Mutational Analysis of Cdc19p, a *Schizosaccharomyces pombe* MCM Protein

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ABSTRACT

The *cdc19*$^+$ gene encodes an essential member of the MCM family of replication proteins in *Schizosaccharomyces pombe*. We have examined the structure and function of the Cdc19p protein using molecular and genetic approaches. We find that overproduction of wild-type Cdc19p in wild-type cells has no effect, but *cdc19-P1* mutant cells do not tolerate elevated levels of other MCM proteins or overexpression of mutant forms of Cdc19p. We have found genetic interactions between *cdc19*$^+$ and genes encoding subunits of DNA polymerase δ and the replication initiator *cdc18*$^+$. We have constructed a series of point mutations and sequence deletions throughout Cdc19p, which allow us to distinguish essential from nonessential regions of the protein. Not surprisingly, conserved residues in the MCM homology domain are required for protein function, but some residues outside the core homology domain are dispensable.

CELLULAR DNA replication requires precise regulation to ensure that the genome is replicated once completely in each cell cycle, and that the process of replication is coordinated with proper cell cycle entry and subsequent nuclear division. Genetic analysis in the yeasts has been important in identifying the molecules that are important for the regulation of these cell cycle events. In the fission yeast, a number of genes have been characterized with roles in S phase progression and checkpoint mechanisms (reviewed in FORSBURG 1996). Molecular genetic analysis of these genes not only allows them to be placed in context relative to one another in the cell, but also enables rapid characterization of important structural features in the encoded proteins.

The MCM proteins comprise a family of essential genes required early in eukaryotic DNA replication. There are at least six distinct members, highly conserved in eukaryotes (reviewed in CHONG *et al.* 1996; KEARSEY *et al.* 1996; TYE 1994). The MCM proteins were first identified in the budding yeast *Saccharomyces cerevisiae* because *mcm* mutants have an origin specific defect in minichromosome maintenance (MAINE *et al.* 1984; SINHA *et al.* 1986). Genetic analysis shows an extensive network of synthetic interactions among *mcm* mutations and other mutations affecting origin function in *S. cerevisiae* (GIBSON *et al.* 1990; HENNESSY *et al.* 1991; LI and HERSKOWITZ 1993; YAN *et al.* 1993). Interestingly, despite a core of sequence homology shared among all MCM proteins, each single budding yeast MCM protein is essential (DALTON and WHITBREAD 1995; GIBSON *et al.* 1990; HENNESSY *et al.* 1990; YAN *et al.* 1993; WHITBREAD and DALTON 1995). This implies that they are not completely redundant, although they may share some functions. Structural comparisons of the proteins suggest that they may define a class of DNA-dependent nucleotide binding proteins (KOONIN 1993).

MCM proteins have now been found in eukaryotes ranging from humans and mice to Drosophila, Xenopus, and Arabidopsis (reviewed in TYE 1994; CHONG *et al.* 1996; KEARSEY *et al.* 1996). MCM proteins are thought to function early in the cell cycle, perhaps at the level of origin function (MAINE *et al.* 1984; CHONG *et al.* 1995; KUBOTA *et al.* 1995; MADINE *et al.* 1995a). In a number of systems, it has been shown that MCM proteins interact with one another to form large complexes (BURKHART *et al.* 1995; CHONG *et al.* 1995; MADINE *et al.* 1995a; LEI *et al.* 1996; SCHULTE *et al.* 1996; SU *et al.* 1996). In particular, immunodepletion experiments in Xenopus extracts suggest that some MCM complexes are required for replication initiation (CHONG *et al.* 1995; KUBOTA *et al.* 1995; MADINE *et al.* 1995a). This probably occurs at a step subsequent to the binding of the origin recognition complex (ORC) and the initiator Cdc18p/Cdc6p to the origin (COLEMAN *et al.* 1996; ROMANOWSKI *et al.* 1996; ROWLES *et al.* 1996). In contrast to these biochemical observations, genetic analysis of yeast *mcm* mutants shows that many are able to undergo DNA synthesis under restrictive conditions, which suggests that individual MCM proteins may not be required for initiation per se, but for elongation or processivity (GIBSON *et al.* 1990; YAN *et al.* 1991; COXON *et al.* 1992; FORSBURG and NURSE 1994b; TAKAHASHI *et al.* 1994). These somewhat conflicting findings may reflect the variety of technical differences in the approaches used. Alternatively, these contrasting observations could suggest subtle differences between the behavior of these

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conserved proteins in different systems. Thus, study of the MCM proteins in several cell types is important to determine their general function and specific regulation.

