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Centromere promoter factors (CPF1) of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are functionally exchangeable, despite low overall homology

Wietse Mulder¹, Aaron A. Winkler², Inge H.J.M. Scholten¹, Ben J.M. Zonneveld², Johannes H. de Winde¹, H. Yde Steensma², Leslie A. Grivell¹

¹ Section for Molecular Biology, Institute for Molecular Cell Biology, Biocentrum Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands

² Department of Molecular and Cellular Biology, Leiden University, Wassenaarseweg 64, NL-2333 AL Leiden, The Netherlands

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The *KICPF1* gene, coding for the centromere and promoter factor CPF1 from *Kluyveromyces lactis*, has been cloned by functional complementation of the methionine auxotrophic phenotype of a *Saccharomyces cerevisiae* mutant lacking ScCPF1. The amino-acid sequences of both CPF1 proteins show a relatively-low overall identity (31%), but a highly-homologous C-terminal domain (86%). This region constitutes the DNA-binding domain with basic-helix-loop-helix and leucine-zipper motifs, features common to the myc-related transcription factor family. The N-terminal two-thirds of the CPF1 proteins show no significant similarity, although the presence of acidic regions is a shared feature. In *KICPF1*, the acidic region is a prominent stretch of approximately 40 consecutive aspartate and glutamate residues, suggesting that this part might be involved in transcriptional activation. In-vitro mobility-shift experiments were used to establish that both CPF1 proteins bind to the consensus binding site RTCACRTG (CDEI element). In contrast to *S. cerevisiae*, *CPF1* gene-disruption is lethal in *K. lactis*. The homologous *CPF1* genes were transformed to both *S. cerevisiae* and *K. lactis* *cpf1*-null strains. Indistinguishable phenotypes were observed, indicating that, not withstanding the long non-conserved N-terminal region, the proteins are sufficiently homologous to overcome the phenotypes associated with *cpf1* gene-disruption.

Key words: *Kluyveromyces lactis* – Centromere – Transcription regulation – CPF1

Introduction

Centromere and promoter factor CPF1 was identified via specific binding to the conserved centromere DNA element I (CDEI) of *Saccharomyces cerevisiae* centromeres (Bram and Kornberg 1987; Baker et al. 1989; Jiang and

Philippson 1989). Various named CP1, CBF1, CPF1, the gene for the protein has been cloned and sequenced by several groups (Baker and Masison 1990; Cai and Davis 1990; Mellor et al. 1990). Another reported factor, GF2, turned out to be identical to CPF1 (Dorsman et al. 1991).

The CDEI element is an octanucleotide, with the consensus sequence RTCACRTG, and mutational analysis has shown that this element (Hegemann et al. 1988; Baker et al. 1989), and binding of CPF1 as a dimer to this element, is important for chromosome segregation (Niedenthal et al. 1991). Although the CDEI element has also been found in several promoter regions, e.g., *GAL2*, *TRP1*, *MET25*, *QCR8* (Bram et al. 1986; Dorsman et al. 1988; Thomas et al. 1989), no direct role in transcription activation has been established (Mellor et al. 1991; DeWinde and Grivell 1992; Thomas et al. 1992). Recently, however, Thomas et al. (1992) showed that CPF1, together with MET4, is required for transcriptional activation of several of the *MET* genes and therefore CPF1 may function as an ancillary activator by facilitating factor access. Support for this view is given by results of in-vivo footprinting experiments, which show that CPF1 influences chromatin structure and positioning of the nucleosomes on the promoter regions of the *MET25*, *GAL2* and *TRP1* genes (Mellor et al. 1990).

CPF1, together with ABF1, RAP1 and REB1, belongs to a group of abundant multi-functional DNA-binding proteins (Diffley 1992). Disruption of any one of these latter three genes is lethal in *S. cerevisiae*. CPF1 is exceptional in that gene disruption is not lethal, but results in viable mutants displaying slow growth, partial chromosome loss and methionine auxotrophy (Mellor et al. 1990). Interestingly, this latter phenotype is absent when the strain contains a second mutation in genes which are involved in chromatin organisation e.g., SPT21 or RPD1 (McKenzie et al. 1993), suggesting that CPF1 may be required to overcome the repressive action mediated by these chromatin regulatory factors.

The observation that CPF1 is involved in both chromosome segregation and transcription activation has interested, but also confused, many researchers. Several attempts have been made to allocate these functions to sep-

arate domains of CPF1. Using deletion analysis, Dowell et al. (1992) showed that the C-terminal region, including the basic-helix-loop-helix (bHLH) motif, is sufficient for restoration of methionine prototrophy and normal chromosomal segregation. However, while Mellor et al. (1991) propose that DNA binding is only necessary for accurate chromosome segregation, Masison et al. (1993) showed that DNA binding is also required for restoration of methionine prototrophy and suggest that CPF1 performs the same general chromosome-related function at both centromere and promoter regions and that observed differences may be more quantitative than qualitative. Nevertheless, some aspects of the roles which CPF1 plays in chromosome segregation and transcription activation may be mechanistically distinct as suggested by Foreman and Davis (1993).

