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The fission yeast *cdc19⁺* gene encodes a member of the MCM family of replication proteins

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SUMMARY

We have cloned and characterized the fission yeast *cdc19*$^+$ gene. We demonstrate that it encodes a structural homologue of the budding yeast MCM2 protein. In fission yeast, the *cdc19*$^+$ gene is constitutively expressed, and essential for viability. Deletion delays progression through S phase, and cells arrest in the first cycle with an apparent 2*C* DNA content, with their checkpoint control intact. The temperature-sensitive *cdc19-P1* mutation is synthetically lethal with *cdc21-M68*. In addition, we show by classical and molecular genetics that *cdc19*$^+$ is allelic to the *nda1*$^+$ locus. We conclude that cdc19p plays a potentially conserved role in S phase.

Key words: cdc19, DNA replication, MCM2, fission yeast, cell cycle

INTRODUCTION

START in yeast is a point of commitment to a cycle of division (reviewed by Forsburg and Nurse, 1991). Passage through START requires the p34^{cdc2} protein kinase or a close structural relative, which is presumed to associate with a regulatory cyclin molecule in an analogous complex to that identified in mitotic control (Blow and Nurse, 1990; Nasmyth, 1990; Fang and Newport, 1991; reviewed by Reed, 1992; Sherr, 1993). After commitment to the cell cycle at START, a cell completes G_1, initiates DNA replication and proceeds through S phase. This process needs to be tightly controlled, to ensure that the cell is able to support replication and division before embarking on the cell cycle, and also to ensure that replication occurs only once in a cycle, after M phase is completed. Much of this regulation is likely to be in the timing of initiation of the process of DNA replication. But what actually happens at START of the cell cycle, and how START is coupled to initiation of DNA replication, are still unclear.

Yeast genetics offers one means of identifying the linkages between the structural necessities of the replication process and the regulatory constraints of the cell cycle. The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are evolutionarily highly diverged organisms (Sipiczki, 1989). Comparison of cell cycle regulation in the two yeasts has been uniquely informative in determining which regulatory components are likely to be generally conserved amongst all eukaryotes, and which are likely to be system-specific. An extensive body of work has developed around yeast DNA replication (Bartlett and Nurse, 1990; Campbell and Newlon, 1991; Campbell, 1993; Kelly et al., 1993b). A common regulatory link between START and S phase in both yeasts is provided by the DSC (or MCBF) transcriptional activator, which regulates expression of a number of genes involved in DNA replication (Gordon and Campbell, 1991; Lowndes et al., 1991; McIntosh et al., 1991; Verma et al., 1991). This regulation occurs through a conserved sequence called the MCB element. In fission yeast, this binding complex contains the products of the *cdc10*$^+$ and *sct1*$^+$*(res1*$^+$*)* genes (Lowndes et al., 1992; Tanaka et al., 1992; Caligiuri and Beach, 1993). The process of replication is coupled to both upstream and downstream events, as shown by the phenotype of the fission yeast *cdc18*$^+$ gene (Kelly et al., 1993a), a target of this transcriptional activation complex. Both *cdc18*$^+$ and another gene, *rad4*$^+$/*cut5*$^+$, are required for DNA replication and to generate the downstream signal that prevents the cell from continuing into mitosis unless S phase is completed (Kelly et al., 1993; Saka and Yanagida, 1993). Thus some gene products required for replication are also components of the previously identified checkpoint control that helps maintain the dependence of M phase on S phase (Enoch and Nurse, 1990). Nevertheless, these analyses have shown that *cdc10*$^+$-dependent transcription of *cdc18*$^+$ cannot be the only bridge between START and the beginning of S phase in fission yeast (Kelly et al., 1993a), so additional connections must still be sought.

Another connection between START and S phase in *S. cerevisiae* is provided by the *MCM* gene family, originally isolated because mutants showed origin-specific defects in the maintenance of minichromosomes (Maine et al., 1984; Sinha et al., 1986). These defects are apparently due to failures in replication, rather than in segregation (Maiti and Sinha, 1992). Subsequently, it has been shown that several MCM proteins, MCM2p, MCM3p and CDC46p (MCM5p), are structurally related to one another, forming a large super-family (Gibson et al., 1990; Hennessy et al., 1991; Yan et al., 1991; Chen et

al., 1992). Intriguingly, these gene products all undergo a cell-cycle-specific nuclear localization near the G_1/S transition (Hennessy et al., 1990; Yan et al., 1993). Such controlled nuclear entry could provide another means of regulating the G_1 to S phase transition and maintaining the order of events in the cell cycle. In addition, it fulfils one of the expectations of the inferred activity known as licensing factor (Blow and Laskey, 1988). The *MCM* genes are not redundant; mutation of any one in the budding yeast is lethal, with a final DNA content of either 1*C* or 2*C* (Gibson et al., 1990; Hennessy et al., 1990; Hennessy et al., 1991; Yan et al., 1991). At least two additional members of the family have been identified in *S. cerevisiae* (Coxon et al., 1992; Bussereau et al., 1993). The family is not restricted to budding yeast. Three distinct members have been cloned from *S. pombe* (this work; Coxon et al., 1992; Miyake et al., 1993) and a mouse protein with homology to MCM3 has been identified biochemically by its weak association with the DNA polymerase alpha complex (Thömmes et al., 1992). Other related sequences in metazoan systems have been identified by genome sequencing, by PCR or by screens for conserved epitopes (Hu et al., 1993; Todorov et al., 1994).

We are interested in characterizing the G_1/S transition in fission yeast. We report here the cloning of the *cdc19*$^+$ gene from *S. pombe*. We show that *cdc19*$^+$ encodes an excellent structural homologue of the budding yeast MCM2 protein. Despite its early execution point in the cell cycle (Nasmyth and Nurse, 1981), *cdc19*$^+$ is not required by cells to synthesize DNA. However, in its absence, S phase is delayed. The cells do not show a checkpoint deficiency phenotype when *cdc19*$^+$ is deleted, but arrest with a phenotype similar to the original conditional allele. Expression of *cdc19*$^+$ is constitutive in the cycle. A double mutant between *cdc19-P1* and *cdc21-M68*, which encodes another member of the family, has a synthetic lethal phenotype at the permissive temperature. *cdc19*$^+$ is allelic to the *nda1*$^+$ gene, which was recently cloned (Miyake et al., 1993). Our evidence suggests that cdc19p functions as an effector of S phase, and offers further evidence that this family of proteins is likely to have a conserved role in DNA replication.

MATERIALS AND METHODS

Strains and genetic analyses

All *S. pombe* strains are congenic to 972 *h*$^-$. The *cdc19-P1* and *cdc21-M68* mutations were first described by Nasmyth and Nurse (1981). Crosses and analysis of progeny were carried out as described by Moreno et al. (1991). The restrictive temperature used for *cdc19-P1* and *cdc21-M68* was 36°C, and for *nda1-KM376* was 20°C. The *cdc19*$^+$ gene was mapped physically by hybridizing the clone of *cdc19*$^+$ to P1 and cosmid filters provided by Jörg Hoheisel, ICRF (Maier et al., 1992; Hoheisel et al., 1993). Map location was confirmed by genetic analysis in crosses with *h*$^+$ *ura5-294 ade7* (B. Grallert), and *h*$^-$ *leu1 nda1-KM376* (National Collection of Yeast Cultures, no. 2231, Norwich, UK). Distances were determined using the formula 50×(6NPD + TT)/total (see Table 1 legend). All yeast transformations were carried out using electroporation (Kelly et al., 1993a) or lithium acetate (Moreno et al., 1991).

Cloning

The genomic clone of *cdc19*$^+$ was obtained by transforming a strain *h*$^-$ *cdc19-P1 leu1-32 ura4-D18 ade6-M210* with a genomic library in pUR19 provided by A. M. Carr (Barbet et al., 1992). Cells were plated on selective plates and allowed one day of growth at 25°C before being transferred to 37°C for direct selection of rescue. Thirty five clones were isolated, and plasmid was recovered from five of them using glass bead lysis in the presence of phenol and the Promega Wizard DNA cleanup kit.

