Mol Gen Genet (1991) 229:1–9
002689259100271F

MGG

The *KNS1* gene of *Saccharomyces cerevisiae* encodes a nonessential protein kinase homologue that is distantly related to members of the *CDC28/cdc2* gene family

Ramesh Padmanabha, Sonja Gehrung, and Michael Snyder

Department of Biology, Yale University, New Haven, CT 06511, USA

Received September 17, 1990

Summary. A novel protein kinase homologue (*KNS1*) has been identified in *Saccharomyces cerevisiae*. *KNS1* contains an open reading frame of 720 codons. The carboxy-terminal portion of the predicted protein sequence is similar to that of many other protein kinases, exhibiting 36% identity to the *cdc2* gene product of *Schizosaccharomyces pombe* and 34% identity to the *CDC28* gene product of *S. cerevisiae*. Deletion mutations were constructed in the *KNS1* gene. *kns1* mutants grow at the same rate as wild-type cells using several different carbon sources. They mate at normal efficiencies, and they sporulate successfully. No defects were found in entry into or exit from stationary phase. Thus, the *KNS1* gene is not essential for cell growth and a variety of other cellular processes in yeast.

Key words: Protein kinase – Yeast – *CDC28* – Cell cycle

Introduction

Protein phosphorylation has been implicated in a wide variety of cellular functions as the mechanism by which both enzymatic and non-enzymatic activities of proteins are controlled (Krebs 1986). Events influenced by protein phosphorylation include enzyme regulation, assembly of cytoskeletal components, and cell cycle control. The *cdc2* gene of *Schizosaccharomyces pombe* and its closely related homologue in *Saccharomyces cerevisiae, CDC28*, encode protein kinases that are necessary for cell cycle control at G1/S and G2/M transitions (Wheals 1987). Several additional homologues of *CDC28* have been identified in *S. cerevisiae*. These include *KIN28*, an essential gene in yeast (Simon et al. 1986), *KSS1* and *FUS3*, two genes involved in growth control (Courchesne et al. 1989; Elion et al. 1990), and *PHO85*, a gene involved in negative regulation of the *PHO* genes (Toh-e et al. 1988). More distantly related members of this family include casein kinase II the catalytic subunits of which are encoded by two genes, *CKA1* and *CKA2* (Chen et al. 1988; Padmanabha et al. 1990). All of these proteins are thought to phosphorylate serine and threonine residues and in most cases this has been directly demonstrated.

It is presently unclear just how large the *CDC28* gene family is, and whether or not most members of this family participate in cell cycle regulatory functions is also not known. Described below is a novel protein kinase homologue which was identified while studying the organization of the *SPA2* region on chromosome XII of *S. cerevisiae* (Snyder 1989). This putative kinase gene is a distantly related member of the *CDC28* gene family.

Offprint requests to: R. Padmanabha

Materials and methods

General methods. Yeast growth media and genetic manipulations were as described in Sherman et al. (1986). All bacterial media and manipulations were as described in Maniatis et al. (1982). Yeast strains used are listed in Table 1.

DNA sequence analysis. Individual subclones in either M13mp18 or mp19 (Yanisch-Perron et al. 1985) were sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using the Sequenase kit (United States Biochemical) according to the recommendations of the manufacturer.

Gene disruption. A *Xho*I-*Spe*I DNA fragment containing a full-length *KNS1* gene was cloned into pUC18. The resulting plasmid was designated p217. *kns1-Δ1* was constructed by partially digesting this plasmid with *Sty*I and *Xba*I, the ends were made flush by treatment with the large fragment of DNA polymerase I plus dNTPs, and ligated to a 1.1 kb *Hin*dIII fragment containing the yeast *URA3* gene. The ends of the *URA3* DNA fragment had previously been made flush by treatment with the large fragment of DNA polymerase I. Similarly, *kns1-Δ2* was

Table 1. Yeast strains

Strains genotypes								
Y264	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*		
Y428	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*		
Y270	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*		
	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*		
Y650	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*	*kns1-Δ1::URA3*	
	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*	*KNS1*	
Y651	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*	*kns1-Δ2::URA3*	
	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200trp1-901*		*KNS1*	
Y652	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200trp1-901*		*kns1-Δ3:URA3*	
	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*	*KNS1*	
Y657	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*	*kns1-Δ1::URA3*	
Y659	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*HIS3*	*trp1-901*	*leu2-Δ98*	*KNS1*
	α	*ura3-52*	*lys2-801*	*ade2-101*	*his3-Δ200*	*trp1-901*	*LEU2*	*kns1-Δ1::URA3*
Y660	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
Y661	α	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
Y662	α	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
Y663	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
Y664	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
	α	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
Y665	*a*	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*
	α	*ura3-52*	*lys2-801*	*ade2-101*	*trp1-901*	*leu2-Δ98*	*his3-Δ200*	*kns1-Δ1::URA3*

All strains are congenic and in an S288C background. Y664 and Y665 are derived from different isolates

constructed by replacing the *Sty*I fragment with the *URA3* DNA fragment, and in *kns1-Δ3* the internal *Xba*I fragment was replaced with the *URA3* gene. The orientation of the *URA3* fragment in all the constructs was determined by restriction map analysis. Linearized DNA fragments containing the mutations were generated by digestion with *Eco*RI and *Hin*dIII and transformed into diploid yeast (Y270) (Ito et al. 1983; Rothstein 1983). The heterozygous diploids were then sporulated and the asci dissected (Sherman et al. 1986). Disruption of the *KNS1* genomic locus was confirmed by gel blot analysis of genomic DNA as described by Maniatis et al. (1982).

Phenotype studies. The growth rates of *KNS1* and *kns1-Δ1* strains were determined in YPD rich media. The doubling time was determined by measuring the OD_{600} of exponentially growing cultures every 4–5 h starting at $OD_{600} = 0.2$, for a total of 24 h. Mating efficiencies were determined by mixing equal numbers (2.5×10^6 cells; 1 ml total volume) of exponentially growing *MATa* and *MATα* cells and allowing them to stand upright in an 18 mm tube without agitation at 30° C. After 5 h, the cultures were vortexed vigorously and serial dilutions of the cell suspensions plated on selective media to determine the number of diploids. For each mating experiment between 150 and 280 diploids were counted. Sporulation efficiencies were determined essentially as described by Sherman et al. (1986). Stationary phase cells were washed in 1% potassium acetate and then incubated in 1% potassium acetate at 30° C for 3 days. The percentage of asci present was then determined by examining appropriate dilutions under a microscope.

