mechanism (Lesage *et al*, 1996). It also was shown that *ScSIP4* complements a *Sccat8* deletion when overexpressed, although deletion of *ScSIP4* itself showed no phenotype for growth in *S. cerevisiae* (Vincent and Carlson, 1998).

Thus it was surprising that the *Klsip4* deletion produced a phenotype even more severe than the *Klcat8* deletion. Growth on ethanol or acetate was almost abolished, whereas growth on glycerol was again unaffected. The similar phenotypes of the *Klcat8*, *Klsip4* and *Klcat8 Klsip4* deletion strains and suppression of the *Klcat8* deletion by *KlSIP4* overexpression convincingly locate both genes in the same

regulatory circuit, controlling growth on ethanol or acetate but not glycerol. Moreover it strongly suggests that in *K. lactis* Sip4p functions downstream of Cat8p, rather than in parallel pathways as suggested for Cat8p and Sip4p in *S. cerevisiae* (Vincent and Carlson, 1998). Deletion of either disrupts the cascade as a whole. The stronger effect of the *Klsip4* deletion could be explained as the result of a KlCat8p-independent activity of *KlSIP4*. This reduced activity allows slow growth that is abolished when *KlSIP4* itself is deleted.

Kdf1-binding to the $CSRE_{LAC4}$ was almost completely abolished in the *Klsip4* mutant. The remaining, extremely weak complex suggested that KlSip4p is not the $CSRE_{LAC4}$-binding factor Kdf1, in analogy to the argumentation for KlCat8p. This means that Kdf1 is a KlSip4p-activated factor further down in the cascade. β-Galactosidase activity in the *Klsip4* mutant was measured. Kdf1-$CSRE_{LAC4}$ binding in the *in vitro* assay does not correlate with *LAC4* activation, yet the doubling of enzyme activity that was observed was surprising. One explanation for this behaviour is that Kdf1 is a KlSip4p-controlled $CSRE_{LAC4}$-binding repressor of transcription. An alternative explanation is that Kdf1 is a $CSRE_{LAC4}$-binding activator that counteracts an also KlSip4p-activated repressor of BCR-mediated transcription, which interferes with KlSip4p-dependent and -independent activation. Combined loss of activation and repression at the BCR leads to a net activation of *LAC4* by the KlSip4p-independent mechanism. Interestingly, a number of recent reports show similar contradictory results for ScSip4p. β-Galactosidase activities from CSRE-*lacZ* reporter constructs were stronger activated in *Scsip4* mutants than in the wild-type on ethanol (Walther and Schüller, 2001; Hiesinger *et al*, 2001; Zaragoza *et al*, 2001). In all cases activation was abolished in a *Sccat8* strain.

In combination with the *Klcat8* disruption, which by itself had no effect on *LAC4* expression, the effect of *Klsip4* deletion on β-galactosidase activity was lost and activity was back to wild-type level. Thus, at least three mechanisms must act at the BCR to regulate *LAC4* basal expression: a negative *KlSIP4*-dependent mechanism that is lost in the *Klsip4* strain, a *KlCAT8*-dependent, *KlSIP4*-independent mechanism that causes the difference between the *Klsip4* mutant and the *Klcat8 Klsip4* double mutant and a *KlCAT8*- and *KlSIP4*-independent mechanism that activates *LAC4* basal expression in the double mutant to the level that is observed in the wild-type.

Deletion of *KlGAL4* in the *Klcat8* and *Klsip4* mutants showed that in the single mutants as well as the double mutant, *LAC4* expression was KlGal4p-independent as was the case in the wild-type. Whether this also applies to the ΔBCR strain carrying the $CSRE_{LAC4}$ insertion remains to be tested.