In fission yeast, five members of the MCM family have been identified: Cdc19p (also called Nda1P, MIYAKE *et al.* 1993; FORSBURG and NURSE 1994b), Cdc21p (COXON *et al.* 1992), Mis5p (TAKAHASHI *et al.* 1994), Nda4p (MIYAKE *et al.* 1993), and a PCR fragment defining an Mcm3p homologue (COXON *et al.* 1992). Cdc19p is a member of the MCM2 subclass of MCM proteins, and other members of this class have been cloned from *S. cerevisiae*, Drosophila, Xenopus, and humans (YAN *et al.* 1991; TODOROV *et al.* 1995; TREISMAN *et al.* 1995; MIYAKE *et al.* 1996). We are investigating the role of the *cdc19*$^+$ gene in fission yeast S phase as a model for overall MCM protein function. Previously, we showed that *cdc19*$^+$ is an essential gene, and that mutations of *cdc19*$^+$ confer an S phase delay and a late S phase block (FORSBURG and NURSE 1994b). We also demonstrated that the double mutant *cdc19-P1 cdc21-M68* is synthetically lethal.

Here, we report further analysis of Cdc19p function. First, we have molecularly characterized the protein. Levels of Cdc19p show little fluctuation through the cell cycle. We show that overproduction of Cdc19p is tolerated in both wild-type and *cdc19-P1* mutant cells. In contrast, overexpression of the other fission yeast MCM proteins or Cdc19p mutants is toxic in *cdc19-P1* cells. This "synthetic dosage lethality" may reflect direct molecular interactions (*e.g.*, FORSBURG and NURSE 1994a; KROLL *et al.* 1996). Second, we have analyzed genetic interactions between the temperature-sensitive allele *cdc19-P1* and mutations in other S phase genes to investigate synthetic interactions with known S phase regulators. Intriguingly, there is evidence for genetic interactions with genes encoding subunits of DNA polymerase δ, as well as with the initiator Cdc18p. Finally, we have carried out a detailed mutational analysis of Cdc19p using a series of point mutations and sequence deletions. These mutations identify the essential domains of the protein. Some of the mutant proteins are toxic when overproduced in *cdc19ts* cells. These experiments also suggest that there is a minimum level of Cdc19p required for viability. We have thus completed a detailed molecular characterization of Cdc19p that provides further evidence that this protein acts in a multi-component complex and affects the progression of DNA replication.

MATERIALS AND METHODS

Strains and manipulation: Strains are listed in Table 1. Media and genetic manipulations were as described (MORENO *et al.* 1991). Where required, EMM was supplemented with adenine, leucine, uracil, and histidine. Transformation was carried out by electroporation or lithium acetate treatment (MORENO *et al.* 1991). The *nmt* promoter was repressed by the presence of 5 μg/ml thiamine in the media (MAUNDRELL 1993). Induction in liquid culture was carried out for 24 hr following the removal of thiamine from the media. Images of plates were digitized directly using a flat bed scanner and Adobe Photoshop, assembled in Canvas, and printed on a Phaser DX printer.

Cell cycle blocks were induced in the appropriate strains by growing the cells to early exponential phase at 25° in minimal media with appropriate supplements, shifting to 36° for 4 hr, and harvesting. The synchronous culture was carried out using strain FY117 (*h- cdc25-22 ura4-D18*). Cells were grown to early exponential phase in EMM+ura, shifted rapidly to 36° and incubated for 4 hr, and then rapidly returned to 25°. Following the down shift, samples were taken every 20 min to determine septation index and preparation of crude lysates.