The induction of thermotolerance in the different strains was determined in the following manner. Exponentially growing cells were diluted into fresh YPD media to $OD_{600} = 0.5$ and then grown at 30° C. Aliquots were periodically removed and appropriate dilutions plated on YPD rich media either with or without a heat treatment. The portions that were heat-treated were transferred to glass 18 mm test-tubes and incubated at 53.5° C for 5 min. The cells were then cooled rapidly on ice and plated. Between 500 and 1500 cells were counted at different incubation times and the percentage survival determined.

RNA blot analysis. Total RNA was prepared by K. Voelkel-Meiman from vegetatively growing diploid yeast cells by the method of Schmitt et al. (1990). Between 7 and 15 μg of the RNA was then subjected to electrophoresis in a 1.5% agarose gel in the presence of formal-

dehyde (Maniatis et al. 1982), transferred to a nylon membrane according to the recommendations of the manufacturer (Zeta-probe, Bio-Lab Laboratories), and probed with a 1.5 kb *Bgl*II *KNS1* DNA fragment labelled with [α-^{32}P]ATP (Feinberg and Vogelstein 1983). After autoradiography, the probe was removed from the filter by incubation in 0.015 M NaCl, 0.0015 M sodium citrate at 95° C, and the blot was rehybridized with a 0.5 kb yeast actin probe (a gift from S. Roeder, Yale University). The actin probe hybridized with a 1.5 kb yeast RNA at less than one-third the intensity of the *KNS1* probe.

KNS1-lacZ *fusions*. In order to determine whether the kinase protein was expressed, fusions between *KNS1* and the *lacZ* gene of *Escherichia coli* were constructed. A 1.5 kb *Bgl*II fragment which contained 341 nucleotides located upstream of the putative initiator methionine and extended into the kinase domain of *KNS1*, was inserted into the *Bam*HI site of plasmid B5 (a gift from S. Roeder, Yale University; plasmid B5 contains a truncated *lacZ* gene inserted into YCp50). In the proper orientation, this produces an in-frame fusion between *KNS1* and *lacZ*. Recombinants containing the *Bgl*II *KNS1* fragment inserted in both orientations were isolated and transformed into yeast.

β-Galactosidase assays were performed as follows. Patches of yeast cells containing the plasmids were allowed to grow overnight at 30° C, after which a piece of Whatman 3 mm filter paper was placed over the patches until the paper was completely moist. The filter paper was removed with the yeast cells adhering to it; the paper was then exposed to chloroform vapours for 5 min to lyse the cells. The paper was then placed on a petri dish containing the assay medium [0.7% agar, 0.1 M sodium phosphate, pH 7.0, 1 mM $MgSO_4$, 120 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)], and incubated at 30° C for 20 h until color development was complete. The assay was performed in duplicate.

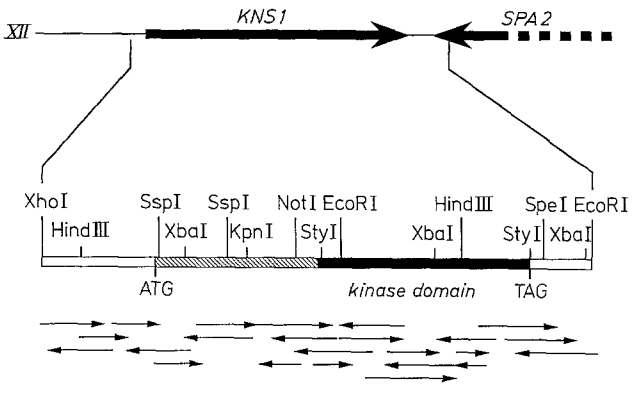

Fig. 1. Restriction map of *KNS1* region and sequencing strategy. *Large arrows* indicate the direction of transcription of the *KNS1* and *SPA2* genes respectively. *Arrows* beneath the map indicate the direction and extent of DNA sequencing as determined by the dideoxy chain-termination method (Sanger et al. 1977), using M13 vectors (Yanisch-Perron et al. 1985)

Results

A protein kinase homologue lies 3′ to the SPA2 *gene of yeast*

During the sequencing of the *SPA2* gene of *S. cerevisiae*, our analysis extended into the 3′ flanking region (Figs. 1 and 2). An open reading frame was identified which could potentially code for a protein the sequence of which shows significant similarity to those of a number of protein kinases. This gene was named *KNS1* for *k*inase *n*ext to *SPA2*. The *KNS1* open reading frame is 2208 bp in length and lies 339 bp away from the *SPA2* open reading frame. There are two methionine codons located at the beginning of the *KNS1* ORF, one at nucleotide 655 and the other at 703. It is likely that the second methionine is the one used for translation initiation since sequences near the second methionine more closely resemble the consensus sequences surrounding the initiator methionine of other eukaryotic genes (Kozak 1983). If this is the case, then the *KNS1* translation product is 720 amino acids long. It is unlikely that the *KNS1* gene is spliced within the coding region, as this sequence lacks the TACTAAC sequence found close to the 3′ end of all yeast introns (Teem et al. 1984). The predicted amino acid sequence of the *KNS1* gene was compared with those in the Genbank data base using the FASTA programs (Pearson and Lipman 1988); the last 420 amino acids of the putative *KNS1* gene product have significant amino acid sequence similarity to many protein kinases. A more detailed comparison of the *KNS1* sequence with the data of Hanks et al. (1988) confirmed that the protein encoded by *KNS1* contained every invariant and semi-variant residue present in all known protein kinases (Figs. 3A and 4). These conserved residues span ten domains (Hanks et al. 1988).

The KNS1 *protein kinase is most similar to the* CDC28/cdc2 *kinase family*

The sequences of a large number of protein kinases have been grouped into families based on their sequence similarities (Hanks et al. 1988). The closest matches of the *KNS1* predicted sequence were with the *CDC28* and *FUS3* genes of *S. cerevisiae*, and *cdc2* gene of *S. pombe* (Fig. 4). The *KNS1* protein shares a number of residues characteristic of the members of this family (indicated by asterisks in Fig. 4). The number of identical residues shared with the CDC2, CDC28, and FUS3 proteins is 36%, 34%, and 32%, respectively over a ca. 350 amino acid stretch. The number of similar residues between the KNS1 protein and these three proteins is 60%–70%. The KNS1 protein, like other members of the cdc2/CDC28 family, contains a large domain X in its carboxy-terminus (see Figs. 3 and 4).