4.4 Target genes of *SIP4* in *K. lactis*

The postulated regulation of *KlSIP4* by *KlCAT8* is likely to occur at the transcriptional level. In *S. cerevisiae SIP4* is autoregulated through a CSRE in its promoter (Vincent and Carlson, 1998; Hiesinger *et al*, 2001) and in the *KlSIP4* upstream region four sequences were identified that show similarity to the $CSRE_{LAC4}$ and *S. cerevisiae* CSRE consensus sequences. Two of those, $CSRE1_{KlSIP4}$ (-468 / -458) and $CSRE2_{KlSIP4}$ (-599 / -609), showed complex formation *in vitro* very similar to the $CSRE_{LAC4}$. $CSRE2_{KlSIP4}$ differs from $CSRE_{LAC4}$ only in the 5'- and 3'- sequences adjacent to the CSRE core, 5'-CGGATGAAAGG-3', and identical complexes are formed by the two oligonucleotides, yet the Kdf1-$CSRE_{KlSIP4}$ complex is considerably less abundant than the Kdf1-$CSRE_{LAC4}$ complex. This means that bases outside the CSRE core affect the affinity of the binding factor. This notion was strengthened by the behaviour of the $CSRE1_{KlSIP4}$ oligonucleotide, which contains a C instead of an A at the less conserved position 4 in the CSRE and differs in the positions directly 3' from the CSRE core. The Kdf1-complex was also observed with this sequence but in addition an extra complex, C_4, formed with this probe. This shows that *K. lactis* has multiple CSRE -binding proteins. Another possibility is that different complexes can form on a CSRE that share the same DNA-binding subunit. The exact sequence of CSRE core and adjacent DNA governs which protein binds or which complex forms. Comparison of complex-formation on $CSRE_{LAC4}$, $CSRE1_{KlSIP4}$ and $CSRE2_{KlSIP4}$ show that single nucleotide differences are decisive.
S. cerevisiae CSREs are targets of both ScCat8p (Rahner *et al*, 1996; Caspary *et al*, 1997; Rahner *et al*, 1999) and ScSip4p (Vincent and Carlson, 1998; Roth and Schüller, 2001) but the proteins bind with different affinities (Hiesinger *et al*, 2001; Roth and Schüller, 2001; Walther and Schüller, 2001). Interestingly, *A. nidulans* FacB, related to Cat8p and Sip4p, binds two different DNA sequences (Todd *et al*, 1998). Half of the binding sites from one class contain the sequence $CGGN_6GG$. From the other class none does. The central basepairs seem random but determine affinity of FacB-binding. Similar observations for the linker sequences were made for Gal4p and Ppr1p binding sites (Liang *et al*, 1996).
Intensity of the fuzzy complex C_3 was both carbon source- and sequence-dependent. This band appears not to represent a defined complex. An undefined modification of the probe is the likely explanation for the appearance of this band. The sequence $KlSIP4_{-467/-477}$ formed no complex with Kdf1. It formed complex C_2, but this complex is not carbon source-regulated. Sequence $KlSIP4_{-137/-127}$ was not tested, as strong similarity to $KlSIP4_{-467/-477}$ suggested similar behaviour.

Transcription of *KlSIP4* was increased on ethanol but not on glycerol. In *S. cerevisiae* transcription of *SIP4* is activated at the diauxic shift from glucose to ethanol (DeRisi *et al*, 1997) in a *ScCAT8*-dependent manner (Haurie *et al*, 2001). β-Galactosidase activity of *ScSip4-lacZ* fusions was derepressed 10-fold (Lesage *et al*, 1996; Hiesinger *et al*, 2001) or 100-fold (Vincent and Carlson, 1998; Zaragoza *et al*, 2001), depending on the growth conditions (Table 4-1).

	β-galactosidase activity (mU/mg)			
Fusion gene	2% Glu	0.2% Glu	3% Gly + 2% Et + 0.2% Glu	3% Gly + 2% Et
ScSIP4-lacZ	15 ± 6	20 ± 8	29 ± 5	1090 ± 250
ScCAT8-lacZ	2 ± 1	24 ± 8	74 ± 23	985 ± 220
ScFBP1-lacZ	3 ± 2	5 ± 1	5 ± 1	1050 ± 80

Tabele 4-1: Influence of low concentrations of glucose on derepression.
β-Galactosidase activities expressed from the fusion genes indicated. Values are means ± SD. Glu = glucose, Gly = glycerol and Et = ethanol. Reproduced from Zaragoza *et al*, 2001.

Deletion of *ScCAT8* strongly interfered with derepression of the *ScSip4-lacZ* fusion whereas deletion of *ScSIP4* did not. Overexpression of *ScSIP4* activated expression of the fusion (Vincent and Carlson, 1998).
Equal amounts of *KlSIP4* mRNA on glucose and glycerol suggest similar amounts of protein, unless translation were regulated. If KlSip4p is present both on glucose and glycerol, induction of Kdf1-CSRE$_{LAC4}$ binding indicates activation of KlSip4 function. KlSnf1p-dependent phosphorylation, in analogy to ScSip4p, is the most obvious possibility.
Phosphorylation of ScSip4p is required for activity (Lesage *et al*, 1996) and involves interaction of ScSip4p with the Snf1 kinase complex (Vincent and Carlson, 1999). Although it has not been tested whether Sip4p interacts with Snf1p (Fog2p) or whether transcription or activity of Sip4p are Snf1p-dependent in *K. lactis*, this is not unlikely. Mutations in genes involved in carbon source regulation lead to very similar phenotypes and Snf1 is of central importance for derepression in both yeasts.
In *K. lactis*, however, whereas Snf1p-activity is required for growth on glycerol as well as ethanol, KlSip4p-activity is not.

