Mol Gen Genet (1994) 245:716–723

ORIGINAL PAPER

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Molecular cloning and analysis of *CDC28* and cyclin homologues from the human fungal pathogen *Candida albicans*

Received: 13 May 1994 / 10 June 1994

Abstract In the budding yeast *Saccharomyces cerevisiae*, progress of the cell cycle beyond the major control point in G1 phase, termed START, requires activation of the evolutionarily conserved Cdc28 protein kinase by direct association with G1 cyclins. We have used a conditional lethal mutation in *CDC28* of *S. cerevisiae* to clone a functional homologue from the human fungal pathogen *Candida albicans*. The protein sequence, deduced from the nucleotide sequence, is 79% identical to that of *S. cerevisiae* Cdc28 and as such is the most closely related protein yet identified. We have also isolated from *C. albicans* two genes encoding putative G1 cyclins, by their ability to rescue a conditional G1 cyclin defect in *S. cerevisiae*; one of these genes encodes a protein of 697 amino acids and is identical to the product of the previously described *CCN1* gene. The second gene codes for a protein of 465 residues, which has significant homology to *S. cerevisiae* Cln3. These data suggest that the events and regulatory mechanisms operating at START are highly conserved between these two organisms.

Key words *Candida albicans* · *CDC28* · G1 cyclins

Introduction

In budding yeast, as in all eukaryotes, the mitotic cell cycle can be divided into four intervals, G1-, S-, G2- and M phase. Overall control of cell division is achieved principally by regulating entry into S phase, the period of DNA synthesis, or into M phase when nuclear division and mitosis occur. In *Saccharomyces cerevisiae*, the major controlling event, termed START, occurs late in the G1 phase (Pringle and Hartwell 1981). At START, environmental signals such as nutrient availability or the presence of mating pheromone are monitored; only under appropriate conditions will cells traverse START and become committed to a round of mitotic division (for recent reviews see Sherlock and Rosamond 1993; Nasmyth 1993).

Passage through START requires the activation of a 34 kDa serine/threonine protein kinase, which in *S. cerevisiae* is encoded by the *CDC28* gene (Piggot et al. 1982). This protein is the functional homologue of the $cdc2^+$ gene product of the fission yeast *Schizosaccharomyces pombe* (Beach et al. 1982) and these two proteins serve as the paradigm for the *cdk* family of protein kinases in higher eukaryotes (Nurse 1990). The enzymic activity of Cdc28 at START is regulated at least in part by assembly of the kinase catalytic subunit into a complex with members of a family of labile proteins, the G1 cyclins (Richardson et al. 1989). In *S. cerevisiae*, at least nine proteins with potential G1 cyclin function have been identified and, although the roles of the different cyclins is unclear, it is thought that they may provide substrate specificity for the Cdc28 kinase complex (for example, see Cvrčková and Nasmyth 1993).

We have used *S. cerevisiae* as a surrogate genetic system to investigate the molecular mechanism of cell cycle control in the evolutionarily related yeast *Candida albicans* (Chen et al. 1984; Hendriks et al. 1989). *C. albicans* is an asexual diploid opportunistic human pathogen that is capable of growing with either a yeast or a hyphal morphology (for review see Scherer and Magee 1990). The factors that determine and regulate the morphogenetic choice seem likely to be important pathogenic determinants; although both morphologies are generally observed in disseminated infections (Odds 1987), various lines of evidence suggest a specific role for the yeast-hyphal transition in pathogenesis (Soll 1988).

Communicated by B. J. Kilbey

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As a first approach to the analysis of the *C. albicans* cell cycle and the relationship between cell cycle regulation and the yeast-hyphal dimorphic transition, we have screened a library of *C. albicans* genomic DNA for genes that rescue conditional lethal mutations in genes needed for the completion of START in *S. cerevisiae*. In this paper, we describe the isolation and molecular characterisation of *CDC28* and two cyclin homologues from *C. albicans*.