Haploid strains containing the chromosomal deletion Δ*cdc19::LEU2* and a complementing plasmid were isolated by transforming the diploid FY362 (Table 1, FORSBURG and NURSE 1994b) with plasmids carrying *ura4*$^+$ and derivatives of *cdc19*$^+$ (Table 2). The transformed diploids were induced to undergo meiosis, which was followed by a random spore analysis. The spore preparation was plated to identify Ura$^+$ clones containing the plasmid and wasscreened for Leu$^+$ isolates containing the Δ*cdc19* allele.

Protein analysis and antibodies: Lysates were prepared as described using glass bead lysis (MORENO *et al.* 1991). For Western blot analysis, cells were lysed in HB15 buffer (60 mM B-glycerophosphate, 150 mM p-nitrophenyl phosphate, 25 mM HEPES pH 7.9, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na-vanadate and protease inhibitors). Except where indicated, equivalent amounts of total cell protein as determined by a BCA protein assay (Pierce) were fractionated by SDS-PAGE (National Diagnostic Protogel) and transferred to Immobilon-P (Millipore). Detection was carried out using anti-rabbit or anti-mouse conjugated secondary antibodies (Sigma or Jackson Labs) and ECL (Amersham) followed by film exposure. Exposure time varied according to the dilution of the antibody and the secondary, typically 10 sec to 5 min. Films were digitally scanned into the Macintosh program Adobe Photoshop; composite images were printed on a dye substitution printer. Beta galactosidase assays were carried out as described previously (FORSBURG 1993).

Polyclonal sera 5616 was raised to a synthetic peptide of sequence LPPSSPPPEFC corresponding to residues 31–40 of Cdc19p (FORSBURG and NURSE 1994b). This sequence is not found in other MCM proteins. The peptide was coupled to KLH via the additional C residue, using manufacturer's instructions (Pierce Imject maleimide activated carrier) and this was used in rabbit immunization. The antibodies were affinity purified from a Western blot using full length bacterially produced Cdc19p. Monoclonal anti-HA antibody 12CA5 was a generous gift of JILL MEISSENHOLDER and TONY HUNTER. Monoclonal anti-tubulin antibody was purchased from Sigma (T5168). Anti-*cdc2* antibody C2 recognizes the C terminus of fission yeast p34^{cdc2} (SIMANIS and NURSE 1986).

Plasmid and mutant construction: Plasmid features are described in Table 2. The *cdc19*$^+$ cDNA was constructed using PCR to splice out the predicted intron, placing a *Sal*I site 22 nucleotides upstream of the ATG. A *Sal*I-*Bam*HI fragment was cloned into the *nmt1*$^+$ expression vectors REP4X, REP42X, and REP82X to give plasmids pSLF160, pSLF161 and pSLF162, which express *cdc19*$^+$ under control of three different strength *nmt1* promoters (BASI *et al.* 1993; FORSBURG 1993). The constructs were tested to determine their ability to complement a mutant *cdc19-P1*. A *Sal*I-*Pvu*II fragment was cloned into the expression vector REP4X cut with *Sal*I-*Sma*I to give plasmid pSLF193, containing a shorter region of 3′ UTR; this was phenotypically indistinguishable from pSLF160. Plasmid pSLF176 contains *cdc19*$^+$ with a triple HA tag at the