The *KlCAT8* promoter contains one sequence similar to the CSRE$_{LAC4}$. This sequence, *KlCAT8*$_{-101/-111}$, shows mismatches in positions 4 to 6, TAC instead of ATG. Kdf1 did not bind to *KlCAT8*$_{-101/-111}$. Positions 4 to 6 can therefore not be exchanged randomly. This finding suggests that *KlCAT8* is not regulated through a CSRE. CSRE-independence of *KlCAT8* would correspond to the situation in *S. cerevisiae* where *CAT8* is also not CSRE-dependent (Haurie *et al*, 2001). In fact,

ScCAT8 appears to be negatively autoregulated, as expression of a *ScCAT8-lacZ* fusion under derepresssing conditions is higher in a *Sccat8* mutant than in the wild-type (Rahner *et al*, 1996; Hiesinger *et al*, 2001). This might be due to loss of the *SIP4*-dependent repression mentioned before. If so, however, this repression should be mediated not only through CSREs, but through different sequences as well.

The *KlICL1* promoter contains three sequences similar to the $CSRE_{LAC4}$. Kdf1binds to $CSRE_{KlICL1}$ but not to sequences $KlICL1_{-287/-297}$ and $KlICL1_{-570/-560}$. Binding to $CSRE_{KlICL1}$ was much weaker than to $CSRE_{LAC4}$, probably due to exchanges in positions 4, 6 and 9. Lack of binding to sequences $KlICL1_{-287/-297}$ and $KlICL1_{-570/-560}$ is most likely caused by mismatches in the strictly conserved position 1. Induction of isocitrate lyase activity on ethanol was completely lost in the *Klsip4* mutant, as in the *Klcat8* mutant (Georis *et al*, 2000). Thus *KlICL1* is both *KlCAT8*- and *KlSIP4*-dependent. The reduction in enzyme activity was slightly stronger in the *Klsip4* than in the *Klcat8* mutant. This is in line with the stronger growth defect of the *Klsip4* strain on ethanol and acetate and strengthens the *KlCAT8-KlSIP4*-CSRE cascade model. As expression of *KlICL1* is *KlCAT8*- and *KlSIP4*-dependent and the *KlICL1* promoter contains a $CSRE_{KlICL1}$ that is functional in the EMSA assay, *KlICL1* is postulated to be a *bona fide* target of this regulatory mechanism. In *S. cerevisiae* expression of *ICL1* is *CAT8*- and CSRE-dependent as well, but it is *SIP4*-independent and is activated on glycerol as on ethanol (Schöler and Schüller, 1994; Hedges *et al*, 1995; Rahner *et al*, 1996; Bojunga *et al*, 1998; Vincent and Carlson, 1998; Rahner *et al*, 1999; Haurie *et al*, 2001; Hiesinger *et al*, 2001).

Comparison of four RST sequences similar to the malate synthase genes from *S. cerevisiae* suggests that *K. lactis* has a single malate synthase gene. Transcription of *KlMLS1* is activated on ethanol but not glycerol and activation is *KlSIP4*-dependent. The level of activation correlates with the increase in enzyme activity in the wild-type (Georis *et al*, 2000). In the *Klsip4* mutant mRNA abundance generally appears to be higher than in the wild-type. This may be a manifestation of the apparent activating effect of *Klsip4*-deletion also observed for basal expression of *LAC4*. As for *ICL1*, *MLS1* is *CAT8*- and CSRE-dependent, but *SIP4*-independent and activated on glycerol in *S. cerevisiae* (Caspary *et al*, 1997).

Two genes, *KlACS1* and *KlACS2*, encode acetyl-CoA synthetases in *K. lactis* (Zeeman *et al*, 2000). The promoter of *KlACS1* contains a $CSRE_{LAC4}$-like sequence. This sequence, $KlACS1_{-766/-776}$, did not bind Kdf1, however, due to substitution of TG for AA in positions 7 and 8. It is more similar to $KlSIP4_{-467/-477}$ and $KlSIP4_{-137/-127}$ than to $CSRE_{LAC4}$. Transcriptional activation of both genes, *KlACS1* on lactate and acetate

and *KlACS2* on ethanol, is *KlCAT8*-dependent, however (Lodi *et al*, 2001). This implies that *KlCAT8* regulates transcription through an alternative route besides *KlSIP4* and CSREs in *K. lactis*. *ScACS1* that encodes the cytosolic acetyl-CoA synthetase is *ScCAT8*- and CSRE-dependent (Kratzer and Schüller, 1997).