CPF1 possesses a bHLH motif as a DNA-binding domain (Cai and Davis 1990; Mellor et al. 1990), a feature common to the myc-related protein family. More recently, an additional motif, similar to the leucine zipper (ZIP), was identified at the carboxy-terminus (Dowell et al. 1992). Deletion of this latter region blocks both dimerisation and DNA binding, and also leads to an inability to complement a *cpf1*-null strain. The dimerisation capacity of this region was further demonstrated by using the two-hybrid system (Fields and Song 1989). In this experimental system, this region was sufficient to promote dimerisation of the DNA-binding domain with the activation domain of GAL4. Moreover, when this motif was replaced with the heptad leucine repeat of the related human USF, the CPF1-ZIP(USF) hybrid protein complemented the methionine auxotrophic phenotype in a *S. cerevisiae* *cpf1*-null mutant, demonstrating that dimerisation is required for function and Dowell et al. (1992) suggest that this probably occurs via a coiled-coil interaction (O'Shea et al. 1989).

To obtain more information about CPF1 and its roles in chromosome segregation and transcription activation we decided to study this protein in *Kluyveromyces lactis*. This yeast is related to *S. cerevisiae* and generally shows a high conservation of both sequence and function within structural genes (Stark and Milner 1989; Bergkamp-Steffens et al. 1992), but tends to display an informative divergence of both features in regulatory factors, e.g., TFIIB (Na and Hampsey 1993), ABF1 (Gongalves et al. 1992), REB1 (Morrow et al. 1993) and HAP3 (Mulder et al. 1994).

Mobility-shift experiments carried out by Dorsman et al. (1990) demonstrated that *K. lactis* contains a CPF1-like activity and Heus et al. (1993b) have shown that the DNA-sequence of the CDEI element of *K. lactis* centromeres is also conserved. We report here the isolation of the *K. lactis* CPF1 gene by functional complementation of the methionine auxotrophic phenotype of the *S. cerevisiae* *cpf1*-null strain. A striking observation is that, in contrast to the situation in *S. cerevisiae*, disruption of the *KICPF1* gene in *K. lactis* is lethal. The homology of the CPF1 counterparts is restricted to the C-terminal domain, which is involved in DNA binding and dimerisation. Nevertheless, despite the low overall sequence homology between ScCPF1 and KICPF1 the genes are functionally interchangeable between *S. cerevisiae* and *K. lactis*.

Materials and methods

Strains and media. The yeast strains used are listed in Table 1. To obtain diploid strain GG1901, which is homozygous for the *ura3-100* allele, two spores derived from the diploid GG1900, with genotypes (*ura3-100*) and [*ura3-100, ade1-88, trp1, Kicpf1::Tn5-ble* (pRS/K1-APT1)], were crossed. Both *S. cerevisiae* and *K. lactis* cells were grown in YPD (1% yeast extract, 2% bacto-peptone and 2% glucose), YPEG (1% yeast extract, 2% bacto-peptone, 2% w/v ethanol and 2% glycerol), or WO (0.67% Yeast Nitrogen Base without amino-acids and 2% glucose) supplemented with the appropriate amino-acids and nucleotides. The *Escherichia coli* strains used for DNA manipulations were NM522 (Stratagene), JF1754 and XL-1 blue (Stratagene). Bacterial cultures were grown in Luria-Bertani medium (1% Bacto-Tryptone, 0.5% Yeast Extract, 1% NaCl), supplemented when necessary with 50–200 µg/ml of ampicillin. For plates 2% Bacto agar was added. If required, the media were supplemented with phleomycin (Cayla) or geneticin (G418-sulphate, Gibco-BRL) to a final concentration of 7.5 µg/ml and 150 µg/ml respectively. Standard methods were used for genetic analysis (Campbell and Duffus 1988), yeast transformation (Dohmen et al. 1991), and *E. coli* transformation (Sambrook et al. 1989).

Plasmid shuffling. Plasmid shuffling was performed to obtain strains containing the *KICPF1* or *ScCPF1* gene in the same genetic background. Briefly, yeast cells, harbouring a vector (A) containing a *URA3* gene were transformed with a vector (B) containing, besides other information, *URA3* and the *APT1* gene, providing geneticin resistance. Several geneticin-resistant transformants were grown overnight in rich geneticin-selective medium at 30 °C. From each culture diluted samples were spotted on rich non-selective medium, resulting in roughly 200 individual colonies. Several of these colonies were grown overnight in non-selective rich medium, and successively spotted on non-selective medium. After 2–3 days growth these plates were replica-plated on geneticin-selective, uracil-selective and non-selective medium. Shuffling was scored as successful when all uracil prototrophic colonies were also geneticin-resistant.