Materials and methods

Yeast strains and methods

The *S. cerevisiae* strains used in this work were: SB860 *cdc28-6 ura3-52 leu2 tyr1 trp1*; SB847 *cdc28-4 his3 leu2 ade2 ura3 metx* from Clive Price, University of Sheffield, UK; and BF305-15dno.21 *MAT***a** *leu2-3,-112 his3 ura3 trp1 ade1 met14 arg5,6 HIS3::cln1 TRP1::cln2 ura3::GAL1-CLN3* from Bruce Futcher, Cold Spring Harbor Laboratory, New York (Xiong et al. 1991). *C. albicans* strain 124 was obtained from Richard Barton, University of Manchester. All strains were grown on media containing 2% peptone, 1% yeast extract supplemented with either 2% glucose (YEPD) or 1% galactose and 1% raffinose (YEPGR). Supplemented synthetic minimal medium (YNB) comprising 0.67% yeast nitrogen base, 2% glucose and appropriate nutritional supplements was used for the selection and maintenance of plasmids in *S. cerevisiae*. Standard yeast genetic and recombinant techniques were used (Sherman et al. 1986). Yeasts were transformed using the lithium acetate procedure with single-stranded carrier DNA (Schiestl and Gietz 1989).

Bacterial strains and methods

Escherichia coli strain HW87 (Patterson et al. 1986) was used as the routine host for maintenance and storage of plasmids. Cultures were typically grown in L-broth (Miller 1972) supplemented when necessary with 50 μg/ml ampicillin. Plasmid DNA was extracted from bacterial cultures either by alkaline lysis (Birnboim and Doly 1979) or by detergent lysis followed by CsCl-ethidium bromide equilibrium density gradient centrifugation (Humphreys et al. 1975). *E. coli* HW87 was transformed either by the method of Warren and Sherratt (1978) or by electroporation (Dower et al. 1988).

Nucleic acid methods

Yeast genomic DNA was prepared from logarithmic-phase cell cultures as described previously (Cryer et al. 1975). Standard recombinant DNA techniques were used throughout (Sambrook et al. 1989). Restriction endonucleases, T4 DNA ligase and Klenow fragment of DNA polymerase I were purchased from Boehringer Mannheim. Sequenase version 2.0 was purchased from United States Biochemical Co. and used according to the manufacturer's recommendations. DNA fragments were radioactively labelled by random oligonucleotide priming (Feinberg and Vogelstein 1983) using a kit from Boehringer Mannheim and [α-^{32}P]dATP from New England Nuclear. DNA sequences were determined using the chain-termination method (Sanger et al. 1977) for direct plasmid sequencing on both strands (Zhang et al. 1988) using [α-^{35}S]dATP. Oligonucleotide primers were synthesised on an ABI381 Synthesiser using phosphoramidite chemistry. Reaction products were resolved and detected as described previously (Patterson et al. 1986). The deduced sequence was analysed using University of Wisconsin Genetics Computer Group (GCG) software on the Daresbury database facility.

Results

Construction of a *C. albicans* genomic library

A high-copy-number library of genomic DNA sequences from *C. albicans* strain 124 was generated using high molecular weight DNA that had been partially digested with *Sau*3A. The digested DNA was size fractionated to 3–12 kb by centrifugation through 5–20% neutral sucrose gradients (Rosamond et al. 1979) and cloned into the *Bam*HI site of the shuttle vector YEp24, which carries the yeast *URA3* gene and 2 μm replication origin (Hurley and Donelson 1980). The library contains 2.5×10^4 independent plasmids of which 70% are recombinant. The average size of the inserts is 7.3 kb. Since YEp24 lacks sequences to direct the expression of cloned DNA, the expression of genes from within the *C. albicans* genomic DNA inserts of the recombinant plasmids relies upon adjacent *C. albicans* regulatory elements.

Cloning and identification of *CaCDC28*

The *CaCDC28* gene was cloned by screening the *C. albicans* genomic library in YEp24 for genes that could suppress the temperature-sensitive lesion in *S. cerevisiae* SB860 (*cdc28-6*). Approximately 6000 Ura$^+$ transformants were obtained initially at 23°C. These cells were recovered in pools of approximately 10^3 transformants and aliquots of each pool were replated on YEPD agar at 37°C. Plasmid DNA was rescued from colonies that grew at the restrictive temperature, amplified in *E. coli* and used to re-transform *S. cerevisiae* SB860 to uracil prototrophy and temperature resistance. From this screen, a single plasmid was isolated that carried a 7.5 kb genomic fragment capable of rescuing both the *S. cerevisiae cdc28-6* and *cdc28-4* mutations. This plasmid was designated pANJ1 (Fig. 1A).