Conservation of the CSRE sequence between *K. lactis* and *S. cerevisiae* appears high but not perfect. The weaker binding of Kdf1 to CSRE1$_{KlSIP4}$ and CSRE 2$_{KlSIP4}$ argues that the consensus sequence is actually longer and more defined in *K. lactis* than in *S. cerevisiae* (Table 4-2). CSRE$_{LAC4}$ has the optimal sequence for Kdf1-binding. The single mismatches in CSRE1$_{KlSIP4}$ and CSRE2$_{KlSIP4}$ reduce Kdf1-binding. In the *S. cerevisiae* CSRE any base is allowed in the corresponding positions. The four mismatches in CSRE$_{KlICL1}$ still allow binding of Kdf1. Conservation in these positions is therefore likely low as it is in the corresponding positions in *S. cerevisiae*. The importance of the flanking sequences cannot be determined from present data. The CSRE$_{LAC4}$ sequence is proposed as *K. lactis* CSRE consensus sequence, until more systematic analysis by single exchanges to the CSRE$_{LAC4}$ sequence allows refinement.

promoter site	sequence	Kdf1-binding
CSRE$_{LAC4}$	5'- GT CGGATGAAAGGG GA -3'	+++
CSRE1$_{KlSIP4}$	5'- TC CGG**C**TGAAAGGG AC -3'	++
CSRE2$_{KlSIP4}$	5'- TC CGGATGAAAGG**C** TA -3'	++
CSRE1$_{KlICL1}$	5'- TT CGG**T**T**T**AA**T**GG**C** GG -3'	+
KlCAT8$_{-101/-111}$	5'- AC CGG**TAC**AAAGGG AT -3'	-
KlSIP4$_{-137/-127}$	5'- AA CGGA**CCTGG**GG**A** AA -3'	N.D.
KlSIP4$_{-467/-477}$	5'- GC CGGAT**CTGG**GG**A** AA -3'	-
KlACS1$_{-766/-776}$	5'- TG CGG**T**TG**TG**AGGG GA -3'	-
KlICL1$_{-287/-287}$	5'- CA **T**GG**T**T**A**AA**T**GG**A** TG -3'	-
KlICL1$_{-570/-560}$	5'- CA **G**GGAT**C**AA**T**GG**A** CG -3'	-
Sc Consensus	5'- CGGNYNAAYGGN -3'	
Kl Consensus	5'- CGGATGAAAGGG -3'	

Table 4-2: Alignment of *K. lactis* CSREs and CSRE-like sequences.
K. lactis CSRE-like sequences identified in this work and *in vitro* affinity for Kdf1. Bold face: nucleotides differing from the CSRE$_{LAC4}$. Underlined: CSRE core sequence. (*): results not shown. N.D.: not tested for Kdf1-binding. Y = T/C, N = any base.

In *K. lactis* gluconeogenesis is not *CAT8*- and *SIP4*-dependent, in contrast to *S. cerevisiae* (Figure 4-2). The glyoxylate cycle is dispensable for glycerol metabolism and its inactivation does not affect growth. This can be explained by a greater intrinsic capacity of the TCA cycle in this yeast.