Plasmids and DNA constructions

Several overlapping clones were isolated and subcloned into the vectors pUR19N or pUR19 (Barbet et al., 1992) to determine the minimum complementing fragment. A gene disruption was constructed by replacing the 1.6 kb fragment between *Xho*I and *Bgl*II with the *S. cerevisiae LEU2* gene on a *Sal*I-*Bam*HI fragment. This construction retains the 5′ and 3′ ends of the *cdc19*$^+$gene and approximately 2 kb of flanking sequence on either side. The plasmid pAC1 containing the *cdc21*$^+$clone was provided by Stephen Kearsey (Coxon et al., 1992).

Sequencing

Restriction fragments of cdc19 were subcloned into pTZ18R or pTZ19R (Pharmacia) and sequenced by cycle sequencing (US Biochemicals). Additional sequencing primers were synthesized and used to complete both strands and all overlaps. Inspection of the sequence identified one possible intron.

Computer analysis

DNA and protein database searches were carried out using a BLAST client to the NCBI database. Alignments were performed using the GAP utility of the GCG package of sequence software. PEST score was determined with the help of Liz Cowe, Oxford University Molecular Biology Data Centre.

Deletion and spore germination

A fragment containing the disrupted *cdc19* gene, with approximately 2 kb of flanking sequence on each side of *LEU2*, was used to transform a diploid strain of genotype *h*$^-$/*h*$^+$ *ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 can1-1/can1-1* to leucine prototrophy. Six clones were analysed further by Southern hybridization. Four showed the expected Southern pattern for a gene disruption. The other two were consistent with non-homologous integration events elsewhere in the chromosome, and one of these was used as a control strain. In random spore analysis, the heterologous integrant gave 2:2 segregation of Leu$^+$ to Leu$^-$ spores and all spores were viable. The four homologous clones gave no Leu$^+$ spores. Tetrad analysis showed each of the homologous clones segregated 2:2 viable spores; all the viable spores were Leu$^-$. However, when the diploid was transformed with plasmid pSLF124 (containing *cdc19*$^+$ and *ura4*$^+$) and sporulated, viable Leu$^+$ Ura$^+$ colonies could be obtained, indicating that the disruption may be complemented by the genomic clone. Spore germination analysis was carried out as follows. Diploid cultures of the homologous integrant and the heterologous integrant (as a control for the single copy *LEU2* marker) were grown to sporulation in malt extract medium. The sporulated diploids were treated with glusalase to kill all the vegetative cells. The spores were washed in sterile water and stored at 4°C. For the germination, the spores were inoculated into complete minimal medium lacking leucine to an absorbance at 595 nm of 0.1 and grown for 21 hours. Samples were taken hourly, beginning at 5 hours, and prepared for flow cytometry analysis.

Flow cytometry

Strains as indicated were grown in Edinburgh minimal medium (EMM). Preparation and fixation of cells for flow cytometry was carried out essentially as described by Sazer and Sherwood (1990), except that cells were stained in a final concentration of 4 μg/ml propidium iodide. Data were analysed using the program MacLysis (Becton Dickinson), and printed from the Macintosh program Canvas.

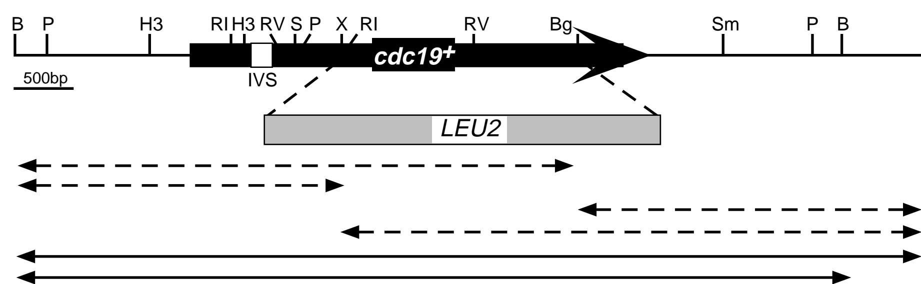

Fig. 1. Structure of the *cdc19*+ genomic locus. The open reading frame is indicated by the large arrow, and the intron by the open box labelled IVS. The stippled box indicates the location of the disruption/deletion with the LEU2 marker. The double-headed arrows underneath indicate the subcloning strategy. Continuous lines: clones that complement the *cdc19-P1* mutant strain. The smallest complementing clone was a *Bam*/*Bam* fragment. Broken lines: clones that fail to complement, which truncated the open reading frame at either *Bgl*II or *Xho*I sites. H3, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV; S, *Sac*I; P, *Pst*I; X, *Xho*I; Bg, *Bgl*II; Sm, *Sma*I.

Photomicroscopy

Ethanol-fixed cells were rehydrated in PBS and stained with DAPI for photography using a Leitz Labolux microscope according to standard protocols (Moreno et al., 1991). The negatives were scanned into a Macintosh computer, where the images were converted to positives using the program Adobe Photoshop and a composite was assembled in the program Canvas. The final results were printed on a Phaser IISDX printer.

Hybridizations and probe

Southern hybridizations using GeneScreen Plus were carried out following the manufacturer's instructions (Dupont/NEN). Probes for Southern blotting were prepared from the cdc19 cDNA clone by random oligo priming a purified fragment containing the entire *cdc19*+ coding region and prepared using a Stratagene Prime-it kit and [^{32}P]dATP. RNA from centrifugally elutriated cells was previously prepared by glass bead lysis in the presence of phenol (Kelly et al., 1993a), and was separated on a formaldehyde gel; transfer to GeneScreen Plus and probing were carried out according to the manufacturer's instructions (Dupont/NEN). The autoradiograph was scanned into a Macintosh computer, and the composite image assembled using the programs Cricket Graph and Canvas. The final results were printed on a Phaser IISDX printer.

RESULTS

Cloning of cdc19

The *cdc19-P1* mutation was originally identified as a single temperature-sensitive allele and characterized as having an early execution point, coincident with or near to the block imposed on DNA synthesis by hydroxyurea, although the cells arrest with an apparent 2*C* DNA content (Nasmyth and Nurse, 1981). This is very similar to the phenotype reported for the original temperature-sensitive allele of the *cdc18*+ gene, which has subsequently been shown to be a partially functional gene product (Nasmyth and Nurse, 1981; Kelly et al., 1993a). We cloned the *cdc19*+ gene by complementation of the mutant *cdc19-P1* strain at 37°C with a genomic library. Several independent, overlapping clones were isolated. We verified that we had cloned the *cdc19*+ gene by integration. The clone on a *ura4*+ marked plasmid was cut in the unique *Xho*I site in the *cdc19*+ open reading frame, and integrated into a *cdc19-P1* strain. We crossed this strain to a wild-type strain and carried out random spore analysis. Out of several hundred progeny, no temperature-sensitive spores were recovered, indicating that integration had taken place at or very close to the *cdc19-P1* locus. A diagram of the clone is shown in Fig. 1, which also indicates the subcloning strategy used to localize the gene.

Table 1. Map location of *cdc19*+

Cross	Tetrad class* PD	NPD	TT	Map distance (cM)†
ura5 × *ade7*	35	1	9	16
ura5 × *cdc19*	36	0	9	10
ade7 × *cdc19*	40	0	4	4.5

cdc19+ was mapped in a cross between *cdc19-P1* and *ura5-294 ade7*.

*Tetrad classes: PD is parental ditype, NPD is non-parental ditype, and TT is tetratype.

†The distances in centiMorgans were determined using the formula 50×(6*N*+*T*)/total, where *N* is NPD tetrads and *T* is TT tetrads.