TABLE 1

Strains

Strain	Genotype	Source
FY117	*h⁻ ura4-D18 cdc25-22*	P. NURSE
FY194	*h⁻ ura4-D18 leu1-32 cdc18-K46*	KELLY *et al.* (1993)
FY243	*h⁻ ura4-D18 leu1-32 ade6-M210 cdc19-P1*	FORSBURG and NURSE (1994b)
FY254	*h⁻ ura4-D18 leu1-32 ade6-M210 can1-1*	FORSBURG and NURSE (1994b)
FY280	*h⁻ leu1-32 nda1-KM376*	NCYC2231[a]
FY310	*h⁺ ura4-D18 leu1-32 ade6-M216 cdc1-7*	This work
FY318	*h⁺ ura4-D18 leu1-32 ade6-M210 cdc25-22*	This work
FY322	*h⁻ ura4-D18 leu1-32 ade6-M216 can1-1 cdc17-K42*	This work
FY362	*h⁻ ura4-D18 leu1-32 ade6-M210 cdc19⁺* *h⁺ ura4-D18 leu1-32 ade6-M216 Δcdc19::LEU2*	FORSBURG and NURSE (1994b)
FY364	*h⁻ cdc21-M68 leu1-32 ura4-D18 ade6 can1-1*	This work
FY368	*h⁻ ura4-D18 leu1-32 cdc23-M36*	This work
FY370	*h⁺ ura4-D18 leu1-32 can1-1 cdc24-M38*	This work
FY440	*h⁺ ura4-D18 rad4-116*	T. CARR
FY458	*h⁺ leu1-32 his2 nuc2*	R. WEST
FY563	*h⁺ ura4-D18 leu1-32 ade6-M210 cdc10-V50*	This work
FY569	*h⁺ ura4-D18 leu1-32 ade6-M216 cdc17-K42*	This work
FY583	*h⁺ ura4-D18 leu1-32 ade6-M216 cdc22-M45*	This work
FY587	*h⁺ ura4-D18 leu1-32 ade6-M216 Δcdc19::LEU2* [pSLF176]	This work
FY598	*h⁻ ura4-D18 leu1-32 ade6 cdc10-V50 cdc19-P1*	This work
FY600	*h⁻ ura4-D18 leu1-32 ade6-M210 cdc19-P1 cdc25-22*	This work
FY603	*h⁻ ura4-D18 leu-32 ade6 cdc22-M45 cdc19-P1*	This work
FY610	*h⁻ ura4-D18 leu1-32 ade6-M210 cdc17-K42 cdc19-P1*	This work
FY612	*h⁺ ura4-D18 leu1-32 ade6-M210 cdc24-M38 cdc19-P1*	This work
FY614	*h⁺ ura4-D18 leu1-32 ade6-M216 polδ-ts1*	T. WANG
FY620	*h⁺ ura4-D18 leu1-32 cdc23-M36 cdc19-P1*	This work
FY634	*h⁺ ura4-D18 leu1-32 ade6-M216 cdc1-7 cdc19-P1*	This work
FY636	*h⁺ ura4-D18 leu1-32 ade6-M216 polδ-ts1 cdc19-P1*	This work
FY700	*h⁺ ura4-D18 leu1-32 ade6-M210 cdc19-P1 cdc18-K46*	This work
FY704	*h⁺ ura4-D18 leu1-32 cdc19-P1 rad4-116*	This work
FY717	*h⁺ ura4-D18 leu1-32 ade6-M210 Δcdc19::LEU2* [pSLF176]	This work
FY718	*h⁺ ura4-D18 leu1-32 ade6-M216 Δcdc19::LEU2* [pSLF196-D1]	This work
FY719	*h⁺ ura4-D18 leu1-32 ade6-M210 Δcdc19::LEU2* [pSLF196-D2]	This work
FY720	*h⁺ ura4-D18 leu1-32 ade6-M210 Δcdc19::LEU2* [pSLF196-D3]	This work
FY721	*h⁺ ura4-D18 leu1-32 ade6-M210 Δcdc19::LEU2* [pSLF196-M7]	This work

Except where noted, *cdc* mutant alleles used to construct these strains were obtained from P. NURSE.
[a] National Collection of Yeast Cultures, Norwich, United Kingdom.

C-terminus. It was constructed by using PCR to insert a *Not*I site before the TAA and cloning the *Sal*I-*Not*I fragment into the triple-HA tagging *nmt* expression vector pSLF172 (FORSBURG and SHERMAN 1997). The cDNA was sequenced to confirm that PCR cloning introduced no mutations. Bacterially produced protein was expressed from plasmid pRY2, containing the *cdc19⁺* cDNA cloned into the 6xHis tagging plasmid RSET2 (Invitrogen) using *Sal*I and *Bam*HI, or a plasmid containing only the *Xho*I-*Bgl*II fragment of *cdc19⁺*.