Although the KNS1 protein sequence is most similar to that of members of the CDC28 family, there are several noteworthy differences. First, the KNS1 protein lacks the conserved tyrosine residue at position 308; a tyrosine residue at this position in the *cdc2* protein of *S. pombe*

```
1     CTCGAGGCTGTGCGGGCCCAGTGCCCGTTTCTTTTTTTTCTAGGGGGCGGCAAACGGGCCAAAGGGGTCAGCGTGATTGGGCGGTTTTCCACAAAGCTTTAGTCTCTCTCCTCAC
116   GCCTTGGCTTAGTGAGTGGCCGGAGTTAAAGTTGCGAGGAAACACACAGCGAAGGGAAAAAGCTTTGGCGTGCTTAACGATATGGCTTATGGCCCGGTTGCAATGCTGGTTTAGT
231   TTCCGATTACGATTTTCGATTTGAATCCTGGCTCCACATCTCCCACCCTCTTTTTTTATCTCTAGCATCGTGCGTTGGATTTTATATTATGAAGTTCCTATATTAGAATAAAGTT
346   TAGTATTTACTTCCCAGATCTCTACCGGATTTACAAAGTATTGTTACCTCACCACCAAGTGAAAGGAAACAGTGCAGTAACTCCTCGTGCTTACGCTAACTAAATAGATATTATA
461   TTCACGAAATACTGCGCCAGATAGCAAGCAAACCCAAAAAAAAAAAAGAAAAAGAAAAAGGAAACAAAGTGGGAGACAGAAAAAGAGTGAAAGAAACATAGTATTTACCAAGTCG
576   AAGATTTAGCACAACAAAGACTAATCTACACGCATACTGCTCTTCTTCATTATTGCTTTATCTTCCGCGTACCTTAGTTATGTCACAGAATATTCAAATTGGCACTAGAAAACGT
691   TCAAGAGCAAAT   ATG AAT AAT TCC ACT ACT ACG GGC CCT GCT AAC AAT ACA AGC TCT AAC AAG ACC TTT TTG GAT AAT TTC GAA GAA
1                   M   N   N   S   T   T   T   G   P   A   N   N   T   S   S   N   K   T   F   L   D   N   F   E   E

778   ACG CGC ACA AAC AAG CTG CTG GAC GAG ATG TTT GCT CGG CAA AAT TCT TTT CTA ACA GAC AAT CTG AGG AAC AGT CTA GAC CTG AAT
26    T   R   T   N   K   L   L   D   E   M   F   A   R   Q   N   S   F   L   T   D   N   L   R   N   S   L   D   L   N

865   CAA GCA GAC AAT CCG CTC CGT CCT CGG CAA CAC CAA CAC CAG TTG TTC TTG GAC AAT GAA AAT GCC ATA GAA CTA GAC GAG GAG CCG
55    Q   A   D   N   P   L   R   P   R   Q   H   Q   H   Q   L   F   L   D   N   E   N   A   I   E   L   D   E   E   P

952   CGT ATC ATT AAC ACC ACA ATC AAT AAC AGT AAT AAT CAT AAC AGT AGT CGA GTG GAC GAG GAT GCT GAC GAT GAC ATC ATT TTT ATC
84    R   I   I   N   T   T   I   N   N   S   N   N   H   N   S   S   R   V   D   E   D   A   D   D   D   I   I   F   I

1039  AAA GAG CAG CCA ATT CAG TTT TCG TCA CCT TTG ATT CTG CCT AGT AGC AGC AGT ATC AAT AAC AAC AAT AAT ATT GTT ACT TCA AAT
113   K   E   Q   P   I   Q   F   S   S   P   L   I   L   P   S   S   S   S   I   N   N   N   N   N   I   V   T   S   N

1126  AAT CCA GGG TGC GGT ACC GCA GCT ACA AGC AAT AGT ACG TAT ATC ACG ACA CCA AAG AAA TTC AAG AAG CAA AGA ACC ATC TCA CTG
142   N   P   G   C   G   T   A   A   T   S   N   S   T   Y   I   T   T   P   K   K   F   K   K   Q   R   T   I   S   L

1213  CCT CAG TTG CCT CTA TCG AAG CTG AGC TAC CAA TCG AAC TAT TTT AAT GTT CCT GAT CAA ACC AAC GCT ATC GTG CCA AGA GTA ACG
171   P   Q   L   P   L   S   K   L   S   Y   Q   S   N   Y   F   N   V   P   D   Q   T   N   A   I   V   P   R   V   T

1300  CAA ACT GAA AAT GAG CTG TTG CAT CTA ACG GGG TCG TGT GCA AAG ACT CTG GAG GGC AAC AAG GCA GTG AAT CTC ACG ATT GCT CAC
200   Q   T   E   N   E   L   L   H   L   T   G   S   C   A   K   T   L   E   G   N   K   A   V   N   L   T   I   A   H

1387  AGC ACT TCT CCC TTT TCC AAC CCA CCA GCA CAA ATA GCT TCC CTG CCT CAA TCC AAC CTC AAG AAA CAA ATT GGT TCT TCA CTA CGG
229   S   T   S   P   F   S   N   P   P   A   Q   I   A   S   L   P   Q   S   N   L   K   K   Q   I   G   S   S   L   R

1474  AAA TTT ACA TCA AAC GGC TCT TCA GAA AGC GCA TCA TCC AAT AAA TCA AAT TTC AAA ACG GAC AAA GAC GGT CAC TAC GTT TAC CAG
258   K   F   T   S   N   G   S   S   E   S   A   S   S   N   K   S   N   F   K   T   D   K   D   G   H   Y   V   Y   Q

1561  GAA AAC GAC ATA TTT GGC AGT GGC GGC CGC TTT GTT GTG AAG GAC CTA TTG GGC CAA GGC ACG TTT GGT AAA GTA CTT AAA TGC ATC
287   E   N   D   I   F   G   S   G   G   R   F   V   V   K   D   L   L   G   Q   G   T   F   G   K   V   L   K   C   I