Complementation of the *S. cerevisiae* *cpf1*-null strain. In five separate experiments, *S. cerevisiae* strain YSS91 was transformed with 5 µg of the multi-copy genomic DNA library of *K. lactis* strain CBS2359 (Mulder et al. 1994). Transformants were selected on medium containing lysine and methionine. After 3 days growth at 30 °C, more than 1000 uracil prototrophic transformants were replica-plated on selective medium containing only lysine. Two transformants complemented the methionine auxotrophic phenotype of the YSS91 strain. Plasmid-DNA was isolated (Soni and Murray 1992) and, after amplification in *E. coli* strain JF1754, retransformed to YSS91 to confirm complementation. Restriction maps were constructed of the complementing plasmids and the *KICPF1* gene was located by Southern blotting, using a 900-bp fragment of *ScCPF1* (*HindIII*-*PstI*) as a probe. Suitable fragments were subcloned in vector pBluescript II⁺ either KS or SK (Stratagene) and the DNA sequence of both strands was determined according to the dideoxy method (Sanger et al. 1977) using either the universal M13 primers or the pBluescript specific primers (Stratagene).

Plasmids. The plasmids used in this study are listed in Table 2. Vector pUC *KICPF1::Tn5-ble* was constructed via several cloning steps resulting in the net cloning of the 4.2-kb *XbaI*-*EcoRI* fragment, derived from pK#3, in pUC19B and the replacement of an internal 713-bp *SalI*-*StuI* fragment of the *KICPF1* coding region by an 1.3-kb *SmaI*-*HindIII* fragment. This latter fragment is derived from pUT332 and contains the *Tn5-ble* gene under the control of the *TEF1* promoter and the *CYC1* terminator (Gatignol et al. 1987). For disruption of the *KICPF1* gene, a 5-kb *SphI*-*KpnI* fragment was subsequently isolated and used for transformation.

Chromosome localisation. The *K. lactis* strain GG718 was used for chromosomal localisation of the *CPF1* gene. This strain has a large deletion in chromosome IV, possibly in the rDNA cluster, allowing

Table 1. Yeast strains

Strain	Genotype	Source
<i>Saccharomyces cerevisiae</i>		
DL1	<i>MATα</i> , <i>his3-11,15</i> , <i>leu2-3,112</i> , <i>ura3-251,328,372</i>	VanLoon et al. (1984)
YPH266	<i>MATa</i> , <i>ura3-52</i> ochre, <i>lys2-801</i> amber, <i>ura3-52</i>	Mellor et al. (1990)
YSS91	as YPH266 but with <i>cpfl::LEU2</i>	Idem
<i>Kluyveromyces lactis</i>		
CBS2359	<i>MATa</i> , K ⁺	CBS, The Netherlands
SD11	<i>lac4-1</i> , <i>trp1</i> , K ⁺	Das and Hollenberg (1982)
JBD100	<i>lac4-1</i> , <i>trp1</i> , <i>ura3-100</i> ; derived from SD11	Heus et al. (1990)
GG777	<i>lac4-1</i> , <i>trp1</i> , <i>ura3-100</i> , <i>ade1-88</i> , derived from JBD100	Idem
GG718	<i>lac4-1</i> , <i>trp1</i> , <i>lys-52</i> , K ⁺ (contains a large deletion on chromosome IV)	Idem
MW149-1C	<i>MATα</i> , <i>uraA</i> , <i>argA1-1</i> , <i>metA1-1</i> , <i>trpA1-1</i> , <i>lysA1-1</i> , <i>ilvA1-1</i> , <i>adeT-600</i>	M. Wésolowski-Louvel
GG754	<i>ilvA1-1</i> (spore derived from cross MW149-1C* SD11)	This study
GG902	<i>MATa/MATα</i> , <i>LAC4/lac4-1</i> , <i>TRP1/trp1</i> , <i>URA3/ura3-100</i> , <i>ADE1/ade1-88</i> , <i>ILVA1/ilvA1-1</i> (cross GG777*GG754)	This study
GG1900	as GG902 but <i>KICPF1/kicpf1::Tn5-ble</i>	This study
GG1901	<i>MATa/MATα</i> , <i>ura3-100</i> , <i>ura3-100</i> , <i>TRP1/trp1</i> , <i>ADE1/ade1-88</i> , <i>KICPF1/kicpf1::Tn5-ble</i>	This study