We mapped the *cdc19*+ gene using the cosmid filters described by Hoheisel et al. (1993) and localized it to the interval between *ura5* and *ade7* on chromosome II. The gene *nda1*+ (Toda et al., 1983) also falls in this interval. To obtain a more precise genetic location, we crossed the *cdc19-P1* strain against *ura5 ade7* and *nda1-KM376* mutant strains. Out of 300 spores plated, we observed no wild-type recombinants in the cross with *nda1-KM376*. In crosses with *ura5 ade7*, we obtained results similar to those observed in the mapping data of *nda1* (Toda et al., 1983), suggesting that *cdc19*+ lies between the two auxotrophic markers (Table 1). We transformed the clone of *cdc19*+, containing 1 kb on either side of the open reading frame (ORF), into the *nda1*^{cs} mutant strain and determined that we could rescue the cold-sensitive phenotype. During the course of this work, the sequence of *nda1*+ was reported, confirming that these two loci are allelic (Miyake et al., 1993), and these sequences agree.

We sequenced the *cdc19*+ clone and found that it potentially encodes a protein of 830 amino acid residues with one deduced intron of 219 nucleotides near the 5′ end, confirmed by Miyake et al. (1993; Fig. 1). There are two clusters of basic amino acids, between residues 5-10 and 114-118, which are similar to nuclear localization signals (NLS) such as those identified in SV40 T-antigen (bold type in Fig. 2; and see Kalderon et al., 1984; Lanford and Butel, 1984). There are no p34^{cdc2} consensus phosphorylation sites (S/TPXK). Database comparison of the predicted *cdc19*+ gene product showed striking homology with the *S. cerevisiae* MCM2 protein (Fig. 3; and see Yan et al., 1991). *MCM2* is a member of a multi-gene family, each member of which is essential for viability in the