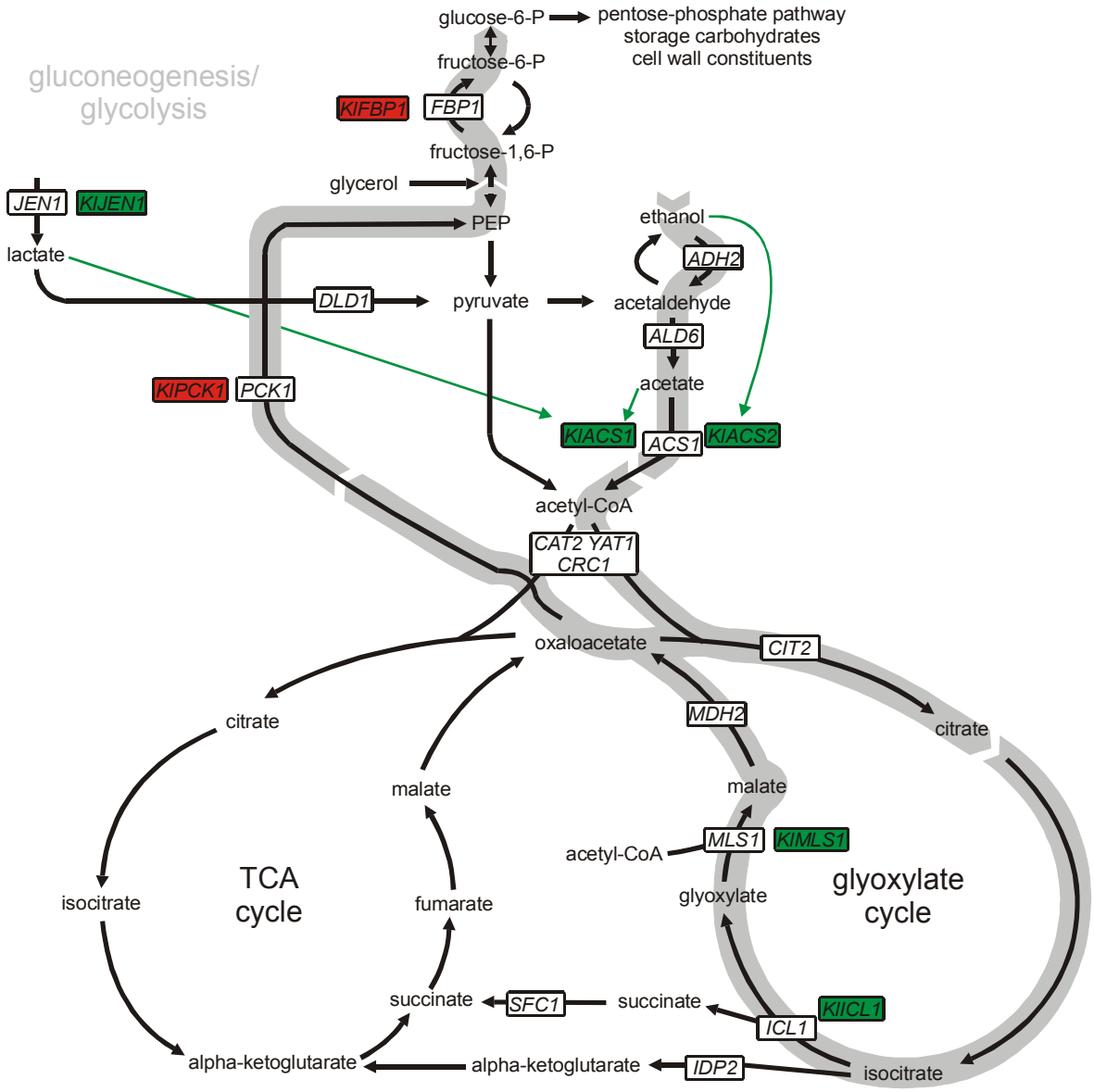

Figure 4-2: In *K. lactis* the glyoxylate cycle is *CAT8*-dependent but, in contrast to *S. cerevisiae*, gluconeogenesis is not.
Open boxes: *CAT8*- and CSRE-dependent genes required for growth on poor carbon sources in *S. cerevisiae*. Boxed green: *CAT8*-dependent genes in *K. lactis*. Boxed red: *CAT8*-independent genes in *K. lactis*. Green arrows: activation of *KlACS1* by lactate or acetate and activation of *KlACS2* by ethanol.
Adapted from Haurie *et al,* 2001.

On ethanol and acetate the glyoxylate cycle is required as is expression of *KlACS2* (ethanol) or *KlJEN1* and *KlACS1* (acetate). Expression of these genes is *KlCAT8*-dependent and at least *KlICL1* and *KlMLS1* are *KlSIP4*-dependent as well. Regulation of at least *KlSIP4* and *KlICL1* is likely regulated through CSREs, although a *KlCAT8*-dependent but CSRE-independent regulation may exist. Basal transcription of the *LAC4* gene is mediated in part through the $CSRE_{LAC4}$ but other promoter elements and factors are involved as well.

The conclusions from this work is that regulation of gene expression and growth has diverged between *K. lactis* and *S. cerevisiae* in two important points. First, *SIP4* is as important as *CAT8* for this regulatory circuit in *K. lactis*, whereas in *S. cerevisiae* it is dispensable. Second, the glyoxylate cycle is *CAT8*- and *SIP4*-dependent but not required for growth on glycerol in *K. lactis*, whereas gluconeogenesis is *CAT8*- and *SIP4*-independent, allowing *Klcat8* and *Klsip4* mutants to grow normally on glycerol.