To delimit the region of the genomic fragment cloned in pANJ1 that was responsible for complementation of *cdc28*, we subcloned portions of pANJ1 and tested the ability of the subclones to rescue growth at the restrictive temperature in *S. cerevisiae* SB860. A subclone carrying the 3.5 kb *Sph*I fragment (pANJ2; Fig. 1A) was unable to complement *cdc28-6*. However, a subclone that carried the 3.8 kb region from the *Sph*I site to the end of the insert (pANJ3) was able to rescue *cdc28* as effectively as pANJ1 (Fig. 1A). We conclude therefore that pANJ3 contains all of the elements essential for complementation of *cdc28*.

Nucleotide sequence of *CaCDC28*

Using synthetic oligonucleotide primers, we have determined the complete nucleotide sequence of *CaCDC28* within the cloned DNA fragment of pANJ3. The se-

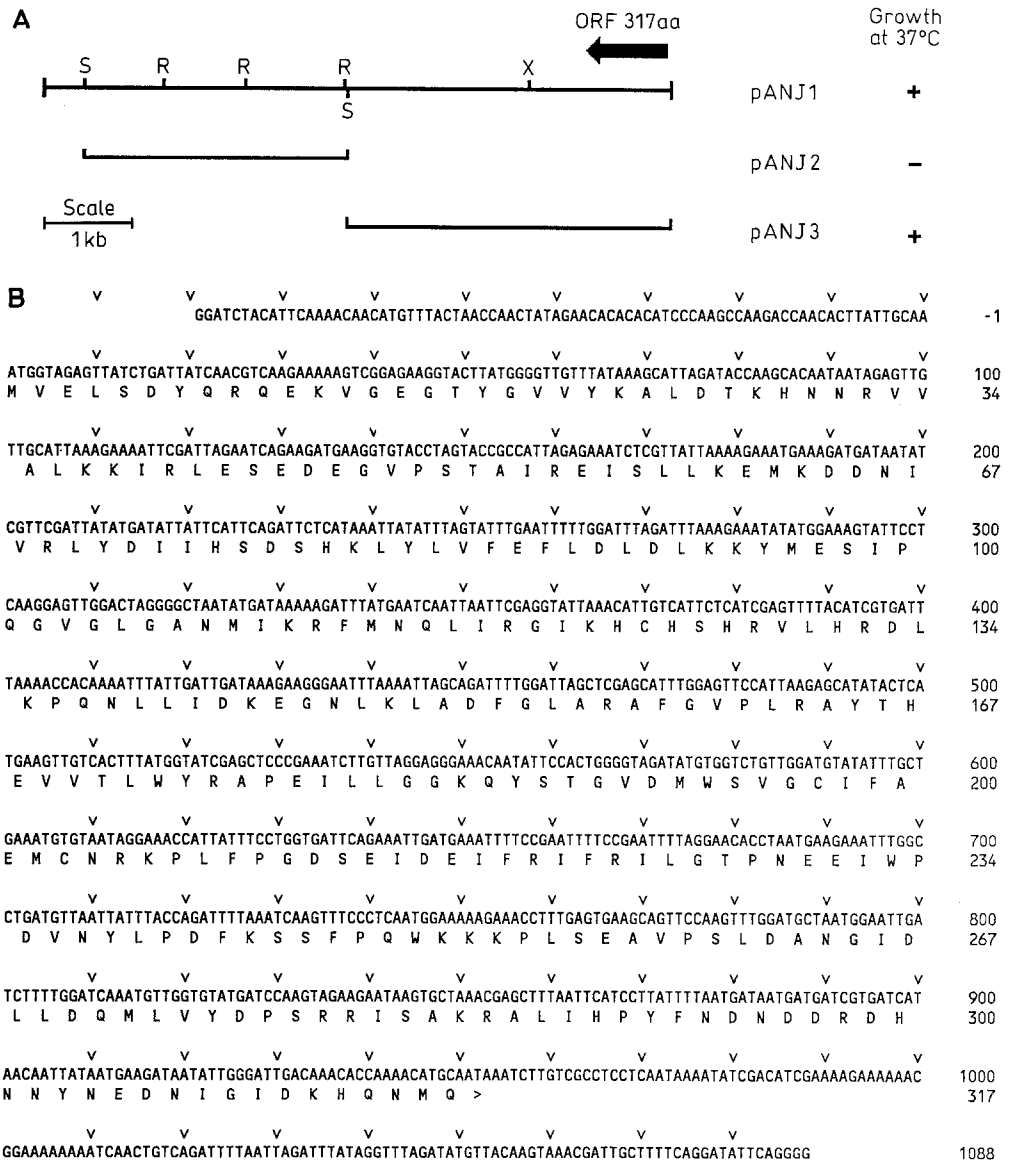

Fig. 1A,B Characterisation of *CaCDC28*. **A** Partial restriction map and complementation analysis of *CaCDC28* subclones. Complementation was assayed by the ability of subclones to restore growth of the *cdc*28-6 strain at 37°C; + indicates growth, – indicates no growth. The *large arrow* shows the location, size and direction of transcription within the cloned DNA of the *CaCDC28* open reading frame. Abbreviations of restriction enzyme sites are as follows: R, *Eco*RI; S, *Sph*I; X, *Xho*I. **B** Nucleotide and deduced amino acid sequence of *Candida albicans CDC28*. Nucleotides are numbered with respect to the first ATG of the open reading frame (ORF). This nucleotide sequence will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X80034