Mutants M1–M8 were constructed using a BioRad site-directed mutagenesis kit according to manufacturer's instructions and a single mutagenic oligonucleotide. Mutants M9, M10, and D1–D3 were constructed using PCR and two mutagenic oligonucleotides. D7 was constructed using PCR to introduce an ATG at residue 204. Restriction fragments containing the mutated regions of the gene were cloned into an otherwise wild-type cDNA and sequenced, before cloning into pSLF172 to epitope tag them and place them under the control of the *nmt1⁺* promoter. D4, D6, D8, D9 and D10 were constructed by restriction manipulation. D4 drops out the internal *Pst*I fragment of pSLF176. D6 deletes the *Bgl*II to *Not*I fragment. D8 deletes the *Eco*RV to *Nco*I fragment; D9, the *Nco*I fragment to *Bgl*II, and D10, the *Eco*RV to *Bgl*II fragment. Digested fragments were filled in with Klenow (NE Biolabs). Where necessary, the reading frame was restored by addition of an 8mer *Kpn*I linker (NE Biolabs). All mutants were cloned into pSLF172, a C-terminally HA tagged expression vector (FORSBURG and SHERMAN 1997) to create the derivatives pSLF196-M1 through pSLF196-D6, and pDS196-D7 through pDS196-D10. Protein expression and reading frame were verified by Western blotting to detect the HA epitope tag. Complementation was assessed by transforming the mutant strains into FY243 and assessing rescue under low (plus thiamine) and high (minus thiamine) levels of expression.

The conditional alleles were cloned from the genome by PCR. Internal sequencing primers were used to allow amplification of either the first third, the middle third, or the last two-thirds of *cdc19*. Duplicate PCR reactions were set up using genomic DNA prepared from the mutant strains to characterize independent isolates and screen out PCR errors. The fragments from each duplicate reaction were cloned into a wild-type *cdc19⁺* gene on a plasmid. These duplicate constructs

TABLE 2

Plasmids

Plasmid name	Features[a]	Source
pSLF160	*nmt-cdc19*$^+$	This work
pSLF161	*nmt*-cdc19*$^+$	This work
pSLF162	*nmt**-cdc19*$^+$	This work
pSLF176	*nmt-cdc19-HA*	This work
pSLF196-M1–M10	*nmt-cdc19-HA* containing point mutations M1–M10	This work
pSLF196-D1–D6	*nmt-cdc19-HA* containing deletion mutations D1–D6	This work
pDS196-D7–D10	*nmt-cdc19-HA* containing deletion mutations D7–D10	This work
pSLF204	*nmt-nda4-HA*	This work
pSLF221	*nmt-HA-cdc21*	This work
pSLF225	*nmt-HA-mis5*	This work
pRY2	6xHis-*cdc19*$^+$ in RSET B	This work
REP4X	*nmt* cloning vector	FORSBURG (1993); MAUNDRELL (1993)
REP42X	*nmt** cloning vector	FORSBURG (1993); MAUNDRELL (1993)
REP82X	*nmt*** cloning vector	FORSBURG (1993); MAUNDRELL (1993)
pSLF172	REP4x + C terminal HA tag	FORSBURG and SHERMAN (1997)
pSLF173	REP4x + N terminal HA tag	FORSBURG and SHERMAN (1997)
REP2-polδ	*nmt-polδ*$^+$	T. WANG
pMF56	*nmt-cdc18-HA*	MUZI-FALCONI *et al.* (1996)

[a] nmt, nmt*, nmt** are strong, medium and weak versions, respectively, of the *nmt* promoter.

were transformed into the mutant strains and tested for complementation of the cognate mutant at its restrictive temperature. The clones from the temperature-sensitive strain containing the first third or the last third of the open reading frame were able to complement the temperature-sensitive allele *cdc19-P1* (NASMYTH and NURSE 1981); the two independent clones containing the last two-thirds of the protein were unable to rescue, localizing the *cdc19-P1* allele to the middle of the protein. The clones containing the last third or last two-thirds of the cold-sensitive protein were unable to complement the cold-sensitive allele *nda1-KM376* (TODA *et al.* 1983), localizing this lesion to the C terminus. The duplicate clones for each allele were sequenced. Because the same lesions were found in duplicate, independent clones from independent PCR reactions, we conclude that they are unlikely to be PCR artifacts and represent the actual mutations. *cdc21*$^+$, *mis5*$^+$, and *nda4*$^+$ were isolated using genomic PCR with primers derived from the published sequences to allow amplification of the full length open reading frame, including the introns. *nda4*$^+$ was ligated into plasmids pSLF172 containing a C-terminal HA tag (FORSBURG and SHERMAN 1997), and *cdc21*$^+$ and *mis5*$^+$ were cloned into pSLF173 containing an N-terminal HA tag (FORSBURG and SHERMAN 1997) to create plasmids pSLF204, pSLF221, and pSLF225, respectively. We verified that pSLF221 complements strain FY364 (*cdc21-M68*).