4.5 Galactose repression of Kdf1-CSRE$_{LAC4}$ complex formation

Glucose represses binding of Kdf1 to the CSRE$_{LAC4}$ and BCR-mediated basal expression of *LAC4*. Galactose induces high level-expression of the *GAL*/*LAC* genes. This induction is independent of the BCR (Schmidt, PhD thesis 1996) but whether Kdf1 binds to the CSRE$_{LAC4}$ under inducing conditions was unknown. In this work it was shown that on galactose Kdf1 does only very weakly bind to the CSRE$_{LAC4}$. This indicated a general "sugar repression" of Kdf1-CSRE$_{LAC4}$ binding.

KlGal1p was considered as the possible effector of "galactose repression" as it has a central role in regulation of galactose metabolism, providing both enzymatic and regulatory activity (Meyer *et al*, 1991; Zenke *et al*, 1996). Here it was shown that in a *Klgal1* deletion mutant galactose repression was lost. In the absence of KlGal1p, transcription of the *GAL*/*LAC* regulon cannot be induced, lactose permease Lac12p is not expressed and galactose cannot be taken up. This shows that extracellular galactose does not exert repression but not that KlGal1p does.

Lack of induction may be overcome by deletion of *KlGAL80*, which encodes the inhibitor. In a *Klgal80* background the *GAL*/*LAC* regulon is constitutively expressed (Zenke *et al*, 1993). The *Klgal1-209 Klgal80-31* background was used for further analysis. The *Klgal1-209* allele encodes a protein that has no enzymatic activity but retains its regulatory function (Riley and Dickson, 1984), which requires interaction with KlGal80p. As the *Klgal1* strain, the *Klgal1-209* strain does not grow on galactose. The KlGal80-31 protein is incapable of interacting with KlGal4p but interaction with KlGal1p is unaffected (Zenke *et al*, 1999). Both mutant proteins must therefore largely retain their structural integrity. In this double mutant background galactose repression of Kdf1-CSRE$_{LAC4}$ binding was completely lost. This deregulation was not due to the *Klgal80-31* mutation that had no influence on complex formation. As the regulatory function of the KlGal1-209 protein is intact, the enzymatic activity of KlGal1p is required for galactose repression of Kdf1-binding. Another regulatory function in KlGal1p, so far unidentified and also affected by the mutation, would be the alternative.

The heterologous *E. coli* galactokinase gene *galK* expressed from the constitutive *ScADH1* promoter complements a *gal1* deletion in *S. cerevisiae* (Bhat *et al*, 1990). In *K. lactis* a *gal1* deletion cannot be complemented by *EcgalK* (Meyer *et al*, 1991), which shows that *E. coli* galactokinase cannot replace the regulatory function of KlGal1p and galactose phosphorylation alone is not sufficient for induction. In the *Klgal80-31* background EcGalK expression restored growth on galactose. Constitutive expression of the *GAL*/*LAC* operon in the *Klgal80-31* strains was evident from high β-galactosidase activity. Repression of Kdf1-CSRE$_{LAC4}$ binding was restored in the *Klgal1-209 Klgal80-31* strain expressing EcGalK. Since it is highly unlikely that *E. coli* GalK could complement a putative regulatory function of KlGal1p, while it cannot complement the known one, this result showed that the enzymatic activity of KlGal1p is the function required for galactose repression of Kdf1-CSRE$_{LAC4}$ complex formation. This function converts galactose to galactose-1-phosphate that is, through glucose-1-phosphate, converted to glucose-6-phosphate for entry into glycolysis. Glucose is directly converted to glucose-6-phosphate by hexokinases and these enzymes are required for glucose repression. This suggests that galactose and glucose mediate repression by the same mechanism that, in the case of galactose, merely requires production of sufficient glucose-6-phosphate.

Abstract

The regulation of carbohydrate metabolism in the dairy yeast *Kluyveromyces lactis* shows that the knowledge that was gained from *Saccharomyces cerevisiae* cannot be projected onto all other yeasts. In the context of this work the mechanism of glukose repression in *K. lactis* was analysed. Initially the transcriptional regulation of the lactose metabolic genes, specifically the β-galactosidase gene *LAC4*, was studied as a model. Basal expression of *LAC4* on poor carbon sources is regulated in part through a Carbon Source-Responsive Element ($CSRE_{LAC4}$) that could replace the Basal Control Region (BCR) in the *LAC4* promoter. Basal expression of *LAC4* is accompanied by binding of a factor Kdf1 to the $CSRE_{LAC4}$. Glucose and galactose repress this binding. It could be shown that repression by galactose merely requires an unimpeded metabolic flux of galactose. Repression by glucose and galactose is therefore probably mediated by the same mechanism.

