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The essential *Schizosaccharomyces pombe cdc23* DNA replication gene shares structural and functional homology with the *Saccharomyces cerevisiae DNA43* (*MCM10*) gene

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Abstract The fission yeast *cdc23* gene is required for correct DNA replication: *cdc23* mutants show reduced rates of DNA synthesis and become elongated after cell-cycle arrest. We have cloned the *Schizosaccharomyces pombe cdc23* gene by complementation of the temperature-sensitive phenotype of *cdc23-M36* and confirmed the identity of the gene by integrative mapping. Analysis of the DNA sequence reveals that *cdc23* can encode a protein of 593 amino acids (M_r=67 kDa) with 22% overall identity and many structural homologies with the product of the *Saccharomyces cerevisiae DNA43* (*MCM10*) gene which is required for correct initiation of DNA synthesis at chromosomal origins of replication. Construction of a *cdc23* null allele has established that the *cdc23* gene is essential for viability, with *cdc23* deletion mutant spores germinating but undergoing arrest with undivided nuclei in the first or second cell cycle. The *S. pombe cdc23* gene on an expression plasmid is able to complement the *S. cerevisiae dna43-1* mutant. These structural and functional homologies between two distantly related species suggest that *cdc23* and *DNA43* may represent genes for a conserved essential eukaryotic DNA replication function.

Key words *Schizosaccharomyces pombe* · *cdc23* · DNA replication · Cell cycle

Introduction

DNA replication is a fundamental cellular process which must be closely co-ordinated with other cell-cycle events in order for division to occur successfully. Biochemical studies in many systems, particularly mammalian viruses, have determined many of the enzymes responsible for DNA replication in eukaryotic cells (Brush et al. 1995). Molecular genetic investigations in yeast have provided a complementary approach useful for identifying and characterising components of control mechanisms regulating the initiation and elongation stages of DNA replication and the co-ordination of these processes with other cell-cycle events. The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have both been extensively used in such studies: these two yeasts are highly diverged in evolutionary terms, and therefore similarities between the two systems are indicative of functions conserved across eukaryotes (Russell and Nurse 1986).

In the fission yeast *S. pombe* many genes encoding DNA replication functions have been identified by the isolation of temperature-sensitive cell-division-cycle (*cdc*) mutants. In particular, genes *cdc17* through to *cdc24* were identified in a screen for *cdc* mutants defective in S-phase functions (Nasmyth and Nurse 1981). Of these, *cdc17* has been shown to encode DNA ligase (Nasmyth 1977; Barker et al. 1987), *cdc22* encodes the large subunit of ribonucleotide reductase (Fernandez Sarabia et al. 1993), and *cdc20* encodes the essential DNA polymerase ε (d'Urso et al. 1997). The *cdc18* gene (homologous to *CDC6* of *S. cerevisiae*) encodes a product required for control of the initiation of DNA replication (Kelly et al. 1993; Nishitani and Nurse 1995). Some other *cdc* genes (Nurse et al. 1976) have also recently been found to encode DNA replication proteins, including *cdc1*, *cdc6* and *cdc27* which code for subunits of DNA polymerase δ (MacNeill et al. 1996; Iino and Yamamoto 1997; Zuo et al. 1997), and *cdc30* (*orp1*) which encodes a subunit of the origin recognition complex (ORC) (Grallert and Nurse 1996).

Two of the S-phase *cdc* genes identified by Nasmyth and Nurse (1981) code for members of the MCM family of DNA replication proteins: *cdc19* (also identified as *nda1*) encodes *S. pombe* MCM2 (Miyake et al. 1993; Forsburg and Nurse 1994) and *cdc21* encodes *S. pombe* MCM4 (Coxon et al. 1992). The MCM proteins are a family of six

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highly conserved eukaryotic proteins (MCM2-7) which are essential for the initiation of DNA replication, which interact with each other to bind DNA in a cell-cycle-dependent manner, and which have been implicated as components of the replication licensing factor (reviewed in Chong et al. 1996; Kearsey et al. 1996). All six MCM genes have been identified in *S. pombe*: the MCM5 and MCM6 homologues are encoded by the *S. pombe nda4* and *mis5* genes (Miyake et al. 1993; Takahashi et al. 1994) and the *mcm3* and *mcm7* genes have been identified by homology (Coxon et al. 1992; Adachi et al. 1997). The products of all these *S. pombe* MCM genes have been shown to form a heterohexameric complex in vivo (Adachi et al. 1997).