1648  GAT AAC AAG TAT GAA CCT AAT TAC GTG GCT GTA AAA GTG ATA AGG GCT GTA GAT AGA TAT AGA GAA GCC GCC AAA ACA GAA CTA AGA
316   D   N   K   Y   E   P   N   Y   V   A   V   K   V   I   R   A   V   D   R   Y   R   E   A   A   K   T   E   L   R

1735  ATT CTA CAG ACT ATC CTG AAT AAT GAC CCT CAA GGT CAG TTC CAG TGC CTC TTG CTA AGG GAG TGC TTC GAT TAC AAA AAT CAC ATT
345   I   L   Q   T   I   L   N   N   D   P   Q   G   Q   F   Q   C   L   L   L   R   E   C   F   D   Y   K   N   H   I

1822  TGT TTG GTG ACA GAT CTA TAC GGC AGG TCC ATT TAC GAT TTT ATG TGC TCC AAC GGC ATT GCA GGT TCC CCG GCT CTC ATA TCA GGC
374   C   L   V   T   D   L   Y   G   R   S   I   Y   D   F   M   C   S   N   G   I   A   G   S   P   A   L   I   S   G

1909  CAT TGC AGA CAG CTA ATC AGA TCT GTC TGC TTC TTG CAC GAT TTG GGC ATA ATA CAC ACG GAT TTG AAA CCA GAA AAT ATC CTG ATT
403   H   C   R   Q   L   I   R   S   V   C   F   L   H   D   L   G   I   I   H   T   D   L   K   P   E   N   I   L   I

1996  TGT GAC GAA ACC CAT ATT GCT CAA AAA TTG CCT TTG AAA ACC GTA CAG TCG CTA AGC AAG AGA CGC CGT GAA GCC AGT AAG GGG AAG
432   C   D   E   T   H   I   A   Q   K   L   P   L   K   T   V   Q   S   L   S   K   R   R   R   E   A   S   K   G   K

2083  CGC AAA ATC TTG AAA AAT CCA GAA ATC AAA ATC ATT GAT TTC GGT AGC GCA ATT TTC CAT TAC GAA TAT CAT CCT CCT GTA ATA TCC
461   R   K   I   L   K   N   P   E   I   K   I   I   D   F   G   S   A   I   F   H   Y   E   Y   H   P   P   V   I   S

2170  ACT CGT CAC TAT AGA GCC CCG GAA ATT GTC CTT GGC TTG GGC TGG TCG TTC CCC TGC GAC ATT TGG TCC ATC GCG TGT GTC CTA GTA
490   T   R   H   Y   R   A   P   E   I   V   L   G   L   G   W   S   F   P   C   D   I   W   S   I   A   C   V   L   V

2257  GAA CTG GTT ATT GGC GAA TCT CTT TAC CCC ATT CAT GAA AAT CTA GAA CAT ATG GCT ATG ATG CAA CGA ATC AAT GGA ACC CCT TTC
519   E   L   V   I   G   E   S   L   Y   P   I   H   E   N   L   E   H   M   A   M   M   Q   R   I   N   G   T   P   F

2344  CCC ACA GAC ATT ATT GAT AAG ATG TTT TAC AAA TCT AAA CAT AAA TTG GGC AAC TCT CCA TCA GAC CTA AAT TCA ACG GTG ATA AAG
548   P   T   D   I   I   D   K   M   F   Y   K   S   K   H   K   L   G   N   S   P   S   D   L   N   S   T   V   I   K

2431  CAT TTC GAC AGA AAA ACT TTA AGC TTA CAA TGG CCT GAA AAA AAT AAA CGC GGA GAT ACC ATA ACC ACC GAA AAG TCA ATG AAA AGG
577   H   F   D   R   K   T   L   S   L   Q   W   P   E   K   N   K   R   G   D   T   I   T   T   E   K   S   M   K   R

2518  GTC TTG CAG TCA TGT GAT CGC TTA GAT ATT TAC ATC TCA AAG GTC TTG AAA CAG GAT TAC GGC GAT AGC TTA AGC ATC AAT TGG AAT
606   V   L   Q   S   C   D   R   L   D   I   Y   I   S   K   V   L   K   Q   D   Y   G   D   S   L   S   I   N   W   N

2605  CTA CCG CCG GAG AAA AAC TGG TCT TTG ATA AAT TCG AAA TTG GCA TGG AAA AGA CAA ACA CAT TCC TCT TCT TCC TCA ACT ACT GAT
635   L   P   P   E   K   N   W   S   L   I   N   S   K   L   A   W   K   R   Q   T   H   S   S   S   S   S   T   T   D

2692  GAA CTC GAT AAG GAA ACT TTC TTG TTT TGG TAC TGG TTC ATC GAT CTA CTC AGG AAA ATG TTT GAG TTC GAC CCA ACA AAA AGG ATT
664   E   L   D   K   E   T   F   L   F   W   Y   W   F   I   D   L   L   R   K   M   F   E   F   D   P   T   K   R   I

2779  ACC GCA AAG GAC GCT CTG GAC CAT GAA TGG TTT AAT CTG GGC ATA CTG GAT GAT GGT ATT GCA ACT TAT AAT AAT ACC CAA GGA TAG
693   T   A   K   D   A   L   D   H   E   W   F   N   L   G   I   L   D   D   G   I   A   T   Y   N   N   T   Q   G   Trm

2866  TGGGTGTTCTCGTTCTACTTTTCTCTTCTTTTTTTCACATCTTTTACTAGTTCCGTTTTTTTTTTTTTTAACTCTTCTTTTTCATTTTCCGGTTTTTAATAATATTTCTTTAAAT
2981  TTCTATATTCAATAAAAATTCTTCCTACATATATATCTTCATATTCAAACATTTATAGTATTTTTACTAATTTTTTGTTGTTTTAATATACTATAATAATATTATTATTACTGCT
3096  TTAACTACTATTATTATTATTATAATTATTATTATTATATGTCATACATAATACATTCTATTGATTGTCTTTGTCTTCCTTTTCTTTCTCCTCTAGA
```