quence contains an open reading frame of 317 codons, potentially encoding a protein of 36645 daltons, the location of which is consistent with the subcloning data (Fig. 1A, B). The regions flanking the open reading frame contain motifs frequently found adjacent to coding regions in yeast, including a TATA box at position −40 relative to the initiation codon, as well as consensus transcription termination and polyadenylation signals in the 3′ flanking region between nucleotides 971–1073 (Fig. 1B; Zaret and Sherman 1982). Comparison of the predicted protein product of *CaCDC28* with *S. cerevisiae* Cdc28 showed that the proteins were 79% identical over 295 amino acids, and that CaCdc28 contains all of the motifs characteristic of protein kinases in general and the Cdc28 protein kinase in particular (Fig. 2; Hanks and Quinn 1991).

Cloning and identification of *CaCLN* genes

Since Cdc28 protein kinase activity is regulated in part by interaction with cyclins (Richardson et al. 1989), we screened the *C. albicans* genomic library for genes encoding putative G1 cyclins. For this purpose we used *S. cerevisiae* strain BF305-15dno.21, which is deleted for *CLN1* and *CLN2* and dependent for viability on the galactose-inducible expression of *CLN3* (Xiong et al. 1991). *S. cerevisiae* BF305-15dno.21 was grown in YEP-GR and transformed with DNA from the *C. albicans* genomic library. Cells were screened for plasmid-borne cyclin genes by plating directly onto minimal YNB medium lacking uracil and supplemented with glucose. Two colonies were obtained (from a total of approximately 5×10^3 Ura^+ transformants); plasmid DNA was recovered from each of these clones, amplified in *E. coli* and used to re-transform BF305-15dno.21 to uracil prototrophy and galactose independence. From this screen