Mutants of the *cdc23* DNA replication gene show slow DNA synthesis at the non-permissive temperature: the *cdc23-M36* mutant requires 4–5 times as long as a wild-type cell to achieve a 2C DNA content and becomes cell cycle-arrested giving elongated uninucleate cells (Nasmyth and Nurse 1981). The execution point falls within S-phase and reciprocal shift experiments indicate that *cdc23* function is completed coincident with or after the block point imposed by hydroxyurea (Nasmyth and Nurse 1981). In this paper we have explored the function of *cdc23* in DNA replication by cloning and characterising the gene, by construction of a deletion mutant, and by an investigation of functional homology.

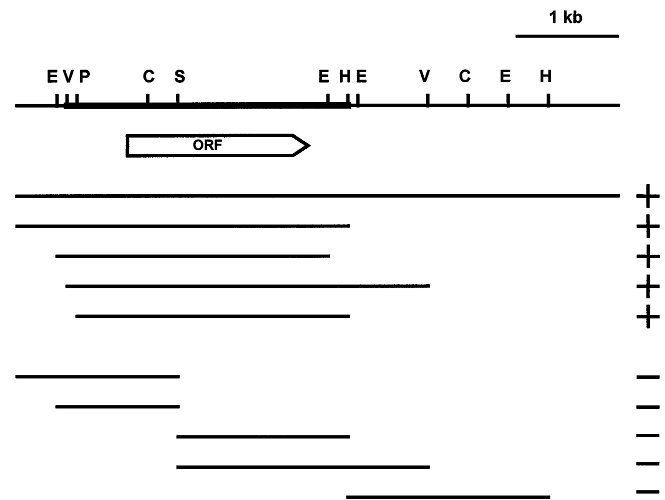

Fig. 1 Restriction map and subclones of the *cdc23* region. The restriction map of the 6.0-kb insert in the *cdc23*-complementing plasmid is shown, with the sequenced region in bold and the 1779-bp ORF indicated. *C*=*Cla*I, *E*=*Eco*RI, *H*=*Hin*dIII, *P*=*Pst*I, *S*=*Sst*I, *V*=*Eco*RV; the sequence contains no sites for *Bam*HI or *Sma*I. Restriction-fragment subclones are shown under the insert map, with their ability to complement *cdc23-M36* indicated to the right: +=able to complement; –=unable to complement. The vector polylinker *Hin*dIII site was utilised in excising the 3.3-kb *Hin*dIII subclone

Materials and methods

Yeast strains and growth conditions. Genetic and microbiological methods were according to Moreno et al. (1991) for *S. pombe* and Sherman et al. (1979) for *S. cerevisiae*. Strains for *cdc23* complementation were derived from *cdc23-M36 h*$^-$ (Nasmyth and Nurse 1981). The auxotrophic *ura4-D18* strain used in gene-deletion experiments has a complete 1.8-kb deletion of the *ura4* gene (Grimm et al. 1988). A *dna43-1 ura3-167 ade*$^-$ *his*$^-$ strain of *S. cerevisiae*, derived from crosses between strains JL8 (Dumas et al. 1992) and INVSC2 (Invitrogen), was used for *dna43* complementation.

Cloning and subcloning of the cdc23 gene. Standard gene-cloning procedures were used for all DNA manipulations (Sambrook et al. 1989). To clone the *cdc23* gene the *S. pombe* strain *cdc23-M36 ade6-704 leu1-32 ura4-D6 h*$^+$ was transformed by the protoplasting method (Beach and Nurse 1981) with the library pURSP2 (a gift from A. M. Carr) which contains partial *Sau*3A-digested genomic fragments from wild-type *S. pombe* cloned in the vector pUR19 (Barbet et al. 1992). Two transformants out of a total of 10 000 showed cdc$^+$ ura$^+$ co-instability, and DNA conferring ampicillin resistance was successfully recovered from one of these by transformation of *Escherichia coli* DH5αF′. This plasmid was checked for *cdc23* activity by re-transformation of the original *cdc23-M36* strain, and restriction fragments of its 6.0-kb insert (Fig. 1) were subcloned in the *S. pombe* replicating vector pUR19N (Barbet et al. 1992) and similarly tested for function. Colinearity of the *S. pombe* cloned and genomic DNA was established by Southern analysis.

Sequence analysis. The DNA sequence of the 2.74-kb *Eco*RV-*Hin*dIII subclone (Fig. 1) was determined in both directions by chain-termination sequencing using Sequenase™ 2.0 (USB) on templates generated by exonuclease III resection or restriction fragment subcloning, using universal or sequence-specific primers. Some reactions utilised ABI PRISM™ dye-terminator cycle sequencing and were resolved on an ABI377 automated sequencer. The DNA sequence was compiled and analysed using GCG programs (Devereux et al. 1984) accessed through the SEQNET facility of the Biotechnology and Biological Sciences Research Council. Coding-region analysis on the basis of codon usage used FRAMESCAN (Staden and McLachlan 1982). Database searches were performed with BLAST and FASTA (Altschul et al. 1990; Pearson 1991). Protein-sequence analysis used GCG programs and DNA Strider (Marck 1988). The nucleotide sequence of the *cdc23* gene has been deposited in the EMBL database under accession number AJ224944.