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   1 ATTATGATTA TAATCATTTT CCTGAGCCAC ATTCATCAAC GGATTTCCGT CAAACTTACA AGGTATGGAG TGGGCTTCTT
  81 GTGGTGCCAC AATCACATCG AAAATGTCGT TTATTGAATA GGTCCACAAG ATCATGGTCA GCATGGCAGC TAATACTGCA
                        HinD III
 161 CTATAGCAAA CACATTTAAG CTTCCGCTAG CTTTTTGACA TGTCAAACAA GCGTATAAAG GTTGCTTCAA ATAGCCCATC
 241 GAATAAGTGC ACGTGTCAAA GCTGTAAGGC AAAACTTCTC GTGCCTCCCT TTCAAGCTCC CTTTGCTGTT CAACATATTG
 321 CTTAACCGTT AATTCATGAG CCGTTTCCTC CATAAGTTAT TAAATGCAAC TAAACGTTGC AAATAGACGG CGTAAACAAT
 401 TGCGAAGAAT GTCTTTTGAA TAATTCAAAA CACTAACTCA TTACGCTCCT CTATTTCATT TACAGGTATC ACTACATTTT
 481 CCAATACAAT TTAACAATAT ACTGTAGCAT TTCTTATTCT TTCATCCTGC AATACTCTAG CTATTCAATG CATTAAAATA
 561 GTTAGAAACA ACATCTTTAA AGTATTTGAG TCCAAAG ATG GAT TCT TTT CGG AAA AGG GGT CGC CGC GAT TCT
                                         M   D   S   F   R   K   R   G   R   R   D   S
 634 GAA AGT TTA CCT TTT GAA TCA GAA AAT TCT TCT CTC GGT GCT ACA CCT CTT TCT TTA CCT CCT TCT
     E   S   L   P   F   E   S   E   N   S   S   L   G   A   T   P   L   S   L   P   P   S
                     EcoR I
 700 TCA CCT CCT CCA GAA TTC TCT GAT GAA GCT GCA GAA GCT CTC GTG GAG GAG GAT ATT GAG GAT CTG
     S   P   P   P   E   F   S   D   E   A   A   E   A   L   V   E   E   D   I   E   D   L
              HinD III
 766 GAC GGA GAA GCT TTA GAT GTA GAG GAT GAA GAA GGT GAA GAT TTG TTC GGA GAA GGA ATG GAA CG
     D   G   E   A   L   D   V   E   D   E   E   G   E   D   L   F   G   E   G   M   E   R
 831 GTAAGTTTTA TAGCTATGCT TGAGACGTGT CTTGTCAATG TTGTGTATAC TAAGTGTTGC CATCAATATA TAAATTACAT
 911 ACTATTTCTT CTCCTTCTTG TAAATAAATA TATTTATTGG ATTCCTAGGT TGCGGTTATA TTGACAGAAC AAATCTTGAT
 991 TTTCAAAAGA GTAGTTTCAC ATTTCGCGTC AAATAAGGTA CTTTTTTCTA ACTGTATAG T GAT TAT CAA CAA AAT
                                                                   D   Y   Q   Q   N
                                                                                EcoR V
1066 TTG GAG CTA GAT CGT TAT GAC ATT GAA GAA CTA GAT GAC GAT AAC GAT CTT GAA GAA TTA GAT ATC
     L   E   L   D   R   Y   D   I   E   E   L   D   D   D   N   D   L   E   E   L   D   I
                                                         Sac I           Pst I
1132 GGG GCC CGA AGA GCT GTT GAT GCC AGG TTG AGA CGA CGT GAT ATT GAG CTC GAT GCT GCT GCA GGT
     G   A   R   R   A   V   D   A   R   L   R   R   R   D   I   E   L   D   A   A   A   G
1198 AGA ACA AAA CCT GCT GCA TTT TTA CAA GAT GAA GAT GAT GAT TTG GAC TCC AAT CTT GGC ACA GGC
     R   T   K   P   A   A   F   L   Q   D   E   D   D   D   L   D   S   N   L   G   T   G
1264 TTC ACT CGT CAT CGA CAT CGA ATT TAC GAC GAA TAT TCA CCT AAT GTT GGC GCA TTG GAC GAA TCT
     F   T   R   H   R   H   R   I   Y   D   E   Y   S   P   N   V   G   A   L   D   E   S
1330 GGT GAA CTT CCT CTT GAA TCA ATT GCC GAC GTA AAG GCC GAT AGT ATT GCC GAA TGG GTT ACT CTT
     G   E   L   P   L   E   S   I   A   D   V   K   A   D   S   I   A   E   W   V   T   L
                              Xho I  EcoR I
1396 GAT CCT GTT CGG CGT ACA ATT GCT CGA GAA TTC AAA AAT TTC CTT CTT GAA TAT ACA GAT GAA AAT
     D   P   V   R   R   T   I   A   R   E   F   K   N   F   L   L   E   Y   T   D   E   N
1462 GGC ACC TCT GTA TAC GGT AAC CGT ATT CGC ACA TTG GGT GAG GTT AAT GCT GAG TCG TTG ATG GTT
     G   T   S   V   Y   G   N   R   I   R   T   L   G   E   V   N   A   E   S   L   M   V
1528 AAT TAT GCC CAT CTT GGT GAA TCT AAA CCC ATT TTG GCT TAT TTC TTG GCC AAT GCG CCT GCA CCT
     N   Y   A   H   L   G   E   S   K   P   I   L   A   Y   F   L   A   N   A   P   A   P
1594 ATT TTT CGC ATA TTT GAT CGT GTT GCT TTG GAA GCC ACT CTT TTG CAT TAT CCA GAC TAT GAA AGA
     I   F   R   I   F   D   R   V   A   L   E   A   T   L   L   H   Y   P   D   Y   E   R
1660 ATA CAC TCT GAT ATT CAT GTT CGT ATC ACT AAT CTT CCT ACT TGT TTT ACT TTA CGC GAT CTA CGA
     I   H   S   D   I   H   V   R   I   T   N   L   P   T   C   F   T   L   R   D   L   R
1726 CAA TCC CAT CTT AAT TGC CTT GTA CGG GTA TCT GGT GTC GTA ACT AGG CGC ACT GGG CTT TTT CCA
     Q   S   H   L   N   C   L   V   R   V   S   G   V   V   T   R   R   T   G   L   F   P
1792 CAA TTA AAA TAC ATT CGG TTT ACA TGT ACC AAA TGT GGT GCT ACT TTG GGT CCA TTT TTT CAA GAC
     Q   L   K   Y   I   R   F   T   C   T   K   C   G   A   T   L   G   P   F   F   Q   D
1858 TCT AGC GTT GAA GTA AAA ATT TCT TTC TGT CAC AAC TGT TCC AGC CGT GGT CCG TTT GTA ATC AAC
     S   S   V   E   V   K   I   S   F   C   H   N   C   S   S   R   G   P   F   V   I   N
1924 TCT GAA CGG ACT GTA TAT AAT AAC TAT CAA AGG ATC ACT TTA CAA GAA TCA CCT GGC ACC GTC CCC
     S   E   R   T   V   Y   N   N   Y   Q   R   I   T   L   Q   E   S   P   G   T   V   P
1990 TCA GGT AGA CTG CCT CGA CAC CGT GAA GTT ATT CTT TTA GCG GAC TTG GTT GAT GTT GCC AAA CCA
     S   G   R   L   P   R   H   R   E   V   I   L   L   A   D   L   V   D   V   A   K   P
2056 GGC GAA GAG ATT GAT GTT ACT GGC ATA TAC CGC AAT AAC TTC GAT GCT AGT CTG AAT ACC AAA AAC
     G   E   E   I   D   V   T   G   I   Y   R   N   N   F   D   A   S   L   N   T   K   N
2122 GGG TTT CCT GTC TTC GCT ACA ATT ATA GAA GCA AAT CAC ATA TCG CAA CTT GAC GGC AGT GGT AAT
     G   F   P   V   F   A   T   I   I   E   A   N   H   I   S   Q   L   D   G   S   G   N
2188 ACT GAT GAT GAT TTC TCT TTA AGT CGG CTT ACT GAT GAT GAG GAA AGG GAA ATT CGG GCA TTA GCA
     T   D   D   D   F   S   L   S   R   L   T   D   D   E   E   R   E   I   R   A   L   A
2254 AAG TCG CCT GAT ATT CAC AAC AGA ATT ATT GCA TCG ATG GCG CCT TCT ATT TAT GGA CAT CGC TCT
     K   S   P   D   I   H   N   R   I   I   A   S   M   A   P   S   I   Y   G   H   R   S
2320 ATC AAA ACT GCT ATT GCT GCT GCT TTA TTC GGT GGT GTC CCC AAA AAT ATT AAC GGT AAG CAT AAA
     I   K   T   A   I   A   A   A   L   F   G   G   V   P   K   N   I   N   G   K   H   K
                 EcoR V
2386 ATT AGA GGT GAT ATC AAT GTT TTA TTG TTG GGA GAT CCA GGA ACC GCC AAA TCT CAA TTT CTT AAG
     I   R   G   D   I   N   V   L   L   L   G   D   P   G   T   A   K   S   Q   F   L   K
2452 TAT GTT GAA AAA ACG GCA CAT AGG GCT GTC TTC GCC ACA GGT CAG GGT GCC AGT GCT GTT GGT CTA
     Y   V   E   K   T   A   H   R   A   V   F   A   T   G   Q   G   A   S   A   V   G   L
2518 ACT GCA TCC GTC CGT AAA GAC CCC ATT ACG AAT GAA TGG ACT TTG GAA GGG GGT GCT TTG GTT TTA
     T   A   S   V   R   K   D   P   I   T   N   E   W   T   L   E   G   G   A   L   V   L
2584 GCA GAT AAA GGT GTT TGT TTG ATT GAC GAG TTC GAT AAA ATG AAT GAT CAG GAT CGT ACT TCT ATT
     A   D   K   G   V   C   L   I   D   E   F   D   K   M   N   D   Q   D   R   T   S   I
2650 CAC GAA GCC ATG GAA CAA CAA AGT ATT TCT ATT TCT AAG GCG GGT ATT GTG ACT ACC TTG CAG GCG
     H   E   A   M   E   Q   Q   S   I   S   I   S   K   A   G   I   V   T   T   L   Q   A
2716 AGG TGC ACT ATT ATT GCT GCC GCT AAT CCC ATC GGT GGA CGT TAT AAC ACT ACA ATT CCT TTT AAT
     R   C   T   I   I   A   A   A   N   P   I   G   G   R   Y   N   T   T   I   P   F   N
2782 CAA AAT GTT GAG CTG ACC GAA CCT ATT TTA TCT CGT TTT GAC ATC CTT CAG GTC GTC AAA GAC ACG
     Q   N   V   E   L   T   E   P   I   L   S   R   F   D   I   L   Q   V   V   K   D   T
2848 GTT AAT CCT GAA ATT GAT GAA CAG CTA GCT AAT TTT GTT GTA TCA AGT CAT ATT CGA TCT CAT CCT
     V   N   P   E   I   D   E   Q   L   A   N   F   V   V   S   S   H   I   R   S   H   P
2914 GCA TTT GAT CCG AAT ATG GAT GTC TTG AAG AAA GTC CCT ACT GAG ACT GGT ATT GAT GCC AAA CCC
     A   F   D   P   N   M   D   V   L   K   K   V   P   T   E   T   G   I   D   A   K   P
              Bgl II
2980 ATT CCC CAA GAT CTT CTT CGT AAA TAT ATC CAT TTC GCT CGT GAA AAA GTT TTT CCT CGA TTA CAG
     I   P   Q   D   L   L   R   K   Y   I   H   F   A   R   E   K   V   F   P   R   L   Q
3046 CAA ATG GAT GAA GAA AAG ATT TCG AGA CTT TAT AGC GAT ATG AGA CGC GAG TCA CTG GCT ACT GGA
     Q   M   D   E   E   K   I   S   R   L   Y   S   D   M   R   R   E   S   L   A   T   G
3112 AGT TAT CCT ATT ACT GTG CGT CAT CTG GAG TCT GCT ATC CGT TTA AGT GAA GCA TTT GCA AAA ATG
     S   Y   P   I   T   V   R   H   L   E   S   A   I   R   L   S   E   A   F   A   K   M
3178 CAG CTC AGT GAG TTT GTG CGC CCT TCA CAT ATA GAC AAA GCC ATT CAA GTG ATT ATT GAT TCC TTT
     Q   L   S   E   F   V   R   P   S   H   I   D   K   A   I   Q   V   I   I   D   S   F
3244 GTG AAT GCC CAA AAA ATG AGT GTT AAA CGA AGT TTG TCA AGA ACA TTT GCT AAA TAT CTT ATT TAA
     V   N   A   Q   K   M   S   V   K   R   S   L   S   R   T   F   A   K   Y   L   I   *
3310 TTTTTTGACA CAATCAGTTT ATGGAGTCCA GTGATTTTGA  TTTCCCCGTT CTTTGACCCT CCTTTTGTTA CTGCATCTGT
3390 TGCATATATC TATCCTTTAA TAATTTGCAA TCAAAATTTG  CTTTACATTT TTATACCTAT CTTTACATTC ATTCTTTCTT
3470 AGTTTTCAAA ATGTCAATCT AAATTATTTA GGTTAATAAC  ACTTGTTTTG AATATCATTA TTGACTATTC GCTATAGATA
3550 CTAGGTGTAT AGATACTTTC TCCGCTGTAT CAATATTATT  AGCTGTTTCA TTAAAGAGCC ACTGCCCATG TGAAATGACA
3630 TTTTTTGAAA CAGCTGAGGT TTTTTACATA TAGAGCTAAA  TTGTCCATTT CCAAATTTAA AGGGAAGTCA ACTTTTGACA
3710 ATCGGAAATC GATCCAGTTC ATATCTGATG TTCCTGAAGA  ATCTCAAATT TCACACTTTA TTATCTAAAA TTATTTATAT
3790 CGTGTTACTC TTTCAAATCA GTCTTTTTAT TTGGTATATA  TTTTGCCCTA CCCTTACCTA CTTATATTCG TACATCCACC
                                           Sma I
3870 AAGTAAACTT GACACAAAAA CAAAATGGCC TATCCCGGG
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Fig. 2. Nucleic acid sequence and presumed protein sequence of cdc19. The genomic sequence is shown. Restriction sites are indicated by bold face in the nucleic acid sequence. There is a single deduced intron of 219 nucleotides, which fits the consensus GTAAGT-*n*-CTAAC-*n*-TAG (Prabhala et al., 1992). The two stretches of basic residues, similar to a nuclear localization signal from SV40 T-antigen (Kalderon et al., 1984; Lanford and Butel, 1984) are indicated by bold type in the protein sequence. There are no $p34^{cdc2}$ consensus phosphorylation sites. GenBank accession number is U08048.