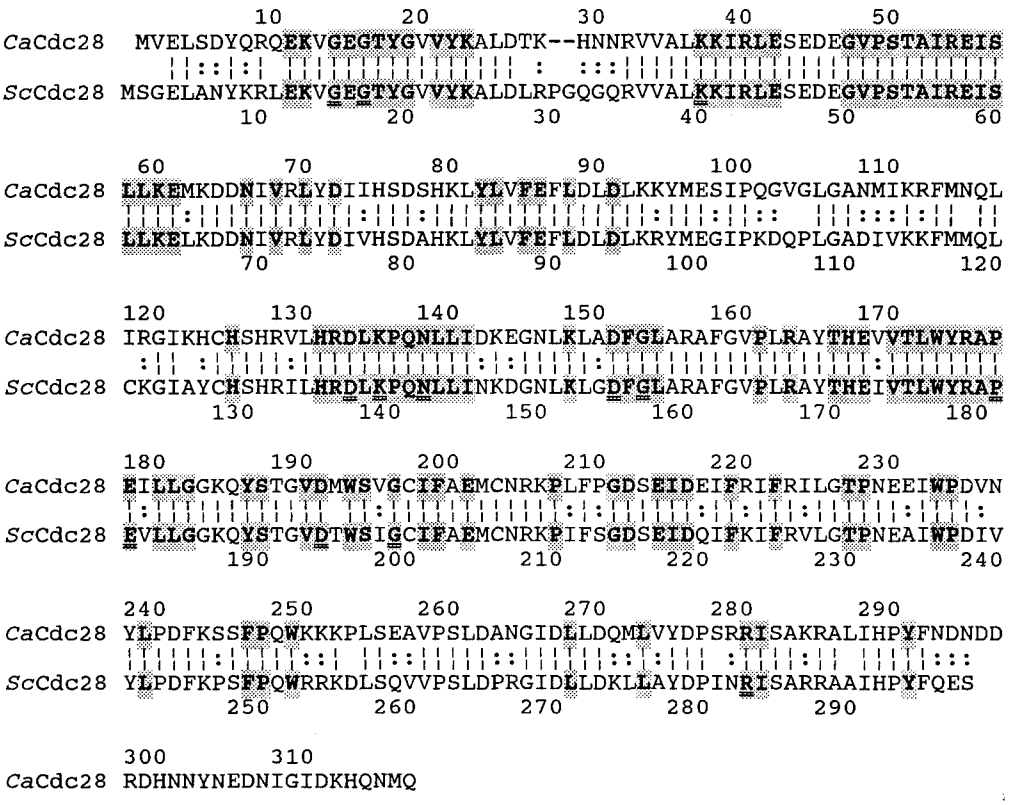

Fig. 2 Comparison of the *Candida albicans* and *Saccharomyces cerevisiae* Cdc28 proteins. Proteins were aligned using FASTA on the Daresbury SEQNET facility. Amino acid identities are indicated by *dashes*; conservative substitutions are indicated by *colons*. Key residues conserved within protein kinases are *underlined*; residues characteristic of Cdc28/cdc2 kinases are *shaded*.

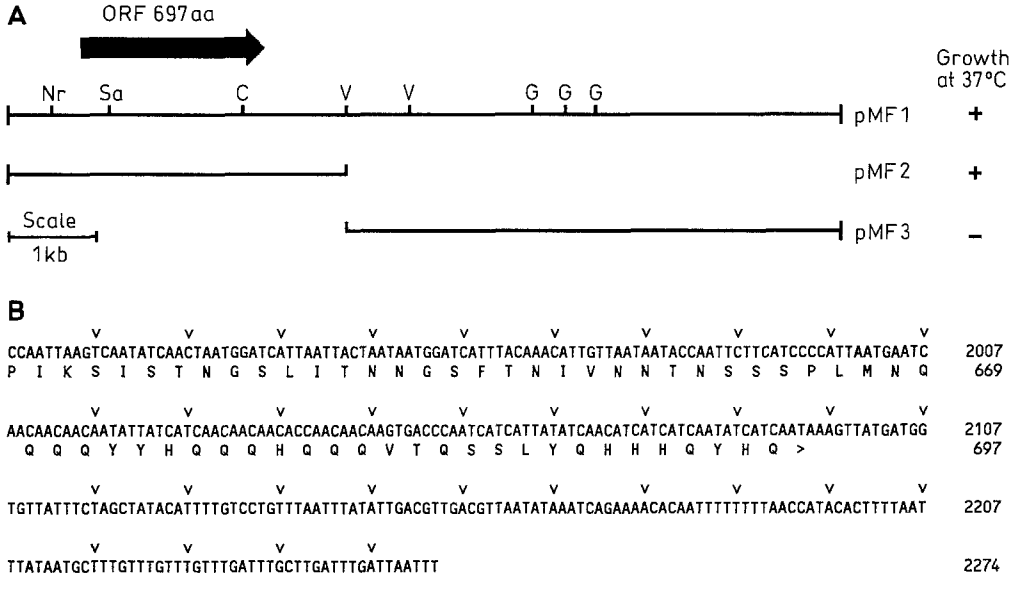

Fig. 3A,B Characterisation of *CaCLN1*. **A** Partial restriction map and complementation analysis of *CaCLN1* subclones, for which symbols used are as described in the legend to Fig. 1. Restriction site abbreviations: C, *Cla*I; G, *Bgl*II; Nr, *Nru*I; Sa, *Sal*I; V, *Eco*RV. **B** Nucleotide sequence of the C-terminal region and 3′-flanking region of *CaCLN1*. This sequence, together with 616 bp of 5′-flanking sequence, will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X80032 as an update to the previously reported partial ORF sequence (accession number M76587; Whiteway et al. 1992)

we obtained two independent plasmids, designated pMF1 and pGS1, which were defined as carrying *CaCLN1* and *CaCLN2*, respectively.