Gene deletion. The *cdc23* subclone comprising the 3.3-kb *Hin*dIII fragment (see Fig. 1) in pUR19N was used for in vitro deletion of the *cdc23* ORF. First, the polylinker *Bam*HI site was removed by cleavage, flush-ending and re-ligation. A PCR amplification was then performed with this clone as template using divergent primers 5′-TCGGATCCTCTTAATCTTAAACCTCC-3′ and 5′-TGGGATCCACAGCGACTGGACTTC-3′. The products were digested with *Bam*HI and self-ligated to give the plasmid pAJF1: this contains a deletion of all but the last 34 codons of the *cdc23* open reading frame (ORF), marked by a *Bam*HI site, with 1135 bp of upstream flanking sequence and 477 bp of downstream flanking sequence. Sequence analysis of the *cdc23* flanking regions in pAJF1 confirmed that no in vitro mutations had been introduced in these sequences during the PCR step. The *ura4*$^+$ gene on a 1.75-kb fragment (Grimm et al. 1988), *Bam*HI linkered, was then cloned into pAJF1 to give plasmid pAJF2. The 3.3-kb *Hin*dIII fragment from pAJF2 containing *ura4*$^+$ flanked by *cdc23* sequences was gel purified and used to transform the stable diploid *S. pombe* strain *ura4-D18/ura4-D18 ade6-704/ade6-704 leu1-32/leu1-32 h*$^+$*/h*$^+$. DNA samples prepared from 12 stable uracil prototrophic transformants were subjected to Southern analysis and seven transformants were found to be heterozygous for the *cdc23* deletion. Sporulation-competent *h*$^+$*/h*^{90} revertants were isolated from two of these for spore-germination analysis.

Spore germination. Sporulation-competent *cdc23* deletion heterozygotes and a *h*$^+$*/h*$^-$ *ura4–27 /ura4*$^+$ *ade6-704/ade6*$^+$ *leu1-32/leu1*$^+$ control strain were inoculated at 10^5 ml^{-1} in EMMG supplemented with 225 mg l^{-1} of adenine, leucine and uracil and incubated with shaking at 29°C for 4 days. Azygotic asci were harvested, washed with water, and vegetative cells were killed by 16-h incubation at 29°C with a 500-fold dilution of β-glucuronidase solution (Type HP-

2S, Sigma) followed by re-suspension in 70% ethanol for 30 min at room temperature. Spores were washed twice with 0.17 M NaCl, inoculated at 1–2×10^7 ml^{-1} in EMM supplemented with adenine and leucine, and incubated with shaking at 36°C. Under these conditions spore germination begins about 8 h after inoculation and 25% of the spores show outgrowth by 10 h. Samples were taken after 16-h incubation, fixed and stained with DAPI (Alfa et al. 1993) and viewed using a Zeiss Axiophot fluorescence microscope. For examination of individual germinating spores, azygotic asci were microdissected and spore germination and growth were monitored at 35°C on yeast extract agar supplemented with 250 mg l^{-1} of adenine, leucine and uracil.

Cross complementation. The *cdc23* gene was PCR-amplified with *Pfu* polymerase using primers 5′-CGCGGTACCCATATGCATGATCCCTTCATTG-3′ and 5′-GCCGGATCCATAATGTATAGAAAAACAACG-3′, cloned into the *Eco*RV site of pBluescript SK$^-$ (Stratagene), and then subcloned as a *Kpn*I-*Bam*HI fragment into the *S. cerevisiae* expression vector pYES2 (Invitrogen) to give pYES2–23. To confirm complementation of *cdc23-M36* the gene was also subcloned as a *Nde*I-*Bam*HI fragment into the *S. pombe* expression vector pREP41 (Maundrell 1993). The *DNA43* (*MCM10*) gene was PCR-amplified with *Pfu* polymerase from genomic *S. cerevisiae* DNA using primers 5′-GGTTCGTGGACATATGAATGATCCTCGTGAAATT-3′ and 5′-TTTGGGGCCCTGAAAAGCACACCAATAC-3′ designed from the yeast genome sequence YIL150c, cloned into the *Sma*I site of pBluescript SK$^-$ and then subcloned as a *Not*I-*Xho*I fragment into pYES2 to give pYES2-43. pYES2, pYES2-23 and pYES2-43 were transformed into a *dna43-1 ura3-167* strain of *S. cerevisiae* by the lithium acetate method (Ito et al. 1983) and ura$^+$ transformants were tested for complementation of *dna43-1* by replica plating to SD-uracil or galactose agar (containing 20 g l^{-1} of galactose in place of glucose) at 37°C.