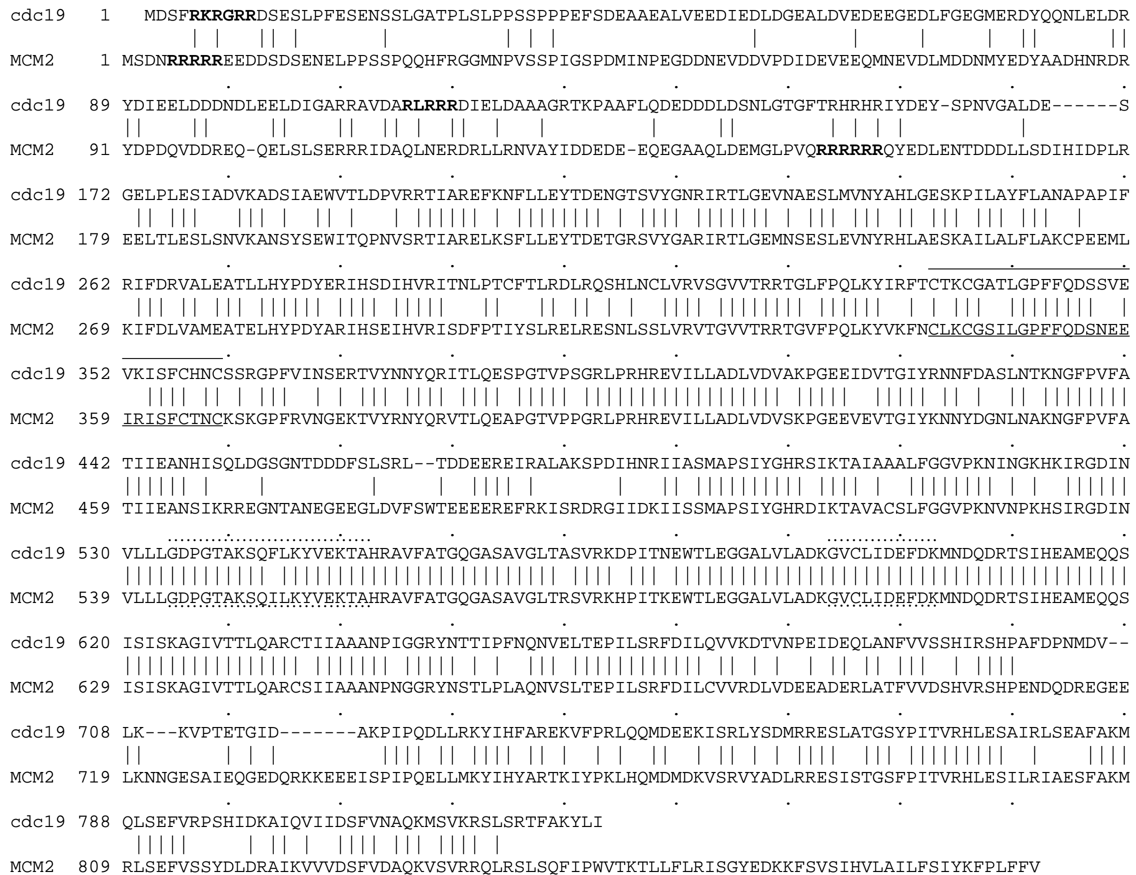

Fig. 3. Identity between MCM2 and cdc19. The two proteins are highly homologous across their entire length (60%). The most striking region of identity is shown in the figure. A putative zinc finger domain shared between MCM2p (Yan et al., 1991) and cdc19p is indicated by continuous lines. The conserved motifs identified by Koonin (1993) as indicative of a superfamily of putative DNA-dependent ATPases are indicated by dotted lines. Bold type identifies potential nuclear localization sequences (Kalderon et al., 1984; Lanford and Butel, 1984).

budding yeast (Gibson et al., 1990; Hennessy et al., 1991; Yan et al., 1991). The original *mcm* mutations were isolated due to an autonomous replication sequence (ARS)-specific defect in the maintenance of minichromosomes, suggesting that they play a role in replication, perhaps by origin recognition (Maine et al., 1984; Sinha et al., 1986). Whilst cdc19p is also structurally related to all members of the MCM family, its identity to MCM2p is most striking, being over 60% across the entire length of the protein (determined using the GAP program of the GCG package). The similarity is also apparent at the nucleic acid level (data not shown). In contrast, the identity between cdc19p and other members of the family is between 30 and 35%. The MCM2 protein contains a zinc finger motif, which is also found in cdc19p (indicated by double lines in Fig. 3; and see Yan et al., 1991). Additionally, there are short tracts of basic residues in MCM2p that are similar to the NLS seen in cdc19p. The amino terminus of cdc19p is rich in PEST sequences, which may target proteins for degradation (Rogers et al., 1986); the region between residues 10 and 78 has a PEST score of 14.5. Recently, a computer-based comparative analysis suggested that the MCM proteins are members of a diverse class of putative DNA-dependent ATPases, and identified several conserved motifs shared amongst all these proteins (Koonin, 1993). The *cdc19*+ gene also contains these sequences (dotted lines in Fig. 3).

Genetic analysis of *cdc19-P1*

We verified the original observation by Nasmyth and Nurse (1981) that *cdc19-P1* arrests with a 2*C* DNA content by flow cytometry on a culture shifted from the permissive temperature of 25°C to the restrictive temperature of 36°C (Fig. 4A). As shown in the figure, there is no evidence for a G_1 or S phase delay in the mutant after a temperature shift. We also carried out a time-course study on a temperature shift of the *nda1-KM376* allele, which is cold-sensitive (Toda et al., 1983; Miyake et al., 1993). We shifted cells from a permissive temperature of 32°C to 20°C, and observed an S phase delay, although the cells appear to block with an S/G_2 DNA content (Fig. 4B). Thus, the two alleles of *cdc19*+ have different behaviour when shifted to their restrictive temperatures.

Previously it has been shown that the ability to arrest the cell cycle for many mutants depends on an active checkpoint pathway operating in part through the *cdc25*+ gene product (Enoch and Nurse, 1990). Enoch and Nurse (1990) demonstrated that checkpoint control could be bypassed by the *cdc2-3w* mutation, which partly relieves the cell of dependence on *cdc25*+. Kelly et al. (1993a) showed that the *cdc18*+ gene product is required for S phase and to prevent mitosis from occurring, presumably by generating a signal that replication is in progress. In order to determine whether the arrest of the *cdc19-P1* strain depends on checkpoint control, we constructed

a double mutant between *cdc19-P1* and *cdc2-3w*. This strain was still temperature-sensitive, but rather than elongating as does *cdc19* alone, at the restrictive temperature the double mutant cells show aberrant divisions (Fig. 5, compare B and C). Upon longer incubation, the cells become smaller as they die by cutting. Thus, in order for *cdc19-P1* to arrest, the cells must have checkpoint control intact, suggesting that some signal is generated in the *cdc19-P1* strain indicating incomplete or damaged DNA.