The genomic fragment cloned in pMF1, carrying *CaCLN1*, was characterised to localise the region required for complementation. This was achieved by subcloning portions of pMF1 and examining the ability of the subclones to rescue the conditional cyclin deficiency in *S. cerevisiae* BF305-15dno.21. The results (Fig. 3A) established that all of the elements required for complementation were located in the 4.0 kb region extending from an *Eco*RV site to the left vector-insert junction (pMF2; Fig. 3A). In the course of this work, it became apparent that the clone carrying *CaCLN1* was similar to the clone carrying the previously reported partial open reading frame, *CaCCN1*, which had been isolated on the basis of its ability to confer α-factor resistance in *S. cerevisiae* (Whiteway et al. 1992). From DNA sequencing we have established that *CaCLN1* is identical to *CaCCN1* for which a partial amino acid sequence has

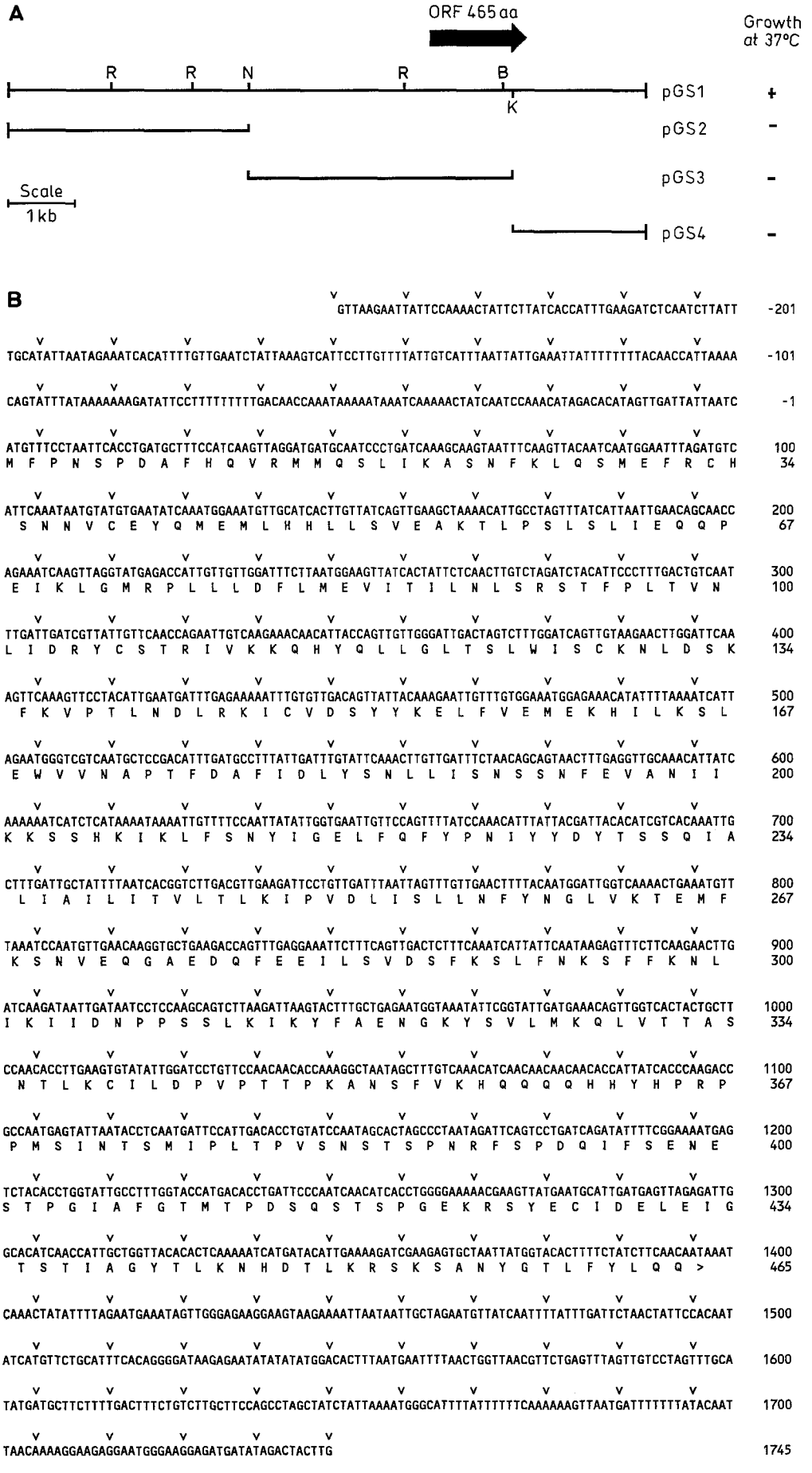

Fig. 4A,B Characterisation of *CaCLN2*. **A** Partial restriction map and complementation analysis of *CaCLN2* subclones, symbols used are as described in the legend to Fig. 1. Restriction enzyme abbreviations: B, *Bam*HI; K, *Kpn*I; N, *Nhe*I; R, *Eco*RI. The *large arrow* shows the location, size and direction of transcription of the *CaCLN2* open reading frame within the cloned DNA. **B** Nucleotide and deduced amino acid sequence of *C. albicans CLN2*. Nucleotides are numbered with respect to the first ATG of the open reading frame. Regulatory features are described in the text. The nucleotide sequence given here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X80033