Results

Cloning of *cdc23*

The *cdc23* gene was cloned by complementation of the temperature-sensitive *cdc23-M36* mutant (Nasmyth and Nurse 1981). Following transformation with a *S. pombe* genomic library and selection of colonies at 35.5°C, two independent clones were isolated and the DNA from these was transformed into *E. coli*. Plasmid DNA recovered from one *E. coli* clone was found to complement *cdc23-M36* when re-transformed back into this yeast strain. A restriction map of the 6.0-kb insert in this plasmid is given in Fig. 1.

In order to confirm that the *cdc23* gene was present in the cloned insert DNA, integrative mapping was performed. Twenty four stable integrants (ura$^+$) were obtained from the re-transformed *cdc23-M36* strain. All of these exhibited a cdc$^+$ phenotype, indicating that a single copy of the cloned gene is sufficient for mutant complementation. All 24 integrant strains were backcrossed to a *ura4-D6 leu1-32 h*$^-$ strain in order to map the site of integration relative to the *cdc23* gene. Three hundred and fifty spores were analysed from each cross and in 23 out of 24 cases no recombinant progeny were detected, indicating that the plasmid had integrated at the same locus as the *cdc23-M36* mutation.

To localise the *cdc23* gene within the 6.0-kb insert, restriction fragments were subcloned into a *S. pombe* replicating vector and subclones were transformed into *cdc23-M36*. Complementation was achieved with the 3.3-kb *Hin*dIII fragment, the 2.7-kb *Eco*RI fragment, the 3.55-kb *Eco*RV fragment and the 2.7-kb *Pst*I-*Hin*dIII fragment (Fig. 1), but subclones cut at the internal *Sst*I site did not complement. This indicates that the *cdc23* gene maps within the 2.5-kb *Pst*I-*Eco*RI fragment of the insert DNA and that it straddles the *Sst*I site.

Sequence analysis of the *cdc23* gene

Both strands of the 2.74-kb *Eco*RV-*Hin*dIII fragment containing *cdc23*$^+$ activity (shown in bold in Fig. 1) were sequenced. The DNA sequence (EMBL accession number AJ224944) revealed a large open reading frame (ORF) of 1.78 kb straddling the *Sst*I site and capable of coding for a protein of 593 amino-acid residues. The ORF corresponds exactly with that part of the DNA sequence predicted as a coding region on the basis of nucleotide composition or codon-usage analysis (see Materials and methods). No predicted introns are apparent when the sequence is scanned for *S. pombe* consensus pre-mRNA splicing signals (Prabhala et al. 1992). To test this, PCR-amplification of the whole ORF was performed using either a genomic or a cDNA library as template. PCR products of identical size were amplified in each case (data not shown) confirming that the whole ORF is expressed and indicating that no introns are present in *cdc23* pre-mRNA. There are no sequences with ≥5/6 identity to the MCB *Mlu*I cell-cycle box (Lowndes et al. 1991, 1992; Johnston 1992) within 550 bp upstream from the ORF, suggesting that the gene is not regulated by a DSC transcription factor. This is in accord with the majority of S-phase genes in fission yeast, which lack MCB sequences and are not subject to regulation at the transcriptional level (Johnston 1992). Four potential polyadenylation sites are present 35–370 bp downstream from the *cdc23* ORF.

Sequence comparisons

Comparison of the *cdc23*-encoded sequence with other sequences in the SwissProt and PIR databases revealed significant similarity only with the product of the *DNA43* (*MCM10*) gene (Blastp 2.0.4: expected value=2×10^{-27}). *DNA43* is required for correct initiation of DNA replication in *S. cerevisiae* and its product has been shown to interact with MCM proteins (Solomon et al. 1992; Merchant et al. 1997). The alignment in Fig. 2 shows that there is 22% overall identity between the two predicted proteins, with highest levels of similarity in the central region of the sequence (41% over 110 residues between 191 and 300). In addition, there are many conserved substitutions and similar motifs (Fig. 2). Both the cdc23 and DNA43 proteins contain a bipartite potential nuclear localisation signal (NLS) at homologous positions near the N-terminus (Fig. 2); the NLS of cdc23 is of the type found in nucleoplasmin and other proteins (Robbins et al. 1991). A short

A

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                                                  NLS
MHDPFIAEEND.............LDLEEKRLQRQLNEIQEKKRLRSAQ 36
MNDPREILAVDPYNNITSDEEDEQAIARELEFMERKRQALVERLKRKQEF 50