Two regulatory genes were identified, *KlCAT8* and *KlSIP4*, that are equally required for growth on poor carbon sources. Both regulators are located downstream of the central protein kinase SNF1. This protein kinase regulates derepression of glucose-repressed genes after a shift to a poor carbon source. It was shown that *KlCAT8* is a regulator of *KlSIP4*. *KlSIP4* activates transcription of the metabolic genes of the glyoxylate cycle *KlICL1* and *KlMLS1*. Moreover, *KlSIP4* is probably autoregulated. CSREs were demontrated in the promoters of these genes. *KlCAT8* and *KlSIP4* are homologues of the *S. cerevisiae CAT8* and *SIP4* genes that encode CSRE-binding transcriptional activators. It is very likely therefore, that KlCat8p and KlSip4p also function through CSREs. Neither KlCat8p nor KlSip4p appears to be identical to the CSRE-binding factor Kdf1, however.

Despite strong similarities, in some respects metabolism of poor carbon sources in *K. lactis* is regulated clearly different from *S. cerevisiae*. The difference is based on the fact that in *K. lactis* gluconeogenesis is *CAT8*-independent and *SIP4* is an equal link in the regulatory chain. In *K. lactis*, *CAT8* and *SIP4* are of similar importance for growth on poor carbon sources, except on glycerol where both are dispensable.
In *S. cerevisiae*, *CAT8* is required for growth on all non-fermentable carbon sources whereas for *SIP4* no function could be identified so far.

Zusammenfassung

Die Regulation des Kohlenhydrat-Stoffwechsels in der Milchhefe *Kluyveromyces lactis* zeigt, dass die an *Saccharomyces cerevisiae* gewonnenen Erkenntnisse nicht auf alle anderen Hefen übertragbar sind. Im Rahmen dieser Arbeit wurde der Mechanismus der Glukose-Repression in *K. lactis* analysiert. Modellhaft wurde zunächst die Transkriptionsregulation der Laktose verwertenden Gene, spezifisch das β-Galaktosidase Gen *LAC4*, untersucht. Basale Expression von *LAC4* auf wenig ergiebigen Kohlenstoffquellen wird teilweise über ein Carbon Source-Responsive Element ($CSRE_{LAC4}$) reguliert, welches die Basal Control Region (BCR) im *LAC4* Promotor ersetzen konnte. Basale *LAC4*-Expression geht einher mit Bindung eines Faktors Kdf1 an das $CSRE_{LAC4}$. Glukose und Galaktose hemmen diese Bindung. Es konnte gezeigt werden dass die Hemmung durch Galaktose lediglich einen unbehinderten metabolischen Fluss der Galaktose erfordert. Repression durch Glukose und Galaktose werden somit vermutlich über den gleichen Mechanismus vermittelt.

Zwei regulatorische Gene wurden identifiziert, *KlCAT8* und *KlSIP4*, die für Wachstum auf wenig ergiebige Kohlenstoffquellen gleichermassen notwendig sind. Beide Regulatoren sind angesiedelt unterhalb der zentralen Proteinkinase SNF1. Dieser regelt die Derepression Glukose-reprimierter Gene nach einen Wechsel zu einer armen Kohlenstoffquelle. Wie gezeigt wurde, ist *KlCAT8* ein Regulator von *KlSIP4*. *KlSIP4* aktiviert die Transkription von metabolischen Genen des Glyoxylatzyklus *KlICL1* und *KlMLS1*. Zudem ist *KlSIP4* wahrscheinlich autoreguliert. In den Promotoren dieser Gene wurden CSREs nachgewiesen. *KlCAT8* und *KlSIP4* sind Homologe der Gene *CAT8* und *SIP4* aus *S. cerevisiae*, die für CSRE-bindende Transkriptionsaktivatoren kodieren. Es ist darum sehr wahrscheinlich dass auch KlCat8p und KlSip4p über CSREs wirken. Allerdings scheint weder KlCat8p noch KlSip4p mit dem CSRE-bindenden Faktor Kdf1 identisch zu sein.