Fig. 2. DNA sequence of the *KNS1* region. The translation of the *KNS1* open reading frame starting at the second methionine is shown beneath the DNA sequence. The first and second methionine residues are indicated in italics. Numbers adjacent to the nucleotide sequence refer to the nucleotide position; numbers in *italics* indicate the respective amino acid residue positions downstream of the second methionine

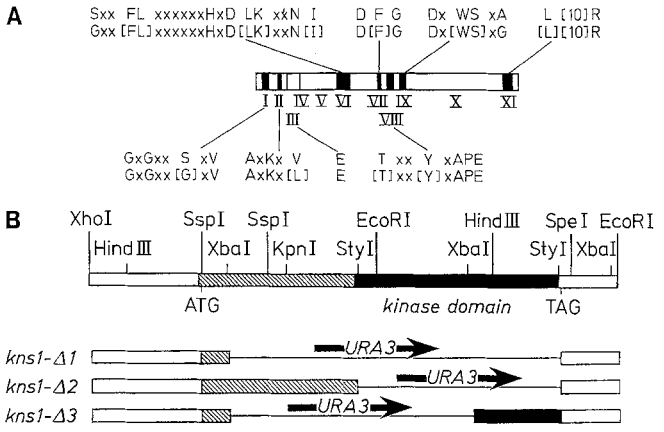

Fig. 3. A Amino acid residues in the predicted KNS1 protein (*top* line) that are present in other protein kinases (*bottom* line). Regions conserved in all known kinases are indicated by the *filled segments*. X = any residue. Residues in the lower line are in all protein kinases; residues in brackets are usually found in other protein kinases, but other residues are sometimes found. **B** *kns1* deletions in which the *URA3* gene has been substituted for portions of the *KNS1* gene. *kns1-Δ1* and *kns1-Δ3* begin at codon 51. *kns1-Δ2* begins at codon 305, which encodes the first conserved residue of the kinase domain indicated by the filled segment; the remaining fragment of KNS1 is indicated by *shading*. *kns1-Δ1* and *kns1-Δ2* deletions end at exactly the last codon of the *KNS1* open reading frame

has been shown to be phosphorylated in vivo (Gould and Nurse 1989). The KNS1 protein also contains 305 amino acids upstream of the putative kinase domain; an upstream domain of comparable size is not present in other members of the CDC28 family. Although we do not yet know its function, this upstream region may be involved in the regulation of kinase activity, via interaction with substrates and/or interaction with as yet undiscovered subunits. This region also contains a possible nuclear localization sequence, KKFKKQR, beginning at residue 176 (see Kalderon et al.1984 for examples of other nuclear localization sequences), suggesting that perhaps the KNS1 protein functions within the nucleus.

Throughout the KNS1 protein sequence there are several homopolymer tracts of amino acids, for example, at positions 142 (SSSS), 147 (NNNNN), and 671 (SSSSS) based on numbering relative to the first methionine of the *KNS1 ORF* (Fig. 2). The latter polyserine stretch is part of a potential recognition sequence for casein kinase II (SSSSSTTDE) (Tuazon and Traugh 1989). This raises the possibility that the *KNS1* protein itself may be regulated by phosphorylation.

The KNS1 *protein is not essential for cell viability in yeast*

To help determine the role of the *KNS1* protein in yeast, three deletion mutations were constructed within the *KNS1* region, and these are shown in Fig. 3B. *kns1-Δ1* is a substitution of *URA3* for a large region of *KNS1*; only 51 codons of *KNS1* coding sequence lie upstream of the *URA3* gene, assuming the second methionine codon is the initiator methionine. The *kns1-Δ1* deletion ends exactly at the last codon of the open reading frame. *kns1-Δ2* contains a precise substitution of the *URA3* gene for the 1274 bp coding segment of the kinase domain; this deletion begins at the codon for the first conserved residue of the kinase domain (residue 305) and also ends exactly at the last codon of the open reading frame. A third deletion, *kns1-Δ3*, removes an intermediate amount of DNA relative to *kns1-Δ1* and *kns1-Δ2*.

Each of the different *kns1* alleles were introduced into diploid yeast strains, and the resulting heterozygotes were sporulated. For all three alleles, four viable spores were obtained, and the markers segregated 2 Ura^+: Ura^-, indicating that the *KNS1* gene is not essential for cell viability. Proper substitution at the *KNS1* locus was determined by gel blot analysis of genomic DNA (not shown). *kns1-1* mutants grow at the same rate as wild-type cells in rich media (YPD) at 30° C and at 11° C (see Table 2 for 30° C results), and have the same proportion of unbudded, small budded, and large budded cells. *kns1* cells also form normal-sized colonies when incubated at either 18°, 30° or 37° C, or when incubated on plates containing either galactose or glycerol as their primary carbon source. *kns1* cells mate at approximately the same efficiency as wild-type cells (Table 3), and *kns1/kns1* diploids successfully undergo meiosis to form four-spored tetrads at equivalent levels to wild-type cells (Table 4).

Since *CDC28* homologues participate in control steps of the cell cycle we also examined entry into and exit from stationary phase. Wild-type yeast cells acquire thermal tolerance upon entry into stationary phase (see Snyder 1989). When cultures of yeast cells were incubated for up to 2 days, *kns1* mutant cells acquired thermal tolerance at a rate equivalent to that of wild-type cells, indicating that entry into stationary phase was normal (Fig. 5). Exit from stationary phase into a proliferative cycle was also tested. Incubation of wild-type cells in the presence of glucose caused a rapid loss of cell viability; the loss of cell viability is suggested to be caused by initiation of cell growth in media that cannot sustain growth (Granot and Snyder 1991). Glucose-induced loss of cell viability occurred in *kns1* mutant cells at a rate similar or faster to that in wild-type cells (not shown), indicating that exit from stationary phase was normal in *kns1* mutant cells. Thus, *kns1* cells appeared normal compared to wild-type cells in all aspects examined.