KEASSENAEVIQVPRSPPQQVRVLTVSSPSKLKSPKRLILGIDKGKTGKD 86
KKPQDPNFEAIEVPQSPTKN.RVKVGSHNATQQGTKFEGSNINEVRLSQL 99

VSLGKGPRGPLPKPFHERLAEARNQERKRSDKLKTMKKNRKQSFQRKRNI 136
QQQPKPPASTTTY.FMEKFQNAKKNEDKQIAKFESMMNARVHTF...... 142

LEDGKSEEEKFPMKCDEIDPYSRQAIVIRYISDEVAKENIGGNQVYLIHQ 186
...STDEKKYVPIITNELESFSNLWVKKRYIPEDDLKRALHEIKILRLGK 189

LLKLVRAPKFEAPEVDNYVVMGIVASNSGTRETVNGN..KYCMLTLTDLK 234
LFAKIRPPKFQEPEYANWATVGLISHKSDIKFTSSEKPVKFFMFTITDFQ 239

WQLECFLFGK.AFERYWKIQSGTVIALLNPEVLKPKNPDIGRF..SLKLD 281
HTLDVYIFGKKGVERYYNLRLGDVIAILNPEVLPWRPSGRGNFIKSFNLR 289

SEYDV..LLEIGRSKHLGYCS.SRRKSGELCKHWLDKRAGDVCEYHVDLA 328
ISHDFKCILEIGSSRDLGWCPIVNKKTHKKCGSPINISLHKCCDYHREVQ 339

VQRSMSTRTEFASSMATMHEPRARREKR.FRGQGFQGYFAGEKYSAIPNA 377
FRGTSAKRIELNGGYALGAPTKVDSQPSLYKAKGENGFNIIKGTRKRLSE 389

VAGLYDAEDAVQTERERKERYKKQRAQAEREREILVRLSKRCCASSSSSS 427
EEERLKKSSHNFTNSNSAKAFFDEKFQNPDMLANLDNKRRKIIETKKSTA 439

NSNNLSTGMSMRTLGHQYLNLQGS..GVKNLHDKGNPTALSKDSEIDSST 475
LSRELGKIMRRRESSGLEDKSVGERQKMKRTTESALQTGLIQRLGFDPTH 489

KKPSVLASFNASIMNPKSSLPSFSNSAILGTNDAASGTPVPQDTTSTKVS 525
GKISQVLKSSVSGSEPKNNL..............LGKKKTVINDLLHYKKE 526

PAVVFTSSPRIFSPQSLRKIGFDPTHSADASTTHSTATGLSRSGSLKNIK 575
KVILAPSKNEWFKKRSHREEVWQKHFGSKETKE................. 559

FRYEFTESDDEDDLEIVP 593
.....TSDGSASDLEII  571
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B

Fig. 2 A, B Comparison of *S. pombe* cdc23 and *S. cerevisiae* DNA43 (MCM10) proteins. **A** alignment of the cdc23 and DNA43 sequences. cdc23 (*upper*) and DNA43 (*lower*) predicted protein sequences were aligned using the GCG GAP program and by eye. Identical residues are indicated by *vertical lines* (|) and similar residues by *colons* (:). The nuclear localisation signal (*NLS*) is shown; also *boxed* are the potential p34^{cdc2} phosphorylation sites (S/TPXK/R) and the CCCH motifs discussed in the text. The DNA43 protein sequence was obtained by translation of the YIL150c genomic open reading frame. The DNA sequence of the *cdc23* gene is available from the EMBL database, accession number AJ224944. **B** diagrammatic representation of similarities between the cdc23 and DNA43 proteins. Nuclear localisation signals (*NLS*), CCCH motifs and potential p34^{cdc2} phosphorylation sites (*p34*^{cdc2} *P~n site*) are shown

distance downstream in both proteins lie consensus phosphorylation sites for the p34^{cdc2} protein kinase (Moreno and Nurse 1990) – at residues 64–67 and 70–73 in cdc23 and at 66–69 in DNA43. A search of both predicted protein sequences using profiles from the BLOCKS and PROSITE databases (Bairoch et al. 1997; Henikoff et al. 1997) recognised no further domains; however, at a homologous central location both proteins contain a $CX_{9-10}CX_{11}CX_2H$ sequence (a 'CCCH motif' – Fig. 2) which is reminiscent of metal-binding sites ('zinc fingers') in nucleic acid-binding proteins. Although the spacing between the cysteine residues differs from known CCCH and other zinc-finger sequences (Berg 1986, 1990; Thompson et al. 1996) it is possible that this region of the proteins has a role in co-ordinating a metal ion. The cdc23 and DNA43 proteins also share many conserved residues and have similar predicted molecular weights (66.7 and 65.8 kDa respectively), isoelectric points (9.4 and 9.7 respectively), amino-acid compositions, hydropathy profiles, and regions of predicted secondary structure (Fig. 2 and data not shown).