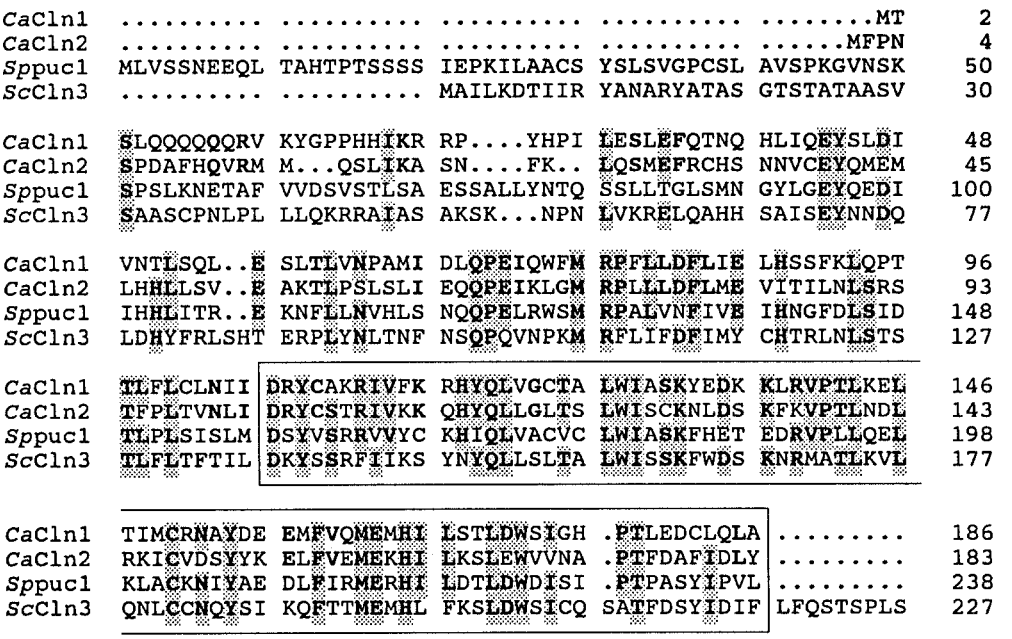

Fig. 5 Comparison of the N-termini of *Ca*Cln1, *Ca*Cln2, *S. cerevisiae* Cln3 (*Sc*Cln3) and *Schizosaccharomyces pombe* puc1$^+$ (*Sp*puc1). Sequences were aligned using the University of Wisconsin Genetics Computer Group (GCG) PILEUP program. *Dots* represent gaps introduced to maximise the alignment. Residues conserved in at least three of the proteins are *shaded*, while amino acids conserved between *Ca*Cln1 and *Ca*Cln2 are shown in *bold*. The cyclin box is *outlined*

been reported (Whiteway et al. 1992). We have confirmed that sequence and extended it to complete the sequence of the entire open reading frame together with 616 bp of 5′-flanking sequence and 158 bp of downstream sequence. Figure 3B shows the nucleotide and predicted protein sequence of the previously unreported C-terminal portion of CaCln1. The predicted gene product thus comprises 697 amino acids with a molecular mass of 79250 daltons.

Characterisation and nucleotide sequence of *CaCLN2*

The genomic fragment in pGS1 was characterised by restriction mapping, which demonstrated that it was unrelated to CaCLN1, and by subcloning to localise the complementing element. All three subclones derived from pGS1 failed to complement the conditional cyclin deficiency in strain BF305-15dno.21 (pGS2-4; Fig. 4A) implying that *CaCLN2* spanned either the *NheI* or *KpnI* site, or both. Using synthetic oligonucleotide primers,initial sequencing experiments demonstrated that the *KpnI* site was contained within an open reading frame, the predicted product of which had significant homology to *S. cerevisiae* Cln3. We have completed the sequence of the *CaCLN2* gene together with 255 nucleotides of 5′- and 347 nucleotides of 3′- flanking sequence (Fig. 4B). The gene encodes a protein of 465 amino acids with a molecular mass of 52000 daltons. In addition, the gene is flanked by typical regulatory motifs including a TATA box at position −90 relative to the initiation codon, and transcription termination and polyadenylation signals in the 3′-flanking region between nucleotides 1670–1750 (Fig. 4).