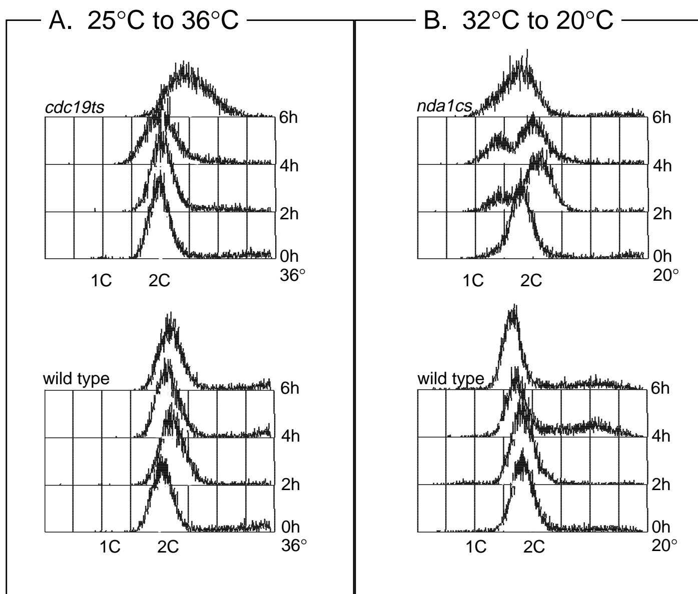

Fig. 4. Flow cytometry time-course of arrested mutant cells. (A) Flow cytometry of shift of *cdc19-P1* strains to restrictive temperature of 36°C, compared to wild-type cells. Cultures were grown in Edinburgh minimal medium and sampled every 2 hours after a shift from 25°C to 36°C. DNA content is shown along the *x* axis. There is no change in the position of the peak, indicating that *cdc19-P1* cells are arresting with a 2*C* DNA content. (B) Flow cytometry of a shift of *nda1-KM376* cells to restrictive temperature of 20°C, compared to wild-type cells. Cultures were grown in EMM and sampled every 2 hours after a shift from 32°C to 20°C. DNA content is along the *x* axis. There is a delay in S phase apparent at 4 hours.

Interaction with *cdc21-M68*

Three members of the *MCM* family have now been identified from the fission yeast (this work; Coxon et al., 1992; Miyake et al., 1993). *cdc21*$^{+}$ is homologous to all members of the family but clearly forms a distinct subclass (Coxon et al., 1992). We were interested to see whether we could identify any interactions between *cdc21-M68* and *cdc19-P1*. We were unable to identify any potential double mutants by tetrad analysis, although there were very few intact tetrads, which could be due to the poor spore viability that we observed in our crosses with *cdc21-M68* (data not shown). In order to confirm that there was a specific synthetic lethality, rather than a non-specific reduction in spore viability, we transformed *cdc21-M68* with a plasmid containing the *cdc21*$^{+}$ gene (Coxon et al., 1992) and then crossed it to *cdc19-P1*. Plasmids are lost rapidly in meiosis but we hoped this would ameliorate the spore viability problem. Our prediction was that if there is a synthetic lethality between *cdc21-M68* and *cdc19-P1*, then for PD tetrads, 4 viable spores should be temperature-sensitive; for NPD tetrads, there should be 2 wild type : 2 dead (non-viable) spores; and for tetratype tetrads, there should be 2 viable temperature-sensitive (ts) spores:1 wild-type spore:1 dead spore. After dissecting 20 tetrads from this cross we found 4 classes of tetrads as shown in Table 2. All the progeny were Leu^{-}, indicating that the plasmid had been lost in meiosis. We posited that the three aberrant tetrads (3 ts:1 dead) might be due to the non-specific spore inviability seen in crosses with *cdc21-M68*, and were thus really defective PD tetrads (PD (b)). We verified

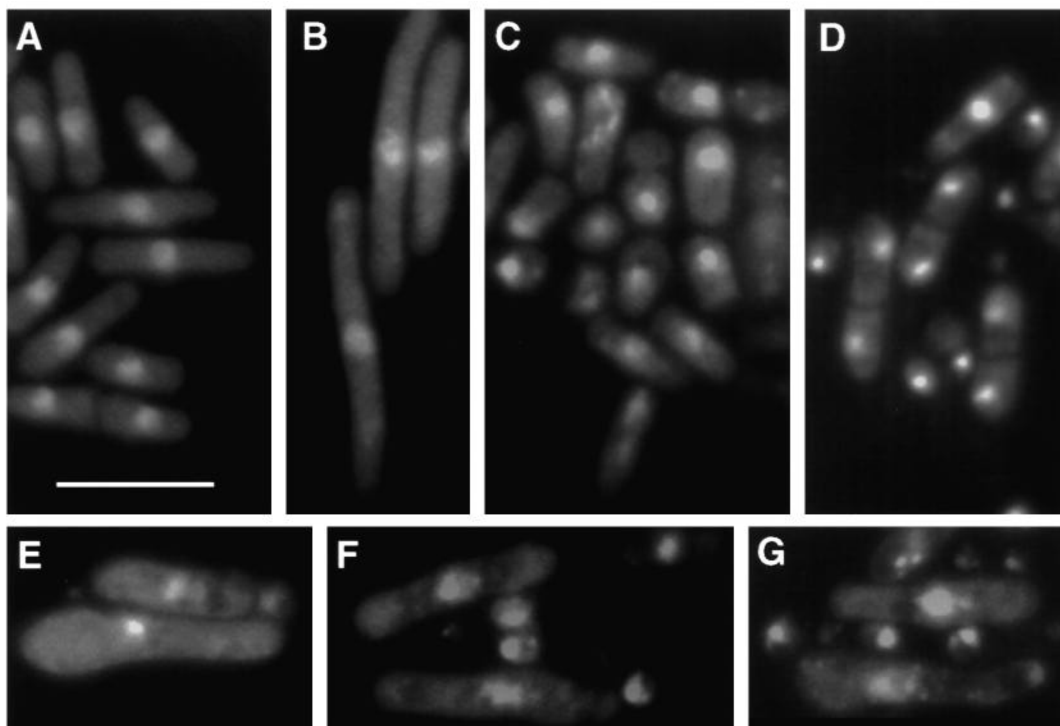

Fig. 5. Photomicrograph of *cdc19* mutant cells. (A) Wild-type cells. (B) *cdc19-P1* after 6 hours at restrictive temperature (36°C). (C) *cdc19-P1 cdc2-3w* after 6 hours at restrictive temperature. Note the number of small, aberrant cells and anucleate fragments, indicating that checkpoint control is abrogated. (D) Wild-type spores after 40 hours in liquid culture. Only spores containing the *LEU2* marker are able to germinate. Note the number of binucleate cells. (E-G) Δ*cdc19::LEU2* spores after 40 hours in liquid culture. Only spores containing the *LEU2* marker are able to germinate. The germinated spores are for the most part elongated and uninucleate. Bar, 10 μm.

Table 2. *cdc19-P1* and *cdc21-M68* are synthetically lethal

Tetrad class	Phenotypes	Number
PD (a)	4 viable, ts : 0 dead	1
PD (b)	3 viable, ts :1 dead	3
TT	2 viable, ts : 1 viable, wt : 1 dead	14
NPD	2 viable, wt : 2 dead	2

Tetrad dissection from a cross between strains of relevant genotypes *h*$^-$ *cdc19-P1*×*h*$^+$ *cdc21-M68* failed to isolate any viable double mutants. Assignment of the tetrad classes is discussed in the text. PD (a) and PD (b) classes of parental ditype tetrads are explained in text. ts, temperature sensitive. wt, wild type.

this by crossing the progeny of two of these tetrads with *cdc19-P1* or *cdc21-M68,* and confirmed that they were all single mutants (data not shown). Of the remaining classes of tetrads, 14 are clear tetratypes (TT), with 2 ts spores, 1 wild type and 1 dead. We inferred that the dead spores were *cdc19-P1 cdc21-M68* double mutants and confirmed this by crossing the surviving progeny of two such tetrads with *cdc21-M68* and *cdc19-P1* strains to verify their genotypes. As expected, both tetrads tested contained one *cdc21-M68* spore, one *cdc19-P1* spore, and one wild-type spore, showing that the *cdc19-P1 cdc21-M68* double mutant was dead. The dead spores in most cases died after germination and a few underwent a single division; two of them managed to make microcolonies of approximately 20 misshapen cells before dying. We were unable to identify any *cdc19-P1 cdc21-M68* double mutant progeny in this cross and therefore conclude that *cdc19-P1* and *cdc21-M68* are synthetically lethal at 25°C.