Construction and study of a *cdc23* deletion strain

To determine whether the *cdc23*$^{+}$ gene is essential for viability a null allele was constructed using the one-step gene-replacement technique (Rothstein 1983). PCR-based in vitro mutagenesis was used to construct a plasmid in which the *cdc23* open reading frame was replaced by the *ura4*$^{+}$ marker gene. The linear insert comprising *ura4* with *cdc23* flanking sequences was then gel-purified and used to transform a *ura4-D18* diploid strain to uracil prototrophy. When tetrad analysis was performed on two ura^{+} diploid transformants, each ascus was found to give rise to two viable and two inviable spores, with all the surviving haploids being ura^{-}. Southern analysis confirmed that the two diploid strains were heterozygous for the *cdc23* deletion and that their ura^{-} haploid progeny were all undeleted for *cdc23* (data not shown); therefore, it can be concluded that *cdc23* is an essential gene for proliferation. In order to investigate the terminal morphology of *cdc23* null cells, we prepared spores from *cdc23*$^{+}$*/cdc23::ura4* diploids and germinated them in ura^{-} minimal medium. All the spores that germinated exhibited cell-cycle arrest, most with an elongated phenotype and with a single nucleus (Fig. 3 a) – a phenotype similar to that of the *cdc23-M36* mutant at the non-permissive temperature. Analysis of individual spores germinating from microdissected tetrads revealed that about half the germinating *cdc23* deletants did not divide at all, but arrested as elongated cells (Fig. 3 c). Those

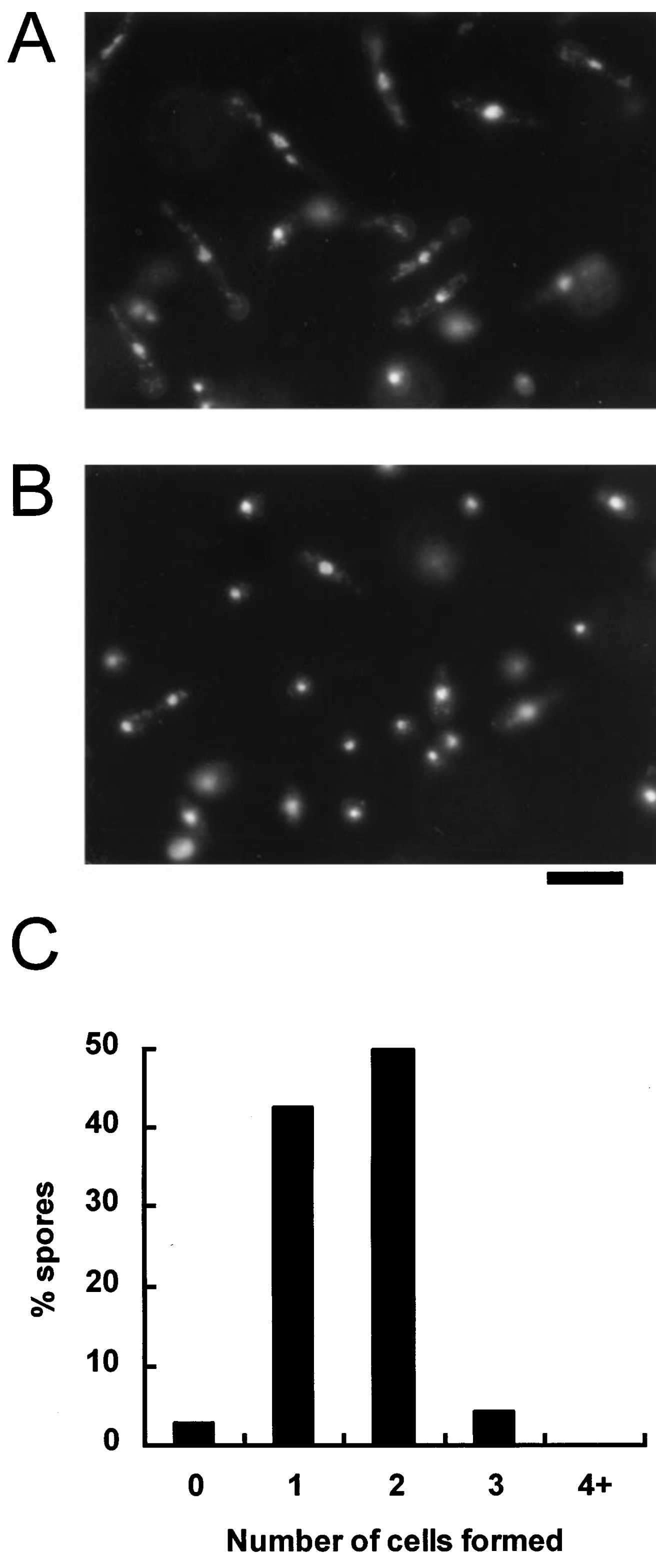

Fig. 3 A–C Analysis of the *cdc23* deletion phenotype. **A** and **B** DAPI fluorescence of germinating spores, 16 h after inoculation into minimal medium lacking uracil, from (**A**) a *cdc23::ura4* heterozygous diploid; (**B**) a diploid heterozygous for *ura4* but wild-type for *cdc23*. Bar=10 µm. **C** numbers of cells formed by germinating haploid *cdc23::ura4* spores as determined from tetrad analysis (*n*=68 spores from 34 tetrads). The frequency of un-germinated spores (zero 'cells formed' bar) is not significantly different from that of wild-type spores

cells which did divide gave rise to a maximum of three cells before arresting.