The KNS1 protein is expressed in vegetatively growing yeast cells

The lack of any observable defect in *kns1* cells might be due to failure of the *KNS1* gene to produce a transcript or protein in yeast. To determine whether the *KNS1* gene is expressed, two types of experiments were performed, RNA blot analysis and *KNS1*::*lacZ* fusions. Total RNA from vegetatively growing yeast cells was prepared and probed with a *KNS1* probe (see the Materials and methods). As shown in Fig. 6, a 2.5 kb tran-

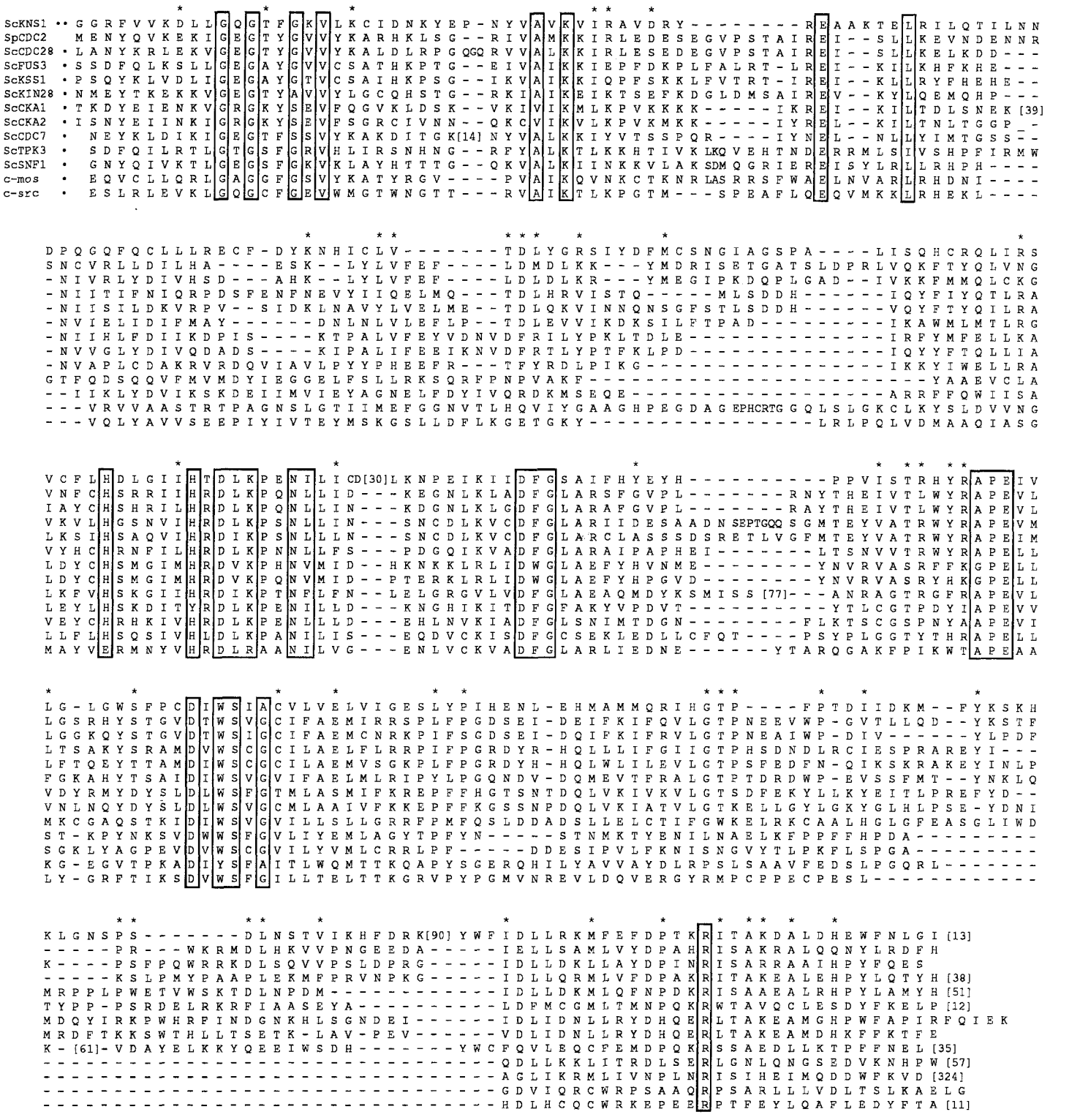

Fig. 4. Comparison of the putative *KNS1* protein sequence with that of other protein kinases. Residues often found in members of the cdc2/CDC28 family and not often present in other protein kinases are indicated by an *asterisk*. Residues common to all family members are enclosed in *boxes*. For comparison *CDC7, TPK3, SNF1, c-mos*, and *c-src* protein sequences are included; these are not members of the cdc2/CDC28 family. The number of amino acid residues upstream of the depicted kinase domains are 291 (KNS1); 0, (cdc2); 4 (CDC28); 9 (FUS3); 9 (KSS1); 4 (KIN28); 36 (CKA1); 46 (CKA2); 5 (CDC7); 85 (TPK3); 52 (SNF1); 57 (c-mos); and 267 (c-src). The protein sequences were obtained from Hindley and Phear 1984 (cdc2); Lorincz and Reed 1984 (CDC28); Courchesne et al. 1989 (KSS1); Elion et al. 1990 (FUS3); Simon et al. 1986 (KIN28); Chen et al. 1988 (CKA1); Padmanabha et al. 1990 (CKA2); Patterson et al. 1986 (CDC7); Toda et al. 1987 (TPK3); Celnza and Carlson 1986 (SNF1); Watson et al. 1982 (c-mos); Anderson et al. 1985 (c-src)

Table 2. Growth rates

Strain	Doubling time at 30° C
MATa KNS1 (Y428)	110
MATα KNS1 (Y264)	120
MATa kns1-Δ1 (Y660)	126
MATα kns1-Δ1 (Y661)	115

KNS1 and *kns1-Δ1* yeast strains were grown in rich media. The doubling time was determined by measuring the OD_{600} of exponentially growing cultures every 4–5 h starting at OD = 0.2 for a total of 24 h

Table 3. Mating efficiencies

MATa	*MATα*	Mating efficiency
KNS1 (Y428)	*KNS1* (Y264)	1.0
KNS1 (Y428)	*kns1-Δ1* (Y661)	0.753
kns1-Δ1 (Y660)	*KNS1* (Y264)	0.825
kns1-Δ1 (Y660)	*kns1-Δ1* (Y661)	0.514
kns1-Δ1 (Y663)	*kns1-Δ1* (Y662)	0.921