FACS analysis: Cells were fixed in 70% ethanol and stained for flow cytometry as described (SAZER and SHERWOOD 1990). We used a Becton Dickinson FACScan and the Macintosh software Cell Quest to analyze the data.

RESULTS

Phenotypes of MCM protein overexpression: As a first step to analyzing the role of Cdc19p in the cell, we characterized the effects of its overproduction in wild-type and *cdc19* mutant cells. We cloned the *cdc19* cDNA from our original genomic isolate by PCR and confirmed its sequence (see MATERIALS AND METHODS). The resulting clone was placed in a series of expression vectors containing the thiamine-repressed *nmt1*$^+$ promoter, so that *cdc19*$^+$ was expressed by either full strength (pSLF160) or attenuated (pSLF161, medium, or pSLF162, weak) versions of the promoter (see Table 2, BASI *et al.* 1993). We also tagged the *cdc19*$^+$ cDNA clone at the C terminus with a triple HA epitope tag and expressed it under control of the full strength *nmt* promoter (pSLF176). To compare the effects of overproduction of Cdc19p with that of other known MCM proteins, we cloned these genes by PCR from the genome and expressed them under the *nmt* promoter with a triple epitope tag, creating plasmids pSLF221 (*cdc21*$^+$, COXON *et al.* 1992), pSLF225 (*mis5*$^+$, TAKAHASHI *et al.* 1994), and pSLF204 (*nda4*$^+$, MIYAKE *et al.* 1993). This panel of plasmids was transformed into wild-type and *cdc19-P1* mutant cells.

As shown in Figure 1A, overexpression of Cdc19p or Cdc19p-HA from the full strength *nmt* promoter had no phenotype in wild-type cells. Colony formation was normal and cell and colony morphology was similar to control cells transformed with vector only. In contrast, overproduction of *cdc21*$^+$, and to a lesser extent, *nda4*$^+$, retarded cell growth: colonies were smaller than control or *cdc19*$^+$ overproducers. This was enhanced at 25° at which temperature *mis5*$^+$ also retarded growth (data not shown). We carried out FACS analysis on these strains at 32° (Figure 1A). The DNA fluorescence profiles of cells overproducing either Cdc19p (pSLF160) or Cdc19p-HA (pSLF176) were indistinguishable from control cells containing the vector REP4; forward scatter also showed no difference in cell size (data not shown). In contrast, overproduction of Cdc21p (pSLF221) had a striking broadening of the peak of DNA content and cell elongation in our assays, similar