Table 2. Plasmids

Name	Characteristics	Source
pBluescript II	<i>E. coli</i> cloning vector; KS/SK	Stratagene
YEplac195	<i>E. coli/S. cerevisiae</i> shuttle vector; <i>URA3</i> gene	Gietz and Sugino (1988)
pKI#3	$\approx$ 4.2-kb genomic <i>Sau3A</i> fragment in YEplac195/ <i>Bam</i> HI	
pUC19/B	Contains an 8-bp <i>Bgl</i> III linker insertion in the <i>Sma</i> I site	Heus et al. (1990)
pUT332	Contains a <i>Tn5-ble</i> gene under control of the <i>TEF1</i> promoter and the <i>Cyc1</i> terminator	Gatignol et al. (1987)
pUC <i>KICPF1::Tn5-ble</i>	Description of construction in text	
pUC ADH/ <i>APT1</i>	Contains an <i>APT1</i> gene under the control of the <i>ScADH1</i> promoter, providing geneticin resistance	S. Das
pRS306	High-copy <i>E. coli/S. cerevisiae</i> shuttle vector; <i>URA3</i> gene	Sikorski and Hieter (1989)
pRS306/K2	Contains an <i>ARS1/KARS2</i> fragment and <i>KICEN2</i>	Heus et al. (1993 a)
pRS306/K2- <i>ScCEN16</i>	Low-copy <i>E. coli/S. cerevisiae/K. lactis</i> shuttle vector; <i>URA3</i> gene	Heus et al. (1993 a)
pRS/S1	A 4-kb <i>Bgl</i> III- <i>Bgl</i> III fragment, containing <i>ScCPF1</i> , from pMA3a (Mellor et al. 1990) cloned in pRS316/K2- <i>ScCEN16/Asp718</i> (blunt)	
pRS/S1- <i>APT1</i>	A 2-kb <i>Sac</i> - <i>Xba</i> I fragment of pUC ADH/ <i>APT1</i> , containing the <i>APT1</i> gene, cloned in pRS/S1 also digested with <i>Sac</i> I- <i>Spe</i> I	
pRS/K1	A $\approx$ 5-kb <i>Xba</i> I- <i>Pst</i> I fragment, containing <i>KICPF1</i> , from pK#3 cloned in pRS316/K2- <i>ScCEN16/Asp718</i> (blunt)	
pRS/K1- <i>APT1</i>	A $\approx$ 2-kbp <i>Sac</i> I- <i>Xba</i> I fragment of pUC ADH/ <i>APT1</i> , containing the <i>APT1</i> gene, cloned in pRS/K1 also digested with <i>Sac</i> I- <i>Spe</i> I	

the suitable separation of chromosomes IV and V (Heus et al. 1990). The chromosomes were separated using a contour-clamped homogeneous electric field (CHEF) apparatus (Bio-Rad). Agarose plugs were prepared in BioRad low-melting agarose essentially as described by DeJonge et al. (1986). The conditions used were: 1% agarose (Seakem, FMC Bioproducts) in 0.5 TBE (0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA, pH 8.3). The gel was electrophoresed for 70 h, at 100 V with the pulse time varying linearly between 100–500 s.

Electrophoretic mobility-shift assay (EMSA). Cell-free extracts were prepared as described (Olesen et al. 1987), and the protein concentrations were determined according to Bradford (1976). EMSA was performed as described by Dorsman et al. (1988). The complementary oligonucleotides MB530 (5'-TCGAG GATCT ACCGT TCCAC GTGAC TAGTC CAAGG -C) and MB531 (5200'-TCGAG CCTTG GACTA GTCAC GTGGA ACGGT AGATC C) were annealed and labelled by filling in the generated *Xho*I sites with the Klenow fragment of the DNA polymerase in the presence of [α^{32} P] dATP, resulting in a double-stranded 40 bp *QCR8* oligonucleotide, containing the overlapping consensus binding sites for the *S. cerevisiae* pro-

teins ABF1 and CPF1. In competition assays, the cell extracts were incubated with an unlabelled *QCR8* oligonucleotide prior to addition of the labelled *QCR8* oligonucleotide.

Miscellaneous. Standard solutions, recombinant DNA methods, isolation of DNA fragments, annealing of oligonucleotides, and labelling of probe fragments were prepared and performed according to standard laboratory procedures (Sambrook et al. 1989).

Accession number. The nucleotide sequence of the *KICPF1* gene will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X76028.

Results

Isolation of the *KICPF1* gene of *K. lactis*

Disruption of the *ScCPF1* gene in *S. cerevisiae* results in slow growth, partial chromosome loss, and methionine

Fig. 1. Cells of the *Sccpf1*-null strain YSS91, the isogenic wild-type YPH266, and YSS91 complemented with pKICPF1#K3, were streaked on rich-glucose medium and grown for 2 days at 30 °C. Thereafter the plate was replica-plated on selective media, supplemented with the necessary amino-acids and nucleotides, but lacking one of them as is indicated. The plates were photographed after 3 days growth at 30 °C

Fig. 2. Restriction map of clone YEplac195/#K3 containing the *KICPF1* gene. The *KICPF1* open reading frame is depicted as a white box and the direction of transcription is indicated by arrow. For disruption of the *KICPF1* gene, the *SalI*-*StuI* fragment was replaced by a 1.3-kb fragment containing the *Tn5-ble* gene under the control of

the *TEF1* promoter and the terminator region of the *CYC1* gene. Relevant restriction sites of the *KICPF1* region and the *Tn5-ble* fragment are indicated above the lines in vertical text. Sites shown in horizontal text belong to either the identical multiple-cloning sites of YEplac195/#K3 or to pUC19 *cpf1*::*Tn5-ble*

auxotrophy (Mellor et al. 1990). We decided to use this latter phenotype to isolate the *CPF1* counterpart of *K. lactis* by functional complementation. We therefore transformed the *Sccpf1*-null strain YSS91 with DNA from the multi-copy genomic DNA library of the *K. lactis* strain CBS2359 (Mulder et al. 1994). First we selected for uracil prototrophy, on growth medium containing methionine, indicating the presence of the *ScURA3* gene that is located on the library vector YEplac195. Thereafter, uracil proto-

trophic transformants were replica-plated onto selective medium lacking methionine. After several days of growth, two transformants were obtained. To confirm complementation, plasmid DNA was isolated, and after amplification in *E. coli*, used to retransform YSS91. Both plasmids appeared to complement the methionine auxotrophic phenotype. In Fig. 1 the complementation is shown for plasmid YEplac195/#K3 in YSS91. Determination of the restriction maps (Fig. 2) revealed that the plasmids contain over-