We transformed both *cdc21-M68* and *cdc19-P1* with the genomic clones of *cdc19*$^+$ and *cdc21*$^+$ (Coxon et al., 1992), to see whether there was any suppression apparent. In both cases, only the cognate gene was able to rescue the appropriate mutant and therefore we found no evidence for cross complementation.

cdc19+ is an essential gene

We constructed a gene disruption of *cdc19*$^+$ by replacing the central core of the protein between the *Xho*I and *Bgl*II sites shown in Fig. 1. The *LEU2* gene was used to replace this region, which shows the greatest identity to *MCM2*, and our subcloning showed that the remaining sequences were incapable of rescuing the mutant (Fig. 1). A diploid strain of genotype *h*$^+$/*h*$^-$*ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 can1-1/can1-1* was transformed with a fragment containing the disruption construct. Leu$^+$ diploid colonies were selected and analysed by Southern blot (data not shown). Four out of six had the structure expected from a gene deletion, and the other two were consistent with non-homologous integration events elsewhere in the genome. One of the non-homologous integration clones was compared with the four disrupted strains by sporulation, and tetrad and random spore analysis. The non-homologous integrant gave approximately 50% Leu$^+$ spores by random spore analysis and all spores were healthy, with no apparent growth defects. For the disrupted strains, no Leu$^+$ colonies were recovered, and in tetrads, there was 2:2 viability. All the viable spores were Leu$^-$. The phenotype of the disrupted, non-viable spores was similar for all four homologous integrants. The spores germinated, and extended a germination tube, so cell growth was not affected. Most arrested as modestly elongated single cells, showing that *cdc19*$^+$ is an essential gene for cell cycle progression. Approximately 10% of spores divided once, after elongation (Fig. 5D-G). We confirmed that the disruption phenotype could be rescued by the genomic clone as follows. We transformed the diploid heterozygote with *cdc19*$^+$ on a plasmid with the *ura4*$^+$ marker, sporulated the strain, and selected spores that contained both the *LEU2* marker of the disruption and the *ura4*$^+$ marker of the plasmid (data not shown). These spores were germinated on selective plates with timing similar to a wild-type control. As well as confirming that the phenotype of the deletion was due to the deficiency of cdc19p, this also verified that cells containing the *LEU2* marker were able to germinate normally if *cdc19*$^+$ was provided.

To determine whether or not the disrupted cells were able to replicate their DNA, we carried out a spore germination experiment in liquid culture. The disruption strain was compared with the non-homologous integrant to control for the single copy of the *LEU2* gene, which complements *leu1-32* poorly in low copy such that growth rate is reduced. Both strains were inoculated in liquid sporulation medium, and treated with glusalase to release the spores. These were washed, and inoculated into selective medium lacking leucine, so that only spores containing the *LEU2* integrant would be able to germinate. Samples were taken hourly for flow cytometry analysis (Fig. 6). The control strain showed replication taking place approximately 10 hours after inoculation of the spore suspension into selective medium, indicated by the appearance of a 2*C* DNA peak. Analysis of the forward scatter also showed cell elongation taking place. The Δ*cdc19::LEU2* strain also underwent DNA replication. However, this took place somewhat after the control, as though the cells were delayed in their progression through S phase. A noticeable shoulder was apparent on the 1*C* peak and moved slowly into an approximate 2*C* position. Thus DNA synthesis takes place, but at this level of analysis we cannot be sure S phase is completed. This observation was repeated several times. There is no evidence for S phase delay in the *cdc19-P1* allele (Fig. 4), which may indicate that the temperature-sensitive allele is somewhat leaky. The Δ*cdc19* and *cdc19-P1* strains have similar terminal phenotypes, both arresting as single, modestly elongated cells (Fig. 5B and E-G).

Expression of cdc19

Regulation of the MCM proteins appears to be by post-translational means, since protein levels of CDC46 are constitutive (Hennessy et al., 1990). It has been suggested that, rather than regulation of expression, it is actually regulation of localization that controls the activity of these proteins (Hennessy et al., 1990; Yan et al., 1993). However, it has also been reported that *CDC46* is a regulated transcript, peaking in G_1 (Hennessy et al., 1990). We determined the cell cycle regulation of the *cdc19*$^+$ transcript by probing a Northern blot of RNA prepared from a synchronous culture (Fig. 7). There was no apparent fluctuation in the expression of *cdc19*$^+$ mRNA. Additionally, sequence analysis of the upstream region showed no likely candidates for a G_1-specific MCB element, the target of the DSC transcriptional activation complex that contains the *cdc10*$^+$ gene product (Lowndes et al., 1992; and see Fig. 2). Thus, it seems unlikely that *cdc19*$^+$ is transcriptionally regulated by the DSC system.

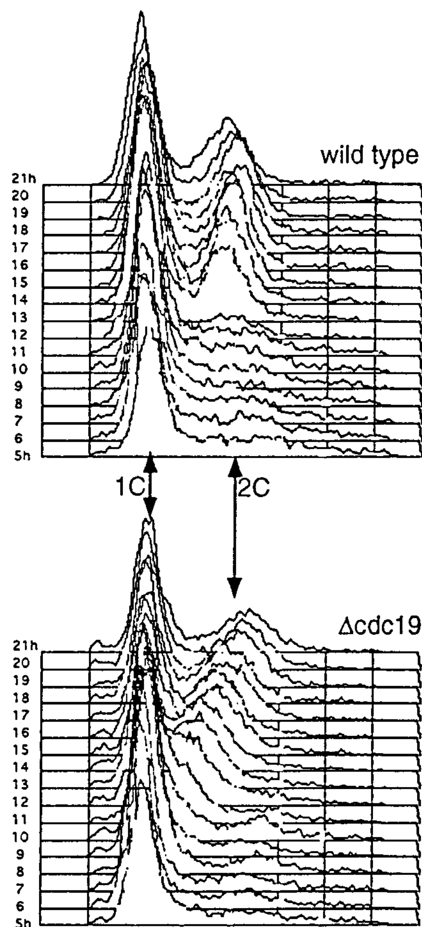

Fig. 6. Flow cytometry analysis of germinating Δcdc19 spores shows a delay going through S phase. Diploid cultures heterozygous for the *Δcdc19::LEU2* disruption, or for a heterologous integrant containing a single copy of *LEU2*, were prepared as described in Materials and Methods and inoculated into minimal medium lacking leucine; only spores containing the *LEU2* marker are able to germinate. The culture was sampled every hour for flow cytometry over 21 hours; the samples were prepared and analysed as described in Materials and Methods.

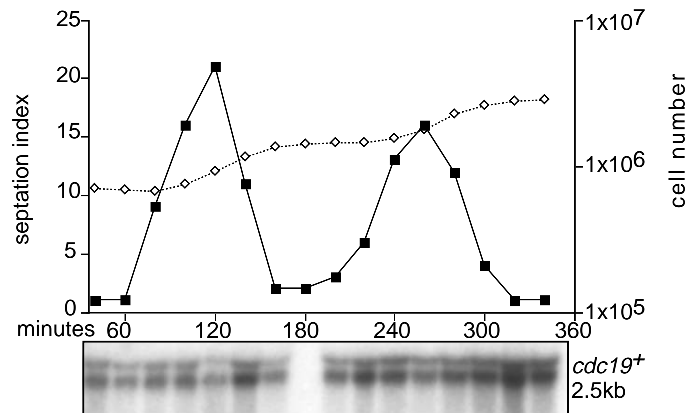

Fig. 7. Expression of cdc19 is constitutive through the cell cycle. A northern blot from elutriated cells was probed with cdc19 (the *Bam*HI fragment; see Fig. 1). The same RNA samples were run separately and probed for the constitutive messages *puc1*$^+$ and *cdc2*$^+$ (Forsburg and Nurse, 1994), and synchrony of the culture was shown previously using a *cdc18*$^+$ probe, which is periodically expressed during G_1/S (Kelly et al., 1993a). One sample was lost during preparation. The top panel shows the septation index (left axis) and the increase in the cell number (right axis) of the elutriated culture. The size of the message was estimated by comparison with the rRNA bands. The upper band is not seen when the probe is the internal *Bgl*II-*Xho*I fragment (data not shown).