Trotz starker Ähnlichkeit ist die Verwertung wenig ergiebiger Kohlenstoffquellen in *K. lactis* in einigen Hinsichten deutlich anders reguliert als in *S. cerevisiae*. Der Unterschied beruht darauf, dass in *K. lactis* die Glukoneogenese nicht unter *CAT8*-Kontrolle steht und *SIP4* ein gleichwertiges Glied in der regulatorischen Kette ist. In *K. lactis* sind *CAT8* und *SIP4* ähnlich wichtig fur Wachstum auf wenig ergiebige Kohlenstoffquellen, ausser auf Glyzerin wo auf beide verzichtet werden kann. In *S. cerevisiae* ist *CAT8* erforderlich für Wachstum auf alle nicht-fermentierbaren Kohlenstoffquellen, während für das *SIP4* bisher keine Funktion gefunden werden konnte.

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Abbreviations

Amp	Ampicillin
ATP	adenosine 5'-triphosphate
bp	basepair
BSA	bovine serum albumin
cpm	counts per minute
dATP	deoxyadenosine triphosphate
cDNA	complementary DNA
DMF	N,N-dimethylformamide
DNA	deoyxribonucleic acid
DNAse	deoxyribonuclease
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FOA	5-fluoro-orotic acid
g	gram
h	hour
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
min	minute
mRNA	messenger RNA
mU	milliunits
OD_{600}	optical density at 600 nanometers wavelength
ONPG	o-nitrophenyl β-D-galactopyranoside
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
SC	synthetic complete
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)aminomethane
Ura	uracil
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
YEP	yeast extract / peptone = rich medium
YNB	yeast nitrogen base = synthetic medium

Danksagung

Ich möchte Frau Prof. Karin Breunig sehr danken für die Überlassung des Themas, für die wertvolle und inspirierende Diskussionen und ihre Betreuung sowohl in wissenschaflicher Sache als in der Kunst der Laborführung.

Vielen Dank ebenfalls den Mitgliedern (manche ehemalig) der Arbeitsgruppe Molekulargenetik, vor allen Karin Sorge, Ulla Klokow, Lutz Kapp, Renate Langhammer, Carsten Milkowski und Raffael Schaffrath, für praktische Hilfestellung, Diskussionen (manchmal erhitzt) zu praktischen und theoretischen Problemen und gute Zeiten im Labor und ausserhalb.

Für das Überlassen von Plasmide mit *KlFBP1* Promotorsequenzen und die Sequenz des *KlICL1* Promotors möchte ich Prof. Jürgen Heinisch und Dr. Rosaura Rodicio danken. Für das Überlassen des Plasmids pGID1 und die Stämme yIG8 und yIG10 und für die gute Zusammenarbeit, vor allem während ihre Aufenthalte in Halle, danke ich Dr. Isabelle Georis.

Für ihre unablässige Unterstützung während meiner Schulzeit und meines Studiums und, aus einiger Entfernung, meiner Doktorarbeit danke ich meine Eltern und meinen Bruder Niels. (Wenn der nächste Fernumzug ansteht kann ich mir für den sperrigen Kram eine Umzugsfirma leisten!).

Zwei Gruppen nicht namentlich genannten Leuten danke ich dafur dass sie mich zu einem Leben ausserhalb der Arbeit verholfen haben:
Den Mitgliedern des Instituts für Genetik mit denen ich grossartige Grillabende erlebte im Innenhof von Domplatz 1 und am Weinbergweg und einige Bierchen trank im Biergarten der Apotheke.
Den Freunden die ich in Halle kennen lernte und die ich oft traff auf ein Bier und eine Diskussion über dieses oder jenes, ein spiel oder zum Grillen im Hinterhof (was ich immer noch mache).

Zu guterletzt (aber eigentlich an erster Stelle) möchte ich Juliane meinen grössten DANK aussprechen dafür dass sie es all diese Jahre mit mir ausgehalten hat, dass sie Willens war mich mit der Wissenschaft zu teilen und mich bei dem Ganzen zu unterstützen.

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Veröffentlichungen

Breunig K.D., Bolotin-Fukuhara M., Bianchi M.M., Bourgarel D., Falcone C., Ferrero I .I., Frontali L., Goffrini P., Krijger J.J., Mazzoni C., Milkowski C., Steensma H.Y., Wesolowski-Louvel M., Zeeman A.M. (2000) Regulation of primary carbon metabolism in *Kluyveromyces lactis*. Enzyme Microb Technol. 26(9-10):771-780.

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Tagungen

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Eidesstattliche Erklärung

Ich versichere an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Halle (Saale), März 2002