Fig. 3A,B. For chromosomal localisation of the *KICPF1* gene, chromosomes of strain GG718 were separated by the pulse-field gel electrophoresis. **A** an ethidium-bromide-stained agarose gel. **B** shows the result of Southern blotting after hybridisation with a labelled 3-kb *EcoRI* fragment of plasmid YEplac19/#K3. The chromosomes are identified by Roman digits

lapping inserts. The location of the *KICPF1* gene on the DNA insert was determined by Southern blotting, using a 900-bp *HindIII-PstI* fragment, containing the *ScCPF1* gene, as probe (data not shown). The DNA insert was subcloned and both strands were sequenced.

In order to identify the chromosome on which the *KICPF1* gene is located, chromosomal DNA was isolated and separated by pulsed-field gel electrophoresis and subsequently Southern blotted. Using a labelled 3-kb *EcoRI* fragment of plasmid pKICPF1#3, the *KICPF1* gene was allocated to chromosome II (Fig. 3).

Features of the *KICPF1* protein

The sequence of the *KICPF1* gene predicts a protein of 358 amino-acids with a calculated molecular weight of approximately 40.5 kDa, and an iso-electric point of 4.4. In

Fig. 4 the amino-acid sequence of KICPF1 is aligned with that of the ScCPF1 protein and identified sequence motifs are indicated. The proteins show an overall identity of 31.3% and are 57.8% homologous. The N-terminal region lacks obvious similarity, whereas the C-terminal region, starting from residue 205 and 231 of ScCPF1 and KICPF1 respectively, is significantly conserved, i.e., 67.2% identical and 86.4% homologous.

The N-terminal region contains a stretch of approximately 40 acidic residues, that are likely to form an α -helix. Such an acidic region is a common feature of the activation domains of many transcription factors and this feature suggests that the KICPF1 protein may also act as a transcriptional activator in *K. lactis*. Immediately N-terminal of the basic region (residues 223–234 in ScCPF1 and 249–260 in KICPF1), a stretch of eight amino acids is strongly conserved between *S. cerevisiae* (aa 205–212) and *K. lactis* (aa 231–238). This region is also basic and is predicted to form a turn, according to the Chou-Fasman and Robson-Garnier algorithms (Chou and Fasman 1978; Garnier et al. 1978). In *S. cerevisiae*, this region separates the second acidic domain and the basic domain, both predicted to form α -helices. Like ScCPF1, KICPF1 possesses a basic-helix-loop helix as a DNA-binding domain, a feature shared by members of the myc-related transcription factor family. In addition, C-terminal of the second helix, a leucine-zipper (ZIP) motif is present. Characteristic for this motif is the presence of an amphiphilic α -helix. This amphiphilic nature is clearly illustrated by the helical wheel representation, as depicted in Fig. 5. Residues at positions **a** and **d**, which are mostly branched-chain neutral amino-acids, like leucine, together form a hydrophobic spine. Charged residues at positions **c** and **g** may help to stabilise the dimer by forming intra-helical salt bridges. Recently, Dowell et al. (1992) have presented experimental evidence that this C-terminal region is involved in dimerisation of the ScCPF1 protein. The similarity in the ZIP region is lower than in the bHLH region, illustrating once more that, as has recently been reported by Pu and Struhl

Fig. 4. Complete amino-acid sequences of ScCPF1 (X52137) and KICPF1 (X76028) were aligned using Clustal V (Higgins and Sharp 1988). Identical residues and homologous substitutions are indicated by asterisks and dots respectively. A gap in the alignment is depicted by a bar. The acidic domains A1 and A2 of ScCPF1 (Mellor

et al. 1990) and of KICPF1 are boxed. Motifs found in the C-terminal region are indicated above the alignment. The amino acids of the ZIP motif located at the **a** position (see Fig. 6) are indicated by a white box and those at the **d** position with a black box

Fig. 5. Helical wheel representation of the ZIP regions of both ScCPF1 and KICPF1. The dimerisation region of a leucine zipper is predicted to form a coiled-coil structure (O'Shea et al. 1989). Align-

ment of the monomers results in formation of a hydrophobic core by residues at positions **d** and **a**. Hydrophobic residues are boxed, whereas charged residues are indicated by a circle