MATa and *MATα* cells growing exponentially were incubated to $OD_{600} = 0.5$ and mating efficiency were determined as described in the Materials and methods. For each mating between 150 and 280 diploid colonies were counted

Table 4. Sporulation efficiencies

Diploids	No. of cells counted	No. of spores	% Asci
KNS1/KNS1 (Y270)	187	43	22.9
kns1-Δ1/kns1-Δ1 (Y664)	149	34	22.8
kns1-Δ1/kns1-Δ1 (Y665)	153	28	18.3

Diploid yeast cells were sporulated in 30° C in 1% potassium acetate, and the % acsi was determined after 3 days

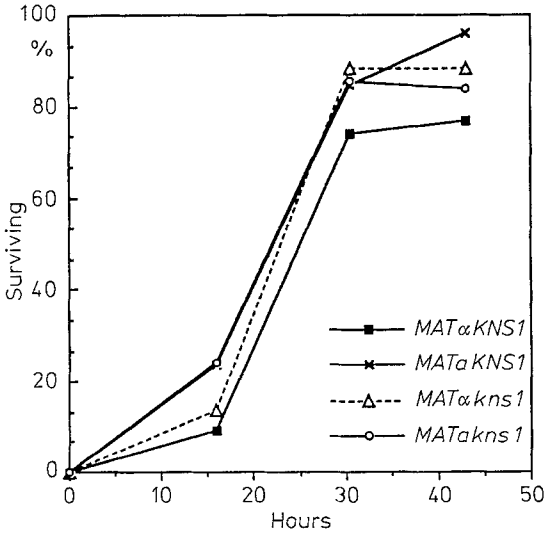

Fig. 5. Test for entry into stationary phase of wild-type cells and of *kns1* mutant cells of the following strains was performed as described in the Materials and methods: *MATα KNS1* (Y264), *MATa KNS1* (Y428), *MATα kns1* (Y661), and *MATa kns1* (Y662)

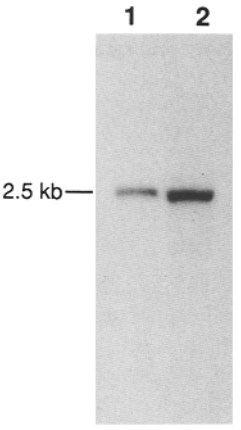

Fig. 6. RNA blot analysis of *KNS1*. Lane 1, 7 μg of total yeast RNA; lane 2, 15 μg of total yeast RNA. The size of the hybridizing band was estimated based on the mobility of the ribosomal RNA bands

script capable of encoding the entire *KNS1* protein was present. The *KNS1* transcript appears to be more abundant than yeast actin RNA (not shown).

In order to determine whether this *KNS1* transcript was capable of being translated, the *lacZ* gene of *E. coli* was fused in frame to *KNS1* coding sequences (see the Materials and methods), transformed into yeast, and assayed for the presence of β-galactosidase. A low level of β-galactosidase activity was observed; this level was at least 50-fold lower than that of the *SPA2::β-gal* fusion protein (Snyder 1989; C. Costigan and M. Snyder, unpublished results). Control strains with *lacZ* present in the opposite orientation relative to *KNS1* failed to produce any detectable β-galactosidase. The low levels of enzyme activity detected in β-galactosidase assays can be explained in several ways: (i) the *KNS1* transcript may be poorly translated, (ii) the presence of the KNS1 protein may be only transient during the yeast cell cycle, (iii) the KNS1 protein or the KNS1::β-gal fusion protein might be particularly sensitive to proteolysis, or (iv) the β-galactosidase activity of this particular fusion construct may itself be intrinsically low. In any event, the *KNS1* gene appears to be expressed as RNA and occurs, at least at a low level, as protein in yeast.

The presence of the KNS1 *gene on a high copy number plasmid in yeast does not suppress the* cdc28-1 *mutation of yeast*

The *KNS1* gene is distantly related to the *CDC28* gene. To determine whether the KNS1 protein might contain overlapping functions with that of the CDC28 protein, we tested whether the *KNS1* gene when present on a high copy number plasmid, YEp24, might suppress the *cdc28-1* mutation of *S. cerevisiae*. Wild-type and *cdc28* mutant cells containing either a *KNS1*/YEp24 high copy number plasmid or YEp24 alone exhibited no difference in colony size when cells were grown at either the restric-

tive temperature (37° C) or at semipermissive temperatures (30° and 32° C). Thus, although we have not determined whether the *KNS1*/YEp24 plasmid overproduces *KNS1* RNA or protein, the *KNS1*/YEp24 plasmid does not suppress the *cdc28-1* mutation.

Discussion

A novel kinase homologue, *KNS1*, which is a distant member of the *CDC28/cdc2* gene family has been identified. The KNS1 protein differs from proteins encoded by other members of the *CDC28/cdc2* gene family in that it contains a 30 kDa segment upstream of the kinase domain, which may regulate the KNS1 kinase activity. An attached regulatory domain might make the KNS1 kinase less versatile than other members of the cdc2/CDC28 family, because the other members of this family can potentially ineract with a number of regulatory domains. The *SPA2/KNS1* region lies on the left arm of chromosome XII, 21 cM from the centromere. The *KNS1* gene contains a *Not*I site in its coding region (Fig. 1), which should allow its precise positioning on a physical map, once it becomes available.

A large deletion of the *KNS1* gene was constructed. *kns1* mutant cells appear similar to wild-type cells in all aspects that were examined. The *KNS1* gene produces a 2.5 kb transcript and *KNS1*::*lacZ* fusions produce β-galactosidase activity indicating that the *KNS1* gene is expressed in yeast. Either we have not found the appropriate conditions under which *KNS1* plays an important function in yeast, or there is another gene or set of genes that has an overlapping function with *KNS1*. There are other examples of dispensable genes in yeast where a second or third homologue exists with a closely related function [examples include cAMP-dependent protein kinase genes *TPK1, TPK2, TPK3* (Toda et al. 1988); *RAS1, RAS2* (DeFeo Jones et al. 1983); histone genes *H2A, H2B, H3, H4* (Hereford et al. 1979; Smith and Murray 1983); and casein kinase II genes *CKA1, CKA2* (Padmanabha and Glover 1987)]. Low stringency hybridizations with *KNS1* probes failed to reveal any related homologue, indicating that if *KNS1* homologues exist, they must be divergent in sequence.