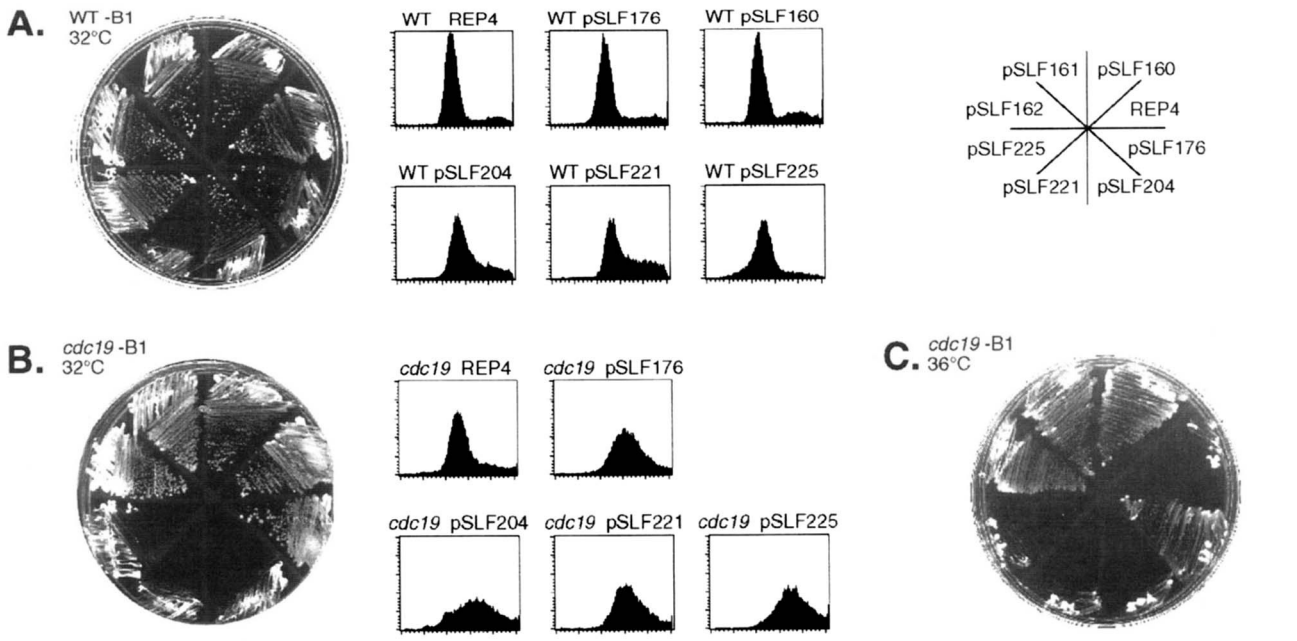

FIGURE 1.—MCM overexpression phenotypes using the *nmt* promoter. (A) Wild-type cells are not sensitive to Cdc19p overproduction but are sensitive to Cdc21p overproduction. Transformants of strain FY254 streaked on EMM plates and grown at 32° without thiamine (-B1) for 2 days. Cells were also grown in liquid culture at 32° under inducing conditions (-B1) for 24 hr and fixed for flow cytometry; shown are histograms for DNA content. (B) *cdc19-P1* cells are sensitive to overproduction of heterologous MCM proteins at the semi-permissive temperature of 32°. Transformants of strain FY243 were streaked on EMM plates at the semi-permissive temperature of 32° without thiamine (-B1) for 3 days. Cells were also grown in liquid culture at 32° under inducing conditions (-B1) for 24 hr and fixed for flow cytometry; shown are histograms for DNA content. (C) Heterologous MCM proteins do not complement *cdc19-P1* cells for growth at 36°. Transformants of strain FY243 were streaked on EMM plates and incubated at the restrictive temperature of 36° without thiamine (-B1) for 3 days. Plasmids used all contain the *ura4*$^+$ marker. REP4 is a vector control. pSLF160, *nmt-cdc19*+. pSLF161, *nmt*-cdc19*$^+$. pSLF162, *nmt**-cdc19*$^+$. pSLF176, *nmt-cdc19*$^+$*-HA*. pSLF204, *nmt-nda4*$^+$*-HA*. pSLF221, *nmt-HA-cdc21*$^+$. pSLF225, *nmt-HA-mis5*$^+$.

to the phenotype previously reported by MAIORANO *et al.* (1996). We verified that our Cdc21p clone was functional by ensuring that it could rescue a temperature-sensitive *cdc21-M68* strain (data not shown), so this dominant negative phenotype is not due to expression of a nonfunctional protein. Nda4p overproduction caused only slight growth retardation and modest cell elongation, resulting in a slightly broadened peak by FACS. Little phenotype was seen with Mis5p at 32°. These observations show that wild-type cells are not uniformly sensitive to MCM protein overproduction.