	<--basic-->	<--helix I-->	<--loop-->	<--helix II-->	
ScCPF1	223	RKDSHKEVERRRRRNTAINVLSDDL	-----VRESS	KAAILARAAEYIQKLKETDEANIEKWT	283
KICPF1	249	RKESHKEVERRRRRQNTAIEKLSDDL	-----VKETS	KAAILSRAAEYIQMKKETETANIEKWT	309
hUSF	199	RRAQHNEVERRRRDKINNWIVQLSKI	DCSMESTKSGQS	KGGILSKACDYIQELRQSN-----	258
XI-TFB1	193	RRAQHNEVERRRRDKINNWIVQLSKI	DCSMESTKSGQS	KGGILSKACDYIQELRQSN-----	251
Xb-TFB1	197	RRAQHNEVERRRRDKINNWIVQLSKI	DCSMESTKSGQS	KGGILSKACDYIQELRQSN-----	255
		* * * * *		* * * * *	
		<-- leucine zipper -->			
ScCPF1		QKLSE-QNASQLASANEKLQEEELGNAYKEIEYMKRVLRKEGIEYEDMHTHKKQENERKSTRSDNPHEA			351
KICPF1		QKLGE-QQVSSLTSAADKLEQLSKAYKNLQELKKLKEAGIEDPTEEE			358
hUSF		HLSEELQGLDQLDNDVLRQVDEKNNKLLLRQLRHHGLEVVIKNSDN			310
XI-TFB1		LRLSEELQNVLDQLMDNELLRQVDEKNNKLLLRQLRHHGVEIISKDGR			303
Xb-TFB1		LRLSEELQNLDDQLMDNEVLRQVDEKNNKLLLRQLRHHGVEIISKDTH			307
		* * * * *			

Fig. 6. Alignment of the relevant C-terminal region of ScCPF1, KICPF1, human USF (P22415), *Xenopus laevis* (B39674) and *Xenopus borealis* (A39674). The positions of the first and last amino acid of each region are indicated, as are also the basic-helix-loop-helix and the leucine-zipper motifs. Identical amino acids are indicat-

ed with asterisks and homologous substitution with dots. A gap in the alignment is depicted by a bar. The residues of position **a** and **d** (see Fig. 6) of the amphipathic α -helix, of the ZIP motif, are indicated by white and black boxes, respectively

(1993), the primary sequence is less important for this motif.

Sequence comparison

The amino-acid sequence was compared with sequences present in databases using (T)FastA (Pearson and Lipman 1988). Not surprisingly, high similarity scores were found with proteins possessing a basic-helix-loop-helix motif. Figure 6 shows the alignment of the relevant C-terminal regions of both CPF1 proteins, together with the transcription factor B1 of *Xenopus laevis* and *Xenopus borealis* (Kaulen et al. 1991), and USF of human (Gregor et al. 1990).

The sequences of the basic domain and the two α -helices are highly conserved. The length and the sequence of the loop between the two α -helices is conserved between the two yeast species; the same is true for the higher eukaryotes. However, no significant sequence similarity is observed between these two groups. Although the distance of the leucine zipper to the C-terminal α -helix is different, the presence of this motif is clear in all proteins depicted.

KICPF1 binds to the ScCPF1 consensus binding site

DNA-binding proteins possessing a bHLH motif all bind as dimers and their binding sites share the common sequence motif CANNTG. Flanking sequences determine the exact binding site for any given protein. The binding sequence of the ScCPF1 protein is RTCACRTG, which is identical to the CDEI element (Hieter et al. 1985). The high degree of amino-acid identity in the DNA-binding motif between ScCPF1 and KICPF1 suggests that the consensus binding site is also conserved in *K. lactis*. To test this hypothesis, we isolated cell-free extracts and incubated these with the QCR8 oligonucleotide (Fig. 7). Except for the XhoI linker, this oligonucleotide is identical to the region from position -253 to -223 of the QCR8 gene (Maarse and Grivell 1987) and contains an overlapping binding site for ABF1 and CPF1 (Dorsman et al. 1988). Dorsman et al. have demonstrated that both these proteins bind to this sequence in a mutually-exclusive fashion (Dorsman et al. 1988; Maarse et al. 1988).

Incubation with an extract derived from *S. cerevisiae* strain YPH266 gave rise to complexes with either ScCPF1 or ScABF1, whereas for the isogenic *cpf1*-null strain

Fig. 7. The *QCR8* oligonucleotide (MB530/MB531) was labelled and incubated with approximately 10 µg of cell-free extract of the strains indicated for 10 min at 30°C. For competition a 50-fold excess of unlabelled *QCR8* oligonucleotide was incubated for 10 min at 30°C, prior to adding the labelled *QCR8* oligonucleotide. Protein-DNA complexes are indicated by the name of the protein that binds to the oligonucleotide

YSS91 only the ScABF1-DNA complex was formed. YSS91, complemented with KlCPF1, gave rise to a prominent KlCPF1-DNA complex, which was also obtained when an extract of the wild-type *K. lactis* CBS2359 was used. Because the *KlCPF1* gene is expressed from the multi-copy vector YEplac195, the amount of CPF1 protein present in the complemented YSS91 strain is higher than in wild-type *K. lactis* and *S. cerevisiae* strains. The ScABF1-, ScCPF1- as well the KlCPF1-DNA complexes are competed when the unlabelled *QCR8* oligonucleotide is added in a 50-molar excess. In addition, a 30-bp oligonucleotide, containing the CDEI element of ScCENIV, was also able to compete both ScCPF1- and KlCPF1-specific complexes (data not shown). These results show that the KlCPF1 and ScCPF1 proteins share the same binding site. Although KlCPF1 has a somewhat higher molecular weight than ScCPF1, it migrates significantly faster when complexed to the DNA as a dimer. This is likely to be the result of differences in charge and/or secondary structure.