Comparison of CaCln2 with the EMBL protein sequence database revealed that the protein was most similar to the *S. cerevisiae* G1 cyclin Cln3, *C. albicans* Ccn1 (CaCln1) and *S. pombe* puc1$^+$, all of which complement the conditional G1 cyclin mutation in BF305-15dno.21. This homology is most obvious within the so-called cyclin box region of the proteins found in the N-terminal region of both CaCln1 and CaCln2 (Fig. 5). Within this domain, the proteins show 36–40% identity in pairwise comparisons. In addition, and in contrast to the parental strain which is unable to grow in the presence of 1 μM α-factor, cells overexpressing either *CaCLN1* or *CaCLN2* are resistant to mating pheromone and grow well in media supplemented with 10 μM α-factor. In both cases, overexpression occurs only as a consequence of the elevated copy number of the gene in YEp24, since both *C. albicans* genes are expressed in *S. cerevisiae* from their native promoters. In *S. cerevisiae*, the pheromone response pathway inhibits G1 cyclin activity to bring about cell cycle arrest (for review see Kurjan 1993). Therefore, while the finding is not altogether surprising that the overexpression of *CaCLN1* and *CaCLN2* results in α-factor resistance, the significance of this with respect to a possible response pathway in *C. albicans* is not clear.

Discussion

We have described the cloning and molecular characterisation of *CDC28* and two putative G1 cyclins from the human fungal pathogen *C. albicans*. The *CDC28* gene from *C. albicans* rescues *cdc28-6* and *cdc28-4* mutations in *S. cerevisiae*, while the protein products of the *C. albicans* and *S. cerevisiae* genes share 79% identity, making these two proteins the most similar within the *cdk* family (Hanks and Quinn 1991). The only difference worthy of note is a short extension of 22 residues at the C-terminus

of *C. albicans* Cdc28, which distinguishes it from other members of the p34 family though the functional significance of this difference is unclear.

In addition to *CaCDC28*, we have isolated two genes that encode cyclins in *Candida*. On the basis of the finding that the expression of these genes can rescue a triple *cln* mutation in *S. cerevisiae*, we suggest that these proteins have a similar function in *C. albicans*, and act as G1 cyclins to regulate Cdc28 protein kinase activity at START. This idea is supported by the observation that, in addition to being homologous to one another, both *Candida* cyclins are most similar to *S. cerevisiae CLN3* and *S. pombe puc1*$^+$, both of which also rescue a triple *cln* mutant (Forsburg and Nurse 1991). However, *puc1*$^+$ now appears to function as a meiotic rather than a G1 cyclin in *S. pombe* (Forsburg and Nurse 1994), and since *Candida* is unable to undergo meiosis, the significance of this similarity is unclear. *CLN1*, *CLN2* and *CLN3* form a functionally redundant gene family in *S. cerevisiae* (Richardson et al. 1989), though there are clear differences in the regulation and function of each of the gene products.

CLN3 is transcribed constitutively and regulated post-translationally by Swi4 and Swi6, whereas the transcription of both *CLN1* and *CLN2* is periodically regulated, peaking in G1 phase, by the Swi4/Swi6 transcription factor, SBF (Nasmyth and Dirick 1991). Also, it has been suggested that Cln3 functions upstream of Cln1 and Cln2 in order to regulate their activity (Tyers et al. 1993). We find that *CaCLN1* has sequences in its 5′-flanking region that are consistent with cell cycle regulation of its transcript in a manner analogous to *CLN1* and *CLN2* in *S. cerevisiae* (G. Sherlock, A. M. Bahman and J. Rosamond, in preparation). However, we have found no such sequence motifs upstream of *CaCLN2*, suggesting that it may in fact have a function more related to that of Cln3 than to Cln1 or Cln2. Such a categorisation requires, however, an analysis of the pattern of expression of these genes during growth and development; such an analysis is currently in progress.

Acknowledgements We would like to thank Clive Price and Bruce Futcher for gifts of plasmids and strains; Anne White, Mark Buddles, Eliot Randle and Penny Johnson for advice, assistance and useful comments. We gratefully acknowledge financial assistance from SERC (to J. R.) and the award of a Wellcome Prize Studentship (to G. S.).

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