Complementation of *S. cerevisiae dna43–1* with the *S. pombe cdc23* gene

In order to test whether *cdc23* and *DNA43* are functional homologues the complete *cdc23* ORF was cloned into a *S. cerevisiae* expression vector and this construct (Fig. 4 a) was transformed into a *dna43-1* strain of *S. cerevisiae* (Dumas et al. 1982). Transformants were screened for mutant complementation by replica plating onto galactose and glucose media at the non-permissive temperature (37°C). Cells containing the plasmid-borne *cdc23* gene were able to grow and divide at this temperature (Fig. 4 b) showing that the *S. pombe cdc23* gene is able to functionally complement the *S. cerevisiae dna43-1* mutant. Microscopic examination of *cdc23*-rescued transformants revealed cells at all stages of the cell cycle, although the cell size was greater than when rescued with the homologous *DNA43* gene, indicating that *cdc23* is unable to completely restore the wild-type phenotype even when expressed at high level.

Discussion

We have cloned the *S. pombe cdc23* DNA replication gene by complementation of the *cdc23-M36* mutation. We have demonstrated that this gene is essential for cell proliferation and that it encodes a structural and functional homologue of the budding yeast DNA43 (MCM10) protein.

Deletion of virtually the whole chromosomal *cdc23* ORF gives rise to a terminal phenotype similar to that of the *cdc23-M36* mutant at the restrictive temperature: elongated cells with a single nucleus. This observation confirms that the *cdc23* gene product is not required to maintain the checkpoints monitoring the integrity of the chromosomal information, which are lost when DNA polymerase α or the cdc18 replication origin-associated factor are disrupted by gene deletion/disruption (Kelly et al. 1993; d'Urso et al. 1995). A *dna43* disruption mutant is similarly lethal (Solomon et al. 1992) and the phenotype of *cdc23::ura4* is directly analogous to the terminal phenotype reported for budding yeast *dna43-1* and *mcm10-1* temperature-sensitive mutants under non-permissive conditions (Solomon et al. 1992; Merchant et al. 1997).

The predicted protein product of the *cdc23* gene has a 22% overall identity to the product of the *S. cerevisiae DNA43* gene with similarity particularly marked in the N-terminal half of the sequence. This is not a high level of identity, even given the great evolutionary distance between the two yeasts, but the *cdc23* and *DNA43* products also share many conserved residues and have similar molecular masses, isoelectric points, amino-acid compo-