The KlCPF1 gene is essential for cell viability in K. lactis

ScCPF1 belongs to a group of abundant multi-functional DNA-binding proteins whose members, like ABF1, RAP1 and REB1, are thought to be involved in the regulation of several metabolic pathways (Diffley 1992). The *ScCPF1* gene is exceptional in this group in that gene disruption is not lethal, but has only minor effects including slow growth, partial chromosome loss, and methionine auxotrophy. To test the effects of gene disruption in *K. lactis* we cloned the *ble* gene, originally derived from transpo-

Fig. 8. Tetrad analysis showing that *KICPF1* gene-disruption using the phleomycin resistance gene, *Tn5-ble*, in *K. lactis* is lethal. All tetrads consist of four spores (*A–D*) of which only two are viable and phleomycin-sensitive. In this figure the result is shown for only ten tetrads out of a total of 40

son Tn5 (Gatignol et al. 1987), into the *KICPF1* gene, as detailed in Materials and methods and schematically depicted in Fig. 2. *K. lactis* diploid strain GG902 was transformed with a 5-kb *SphI-KpnI* fragment, containing the *KICPF1* gene disrupted by the *ble* gene. Transformants were selected for phleomycin resistance. Chromosomal DNA was isolated and used in Southern-blot analysis, which revealed that a high percentage of the phleomycin-resistant transformants contained the disruption fragment integrated at non-homologous positions in the genome (data not shown). This phenomenon of a high integration at non-homologous sites in *K. lactis* has also been observed by others (Stark and Milner 1989; Mulder et al. 1994). Apparently, the process of homologous recombination in *K. lactis* is less sequence-specific than it is in *S. cerevisiae*. Three diploids, having a correctly-disrupted *KICPF1* gene, were sporulated and subjected to tetrad analysis. Forty tetrads per diploid were analysed. All tetrads contained two spores which were not viable, whereas the other two were sensitive to phleomycin (Fig. 8). Non-viable spores did germinate, but stopped growing after 2–5 cell divisions. This result shows that deletion of the *KICPF1* gene is lethal in *K. lactis* but, in order to demonstrate unequivocally that the *KICPF1* gene has been correctly disrupted, we cloned the *APT1* gene, providing geneticin resistance, on the pRS/K1 vector, yielding pRS/K1-*APT1*, and used this to transform the diploid GG1900. Geneticin-resistant transformants were subjected to random spore analysis; 170 spores were tested for both phleomycin and geneticin resistance. All phleomycin-resistant spores were also geneticin-resistant, showing that lethality of *KICPF1* gene-disruption could be overcome by plasmid-borne *KICPF1*.

Thus, in contrast to the situation in *S. cerevisiae*, *KICPF1* gene-disruption is lethal in *K. lactis*, indicating that the KICPF1 protein plays an essential role in cell survival.

CPF1 is exchangeable between S. cerevisiae and K. lactis

Given that in *S. cerevisiae* both CPF1 proteins are interchangeable with respect to the methionine auxotrophic phenotype, we wondered whether ScCPF1 was also able to overcome the lethal effects of *KICPF1* gene-disruption in *K. lactis*. Both plasmids pRS/K1 and pRS/S1 were transformed to diploid GG1901. Several uracil prototrophic

Table 3.

Spore	Gene present	Generation time (h)	$\mu_{\max}$ (h ⁻¹)
A	<i>KICPF1</i>	1.74±0.05	0.404±0.014
A	<i>ScCPF1</i>	1.74±0.04	0.385±0.054
B	<i>KICPF1</i>	1.76±0.06	0.398±0.012
B	<i>ScCPF1</i>	1.73±0.23	0.416±0.065

diploid transformants were sporulated and spores were randomly selected and subsequently tested for phleomycin resistance and uracil prototrophy. All phleomycin-resistant spores were also uracil prototrophic, irrespective of whether the *KICPF1* or the *ScCPF1* gene was present (data not shown), thus confirming that the *S. cerevisiae* CPF1 protein can indeed overcome the lethal effects of *KICPF1* gene-disruption in *K. lactis*.

In order to ascertain whether the presence of either *ScCPF1* or *KICPF1* protein results in a difference in growth rate in *K. lactis*, two different spores, A [*ura3-100*, *Klcpf1::Tn5-ble*, (pRS/K1)] and B [*ura3-100*, *Klcpf1::Tn5-ble*, (pRS/S1)], were used. Both strains were transformed with both the pRS/K1*APT1* and the pRS/*SAPT1* plasmid, resulting in geneticin- and phleomycin-resistant transformants. The pRS/K1 or pRS/S1 plasmids were eliminated through plasmid shuffling, as described in Materials and methods. The four resulting strains were grown in rich-glucose medium and the generation time and the maximal growth rate were determined, as shown in Table 3. No significant difference in growth rates was observed. Despite possible differences in the range of functions, it is thus clear that the homologous CPF1 proteins fulfil similar roles in *K. lactis* with respect to their ability to overcome the lethality of *cpf1* gene-disruption and to maintain wild-type growth rate characteristics. Similar results were obtained for the *S. cerevisiae* *cpf1*-null strain, YSS91, transformed with either pRS/S1 or pRS/K1 (data not shown).