DISCUSSION

After cells pass START, they must initiate DNA replication. A number of components of the regulatory network and of the replication complexes at the G_1/S transition have been identified in a variety of systems. But how these components interact in a pathway to couple START to S phase is still unclear. How general they are to all eukaryotes is also not yet apparent. We wish to examine the nature of the events between the commitment at START and the initiation of replication. To address this regulatory problem, we have begun to characterize a variety of fission yeast genes affecting early S phase. Here we report the cloning of the fission yeast *cdc19*$^+$ gene by complementation of the *cdc19-P1* mutation, and show that it encodes a homologue of the budding yeast MCM2 protein. *cdc19-P1* mutant cells block at the restrictive temperature with an apparent 2*C* DNA content, although the execution point for this function is near to or coincident with the hydroxyurea block at the beginning of S phase (Fig. 4; and see Nasmyth and Nurse, 1981). cdc19p potentially encodes a protein of 830 amino acids, which is over 60% identical to MCM2p, and approximately 30% identical to MCM3p or to cdc21p.

We have mapped *cdc19*$^+$ genetically and physically, and show that it lies between the *ura5*$^+$ and *ade7*$^+$ genes on chromosome II. Additionally, we show that *cdc19*$^+$ is allelic to *nda1*$^+$, a cold-sensitive mutant originally isolated for its elongation at low temperatures (Toda et al., 1984). During the course of this work, Miyake et al. (1993) reported the sequence of *nda1*$^+$, which confirmed that these two genes are allelic.

The *cdc19*$^+$ gene contains a single intron (Fig. 2; and see Miyake et al., 1993). The gene is expressed constitutively in the cell cycle, and there are no MCB elements apparent in the upstream sequence; therefore, *cdc19*$^+$ is apparently not a target for DSC transcriptional regulation. The gene is essential and, when deleted, cells elongate and arrest, and have a late S or G_2 DNA content. Thus the actual process of DNA synthesis is not blocked. Because the mutant cells fail to divide, we conclude either that there is a requirement for *cdc19*$^+$ function throughout S phase, or that the synthesized DNA is somehow defective or incomplete in structure, although in content it is partly replicated. It is impossible at this level of resolution to determine the nature of the defect in DNA replication in a *Δcdc19* mutant strain. Whether the DNA is normally replicated or aberrant repair synthesis is taking place, whether all origins are being used or a few origins are working inefficiently, cannot at this time be determined.

The *cdc19* deletion phenotype differs from those reported for several other fission yeast genes involved in replication. Cells lacking the *cdc18*$^+$ or *rad4*$^+$*(cut5*$^+$*)* gene products not only fail to replicate their DNA but fail in the checkpoint control that blocks mitosis under such conditions, leading to a distinctive terminal morphology in which the cells undergo abnormal

nuclear and cell division, and physically cut the DNA (Kelly et al., 1993a; Saka and Yanagida, 1993). This is similar to the morphological phenotype reported for the deletion of polymerase alpha (polα; Francesconi et al., 1993) suggesting that polα may also be involved in checkpoint control as well as replication. The *cdc19* deletion mutant shows little evidence for cutting, and the cells elongate rather than divide. Therefore, its checkpoint control appears to be intact. In the absence of proliferating cell nuclear antigen (PCNA), an auxiliary factor for polymerase delta, the cells are delayed going through S phase, but they can undergo at least one or two divisions before arresting with a 2*C* DNA content, suggesting that some residual activity is present allowing the cells to proceed through several cycles (Waseem et al., 1992). The Δ*cdc19* strain does not undergo such multiple divisions, showing largely a first cycle arrest. Cells deleted for polymerase delta elongate and arrest in the first cycle (*pol*δ; Francesconi et al., 1993). This is closest to the phenotype seen for Δ*cdc19,* although the Δ*pol*δ cells are more strikingly elongated and *pol*δ^{ts} mutants arrest with an S phase DNA content (Francesconi et al., 1993). While there are features in common, no other replication gene has a deletion phenotype quite like Δ*cdc19*, suggesting *cdc19*+ may fulfil a function distinct from the others.

cdc19+ is the one of three MCM homologues identified from the fission yeast. The first one identified, *cdc21*+, is a member of a sequence subclass distinct from *MCM2*, *MCM3* and *CDC46/MCM5*, and a *cdc21*+-related fragment has been identified in budding yeast (Coxon et al., 1992). An additional novel member from *S. cerevisiae* has recently been sequenced, demonstrating that there are at least five members in the budding yeast (Bussereau et al., 1993). Genetic studies with *S. cerevisiae cdc46* mutants have shown a variety of genetic interactions including synthetic lethality between *CDC46* and a number of other *cdc* genes, *CDC45*, *CDC47* and *CDC54* (Hennessy et al., 1991); it is possible that some of these are allelic to other members of the *MCM* family. We determined that a double mutant *cdc19-P1 cdc21-M68* is not viable, suggesting that similar interactions will be found in fission yeast.

The MCM family of proteins in budding yeast was identified by apparent origin-specific defects in the maintenance of minichromosomes in mutant strains (Sinha et al., 1986; Maiti and Sinha, 1992). Despite their sequence similarity, these genes are not redundant; each one is essential (Gibson et al., 1990; Hennessy et al., 1991; Yan et al., 1991). The biochemical function of the MCM proteins in budding yeast is still not known. It has been suggested that they are ARS-binding proteins, given that they were originally isolated by their defects in maintaining minichromosomes carrying specific ARS elements. Coxon et al (1992) showed an ARS specificity in the maintenance of plasmids in the *cdc21-M68* mutant strain. Recent evidence suggests that the MCM proteins bind tightly to chromatin and may affect the efficiency of origin usage (Yan et al., 1993); with fewer origins firing, DNA synthesis could be retarded, which could also explain the observed phenotype of Δ*cdc19.* Further support for the role of the MCM family in early stages of replication comes from isolation of a metazoan homologue of MCM3; the mouse P1 protein was identified biochemically as part of a polymerase alpha complex (Thömmes et al., 1992). In addition, other approaches have now identified a number of homologues in *Xenopus* and human cells (Hu et al., 1993; Todorov et al., 1994). The MCM proteins in *S. cerevisiae* all undergo regulated nuclear entry (Hennessy et al., 1990; Yan et al., 1993); these findings fulfil some of the expectations of the inferred activity called licensing factor, which was posited by Blow and Laskey (1988) to explain how replication is controlled to occur only once in a cell cycle. Licensing factor has been suggested to act by binding chromatin and allowing replication to occur; as described by Blow and Laskey, the activity would then be lost and only regain access to the nucleus in mitosis, when the nuclear envelope breaks down. Because the yeasts undergo a closed mitosis, with no nuclear envelope breakdown, regulation of nuclear localization would perform the same function. This has led to the speculation that the MCM complex might comprise some form of licensing factor (Hennessy et al., 1990; Yan et al., 1991).

However, mutants in *mcm2* or *mcm3*, as well as mutants in *cdc19* and *cdc21*, apparently arrest with at least a partially replicated genome, although we cannot distinguish normal replication from other forms of synthesis such as DNA repair (Yan et al., 1991; Gibson et al., 1990; Coxon et al., 1992; this work). This result suggests that these proteins do not function as essential replication factors, but may modulate origin usage or processivity of replication enzymes. Interestingly, mutants in *cdc46* in budding yeast, and its homologue *nda4* in fission yeast, have been reported to arrest with a G_1 DNA content (Hennessy et al., 1991; Miyake et al., 1993) so these genes may have different roles. The *nda1-KM376* allele of *cdc19*+ also reportedly arrests with a G_1 DNA content (Miyake et al., 1993) although our results suggest an S/G_2 block (Fig. 4B). There are several possible reasons for this. First, there are interacting genes in budding yeast that show different block points when the temperature is varied (Hennessy et al., 1991), and the point of arrest in fission yeast may also be acutely sensitive to temperature. Different aspects of the process of replication may be inherently cold-sensitive. Second, subtle differences in strains or in growth conditions may affect the perceived block. Further investigation into the nature of the S phase defect in the mutant strains will help determine whether replication or initiation is specifically affected. We are raising antibodies to characterize cdc19p biochemically and determine its intracellular localization. If *cdc19*+ functions analogously in fission yeast to its homologue in budding yeast, a strong case may be made to support the suggestion that the MCM family provides a conserved function in the initiation of S phase in all eukaryotes.

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