Our serendipitous finding of the *KNS1* gene probably reflects the fact that there are many protein kinase genes in yeast. So far, at least 24 yeast protein kinase genes or homologues have been identified. Approximately 520 sequences yeast genes which are expected to be transcribed by RNA polymerase II are present in the GenBank data base (Release 63); thus kinase homologues comprise about 4%–5% of the total, a number slightly higher than that predicted by Hunter (1987). If 4%–5% represents the frequency of protein kinases in yeast, then we predict that the yeast genome contains approximately 400–500 protein kinases among its 10000 genes. We recognize that there are a number of factors that may bias this estimate.

Acknowledgements. We thank K. Voelkel-Meiman for total yeast RNA, B. Grimwade for help with the computer analysis and C. Costigan, B. Grimwade, and B. Page for comments on the manuscript. This work was support by NIH grant GM36494 (90%) and by an Institutional American Cancer Society Grant (10%). R.P was supported by an Anna Fuller Postdoctoral Fellowship. The sequence data are available from GenBank/EMBL/DDBJ under accession number M36980.

References

Anderson SK, Gibbs CP, Tanaka A, Kung H-J, Fujita DJ (1985) Human cellular *src* gene: nucleotide sequence and derived amino acid sequence of the region coding for the carboxy-terminal two-thirds of $pp60^{c\text{-}src}$. Mol Cell Biol 5:1122–1129

Celnza JL, Carlson M (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science 233:1175–1180

Chen J L-P, Padmanabha R, Glover C (1988) Isolation, sequencing and disruption of the *CKA1* gene encoding the alpha subunit of yeast casein kinase II. Mol Cell Biol 8:4981–4990

Courchesne WE, Kunisawa R, Thorner J (1989) A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. Cell 58:1107–1119

DeFeo Jones D, Scolink EM, Koller R, Dhar R (1983) *ras*-Related gene sequences isolated from *Saccharomyces cerevisiae*. Nature 306:707–709

Elion EA, Grisafi PL, Fink GR (1990) *FUS3* encodes a $cdc2^+$/CDC28-related kinase required for the transition from mitosis into conjugation. Cell 60:649–664

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Gould KL, Nurse P (1989) Tyrosine phosphorylation of the fission yeast $cdc2^+$ protein kinase regulates entry into mitosis. Nature 342:39–45

Granot D, Snyder M (1991) Glucose induces cAMP-independent growth-related changes in stationary-phase cells of *Saccharomyces cerevisiae*. Proc Natl Acad Sci (USA) 88:5724–5728

Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241:42–52

Hereford L, Fahrner K, Woolford J, Rosbash M, Kaback DB (1979) Isolation of yeast histone genes H2A and H2B. Cell 18:1261–1271

Hindley J, Phear GA (1984) Sequence of the cell division gene *CDC2* from *Schizosaccharomyces pombe*; patterns of splicing and homology to protein kinases. Gene 31:129–134

Hunter T (1987) A thousand and one protein kinases. Cell 50:823–829

Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153:163–168

Kalderon D, Richardson WD, Markham AF, Smith AE (1984) Sequence requirements for nuclear location of simian virus 40 large T-antigen. Nature 311:33–38

Kozak M (1983) Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol Rev 47:1–45

Krebs EG (1986) The enzymology of control by phosphorylation. In: Krebs EG and Boyer PD (eds) The enzymes, vol 18, Academic Press, London, pp 3–20

Lorincz AT, Reed SI (1984) Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. Nature 307:183–185

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Padmanabha R, Glover C (1987) Casein kinase II of yeast contains two distinct alpha subunits and an unusually large beta subunit. J Biol Chem 262:1829–1835

Padmanabha R, Chen J L-P, Hanna DE, Glover C (1990) Isolation,

sequencing and disruption of the yeast *CKA2* gene: Casein kinase II is an essential enzyme. Mol Cell Biol 10:4089–4099

Patterson M, Sclafani RA, Fangman WL, Rosamond J (1986) Molecular characterization of the cell cycle gene *CDC7* from *Saccharomyces cerevisiae*. Mol Cell Biol 6:1590–1598

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448

Rothstein RJ (1983) One-step gene disruption in yeast. Methods Enzymol 101:202–211

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schmitt ME, Brown TA, Trumpower BL (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res 18:3091–3092

Sherman F, Fink G, Hicks J (1986) Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Simon M, Seraphin B, Faye G (1986) *KIN28*, a yeast split gene coding for a putative protein kinase homologous to *CDC28*. EMBO J 5:2697–2701

Smith MM, Murray K (1983) Yeast H3 and H4 histone messenger RNAs are transcribed from two non-allelic gene sets. J Biol Chem 169:641–661

Snyder M (1989) The SPA2 protein of yeast localizes to sites of cell growth. J Cell Biol 108:1419–1429

Teem J, Abovitch N, Kaufer NF, Schwindinger WF, Warner JR, Levy A, Woolford J, Leer RJ, van Raamsdonk-Duin MMC, Mager WH, Planta RJ, Schultz L, Friesen JD, Fried H, Rosbash M (1984) A comparison of yeast ribosomal protein gene DNA sequences. Nucleic Acids Res 12:8295–8312

Toda T, Cameron S, Sars P, Zoller M, Wigler M (1987) Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell 50:277–287

Toh-e A, Tanaka K, Usono Y, Wickner RB (1988) *PHO85*, a negative regulator of the PHO system is a homolog of the protein kinase gene *CDC28* of *Saccharomyces cerevisiae*. Mol Gen Genet 214:162–164

Tuazon PT, Traugh JA (1989) Casein kinase I and II – multipotential serine protein kinases: structure, function and regulation. In: Greengard P and Robison GA (eds) Advances in second-messenger and phosphoprotein research, vol 23, Raven Press, pp 123–164

Watson R, Oskarsson M, Van de Woude GF (1982) Human DNA sequence homologous to the transforming gene (*mos*) of Moloney murine sarcoma virus. Proc Natl Acad Sci USA 79:4078–4082

Wheals AE (1987) Biology of the cell cycle in yeasts. In: The yeasts, vol 1. Academic Press, London, pp 283–391

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Communicated by N.D.F. Grindley