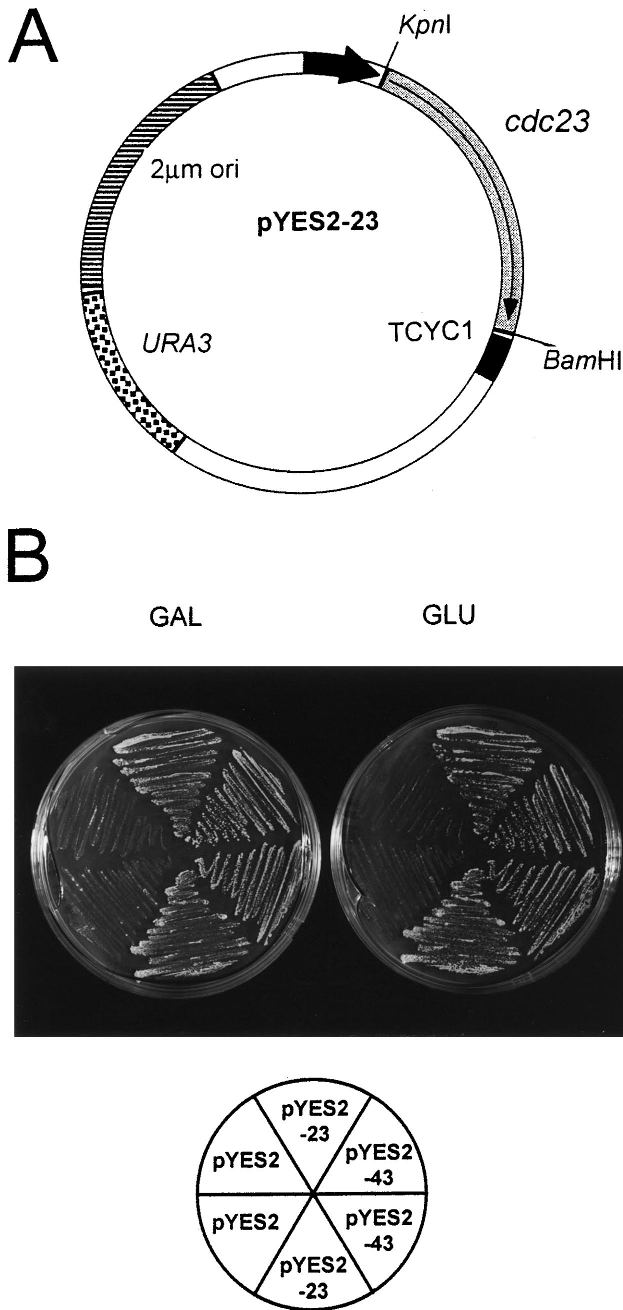

Fig. 4 A, B Complementation of the *S. cerevisiae dna43-1* mutant by the *S. pombe cdc23* gene. **A** diagram of the construct pYES2-23 (7.70 kb) containing the entire *cdc23* ORF under the control of the inducible *GAL1* promoter (*solid arrow*) in *S. cerevisiae* expression vector pYES2. The plasmid pYES2–43, containing the *DNA43* gene, was similarly constructed. **B** transformants of *S. cerevisiae dna43-1* containing pYES2, pYES2-23 or pYES2-43 replica-plated to galactose (*GAL*) or glucose (*GLU*) plates at 37°C to test for complementation

sition, hydropathy profiles and regions of predicted secondary structure. Also conserved are nuclear localisation signals, potential p34^{cdc2} phosphorylation sites, and zinc finger-like domains (Fig. 2). These features are consistent with the view that these proteins are structurally homologous. Completion of the *S. pombe* genome sequencing project will reveal whether *cdc23* is the sole structural homologue of *DNA43* in fission yeast. Conditional mutations in the budding yeast gene give rise to reduced rates of DNA synthesis, as reported also for the *cdc23-M36* mutant (Solomon et al. 1992; Merchant et al. 1997; Nasmyth and Nurse 1981), and gene disruption/deletions are lethal (Solomon et al. 1992, and this study). The similarity between cdc23 and DNA43 from two extremely distantly related yeast species and the phenotypic similarity of the respective mutants, taken together with our demonstration of functional homology between the two genes, suggest a conserved eukaryotic DNA replication function. Merchant et al. (1997) have provided evidence that DNA43 is required for the correct initiation of DNA replication at chromosomal origins and that the protein interacts in vivo and in vitro with at least some of the members of the MCM2-7 family of replication-initiation proteins. It will be of interest to determine if cdc23 functions in a similar manner in fission yeast.

Nuclear localisation signals present in both the cdc23 and DNA43 proteins suggest that both these proteins are targeted to the nucleus: nuclear localisation has already been demonstrated for DNA43 (Merchant et al. 1997) and work is in progress to test this hypothesis experimentally for cdc23. The presence of consensus phosphorylation sites for the p34$^{cdc2/CDC28}$ cyclin-dependent kinase in both homologues suggests that these proteins may be post-translationally regulated in a cell-cycle-dependent manner. Whether the phosphorylation status of either varies through the cell cycle is a key question for future research. The close proximity of the p34^{cdc2} phosphorylation sites to the nuclear localisation signals in the predicted products of both proteins raises the possibility that nuclear localisation is regulated in a cell-cycle-dependent manner: phosphorylation close to nuclear localisation signals within other proteins has been reported to affect the rate of nuclear import (Jans et al. 1991; Rihs et al. 1991). In one case regulation of this type by the p34^{cdc2} kinase was noted (Jans et al. 1991). We report a novel domain present in cdc23 and DNA43 which is reminiscent of the zinc-finger domains described within proteins which bind nucleic acids. Although the motif does not correspond completely to consensus sequences for known zinc-finger structures it is possible that this region of the proteins may have a role in coordinating a metal ion, which suggests a possible protein-nucleic acid interaction.

We conclude that the fission yeast *cdc23* gene product is a structural and functional homologue of budding yeast DNA43 and speculate that these proteins may represent a replication factor conserved throughout eukaryotes. We predict that the protein is nuclear and that it may be subject to post-translational modification in a cell-cycle-dependent manner; specifically it may be a target for phosphorylation by the p34^{cdc2} protein kinase. The future elucidation of the exact role of the cdc23/DNA43 proteins in chromosomal DNA replication can be expected to advance our understanding of the regulation and mechanics of this fundamental process in eukaryotes.

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