Discussion

The yeasts *K. lactis* and *S. cerevisiae* are closely related to one another in terms of the sequences of many of the genes so far analysed. Nevertheless, each displays a characteristic lifestyle and metabolism, indicative of the existence of perhaps widespread differences in regulatory patterns. Such differences have indeed been confirmed both at the level of individual metabolic pathways (Kuzhandavelu et al. 1992; Mazzoni et al. 1992; Wésolowski-Louvel et al. 1992) and of the sequence/structure of an increasing number of regulatory proteins (Gonçalves et al. 1992; Na and Hampsey 1993; Zachariae and Breunig 1993; Mulder et al. 1994). Strikingly, despite sometimes quite extensive differences in sequence and overall organisation, the *K. lactis* proteins often appear to be functionally homologous to their *S. cerevisiae* counterparts, as evidenced by the fact that their coding sequences have been isolated by the technique of functional complementation of the appropriate *S. cerevisiae* null mutant. In many instances, comparison of

the coding sequences of the homologues has yielded valuable information on regions of the protein involved in specific functions. We have used functional complementation to isolate the *K. lactis*-equivalent of the *S. cerevisiae* CPF1 factor and report here on the sequence features displayed by the protein and on the ability of both proteins to substitute for each other in functions related to both chromosome maintenance and transcriptional activation.

The presence of an extended acidic region in the N-terminal part of the *KICPF1* protein suggests that in *K. lactis* the protein may be involved in transcription activation. Whether *KICPF1* itself can function as a transcriptional activator has yet to be established. In *S. cerevisiae*, *ScCPF1* alone does not seem to be capable of activation (Fisher and Goding 1992; Thomas et al. 1992), although the factor is necessary to obtain full levels of activation of several of the *MET* genes (Thomas et al. 1992). How this latter effect is achieved is not yet clear. The factor could possibly influence the positioning of nucleosomes at the promoter region of these genes, as suggested previously (Mellor et al. 1990), and may allow or hinder the binding of other transcription factors to the promoter region.

The bHLH motif is shared by various DNA-binding proteins and has been conserved during evolution from yeast to man. We show that the DNA-binding site of CPF1 is also conserved between *S. cerevisiae* and *K. lactis*. In the light of the result obtained by Fisher and Goding (1992) for the *ScPHO4* protein, it is interesting to note that, within the basic domain, *KICPF1* contains a Glu residue at position 249, while *ScCPF1* contains an Asp at position 225. In the *ScPHO4* protein there is also a Glu present at the homologous position in the basic domain. Substitution of this Glu residue by Asp resulted in a restriction of the DNA sequence-specificity of the *ScPHO4* protein to that of the *ScCPF1* protein. It is conceivable that the DNA sequence-specificity of *KICPF1* is influenced by amino-acids positioned more towards the N-terminus, but it is tempting to speculate that *KICPF1* is playing roles in *K. lactis* which in *S. cerevisiae* are fulfilled by *PHO4*. The findings of Dowell et al. (1992), together with the amino-acid sequence of both *ScCPF1* and *KICPF1*, show that the homologous CPF1 proteins contain a leucine-zipper (ZIP) motif. CPF1 is thus a full member of the myc-related transcription factors family.

Although the N-terminal two-thirds of the CPF1 proteins shows no significant similarity at the amino-acid sequence level, the proteins are functionally interchangeable between *S. cerevisiae* and *K. lactis*, yielding cells with indistinguishable phenotypes. It is thus possible that the C-terminal region, including the bHLH-ZIP motifs, is sufficient for this function of CPF1. This idea is supported by the observation of Dowell et al. (1992) that the N-terminal region of *ScCPF1* is dispensable with respect to the maintenance of methionine prototrophy. The ability of *ScCPF1* to overcome the lethal effects of *KICPF1* gene-disruption suggests that in *K. lactis* this N-terminal region is also dispensable for at least some of the functions of *KICPF1*.

The role *KICPF1* plays in chromosome segregation in *K. lactis* was not separately tested, but the observation that

the DNA sequence of the CDEI element of *K. lactis* centromeres is conserved (Heus et al. 1993) strongly suggests that KICPF1 fulfils a similar role in this process in *K. lactis*.

The reason for the lethal effect of *Klcpf1* gene-disruption in *K. lactis* is as yet unclear. We propose two possibilities. First, homologous factors which can overcome the lethal effect in *S. cerevisiae* may have diverged in, or be absent from, *K. lactis*. Second, KICPF1 may fulfil additional, essential roles, such as transcriptional activation of genes encoding enzymes within major biosynthetic or metabolic pathways or other proteins necessary for cell viability, comparable to, but more important than the activation of the *MET* genes in *S. cerevisiae*.

The more pronounced features of the KICPF1 protein and the lethal effect of gene disruption should, however, facilitate further functional analysis of CPF1 and the demarcation of domains that are involved in transcription activation and chromosome segregation.

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