During the course of these studies, a conflicting report was published that overproduction of Cdc19p is lethal (OKISHIO *et al.* 1996). Although in both cases the full strength *nmt1* promoter was used to drive expression of the cDNA, one obvious difference between the constructs is the plasmid marker: *Schizosaccharomyces pombe ura4*$^+$ for our constructs and *S. cerevisiae LEU2,* which complements poorly in low copy, in the case of the other report (OKISHIO *et al.* 1996). We reasoned that the relative copy number of these plasmids might differ because of the markers, which could affect the amount of protein produced. To assess this, we compared activity of a *lacZ* reporter in the REP3X (*LEU2*) plasmid to a REP4X (*ura4*$^+$) reporter in wild-type cells (see MATERIALS AND METHODS, FORSBURG 1993). Enzyme assays were carried out on duplicate transformants, as described previously (FORSBURG 1993). We found comparable levels of beta-galactosidase produced in cells grown to mid-exponential phase: both REP3X-*lacZ* and REP4X-*lacZ* produced 3500–4000 units of activity in these assays. Thus, we consider that the difference in the markers used is unlikely to explain the variable results of overproduction seen in different laboratories. We will consider these differences further in DISCUSSION.

We investigated whether MCM overproduction conferred a synthetic dosage lethality phenotype in a *cdc19-P1* strain. We transformed the same panel of plasmids into the *cdc19-P1* strain and examined the phenotype at a semi-permissive temperature of 32°. As shown in Figure 1B, *cdc19-P1* cells transformed with the control vector REP4X were able to form colonies at this temperature. Overexpression of the *cdc19*$^+$-containing plasmids conferred no phenotype. In contrast, overexpression of *nda4*$^+$, *cdc21*$^+$, or *mis5*$^+$ caused a striking reduction of colony size in the *cdc19-P1* strain compared to wild-type cells (Figure 1B). At 32°, the *cdc19-P1* transformants grew very slowly with irregular morphology. FACS analysis showed a single, broadened peak in each case at approximately 2C. This phenotype was also observed when expression was induced at 25°. This sug-

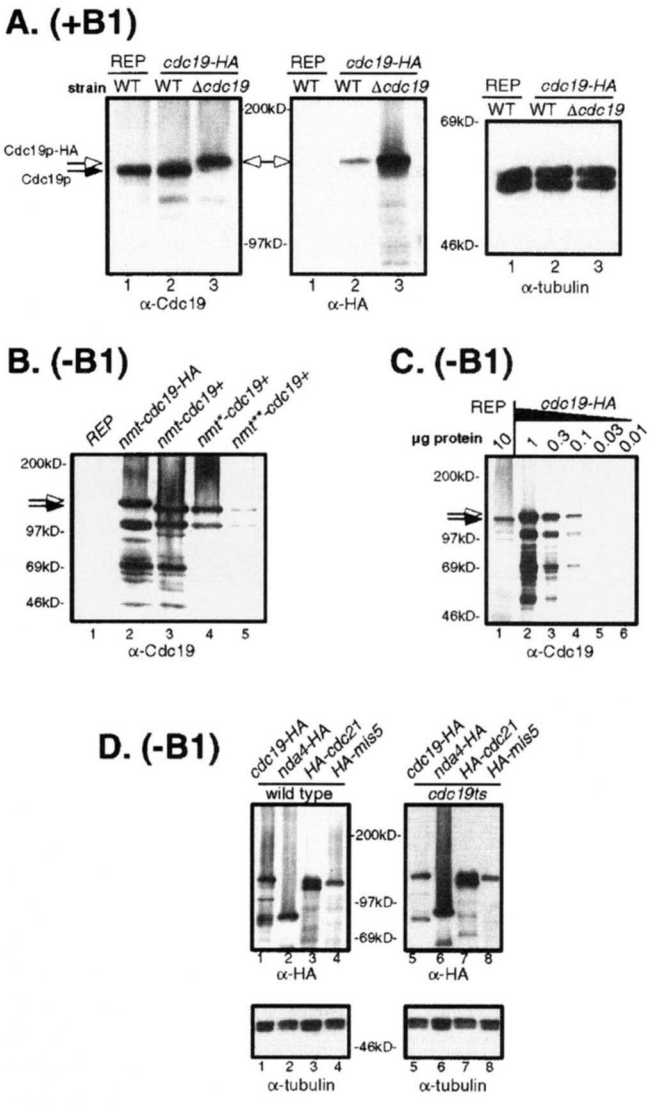

FIGURE 2.—Immunological detection of Cdc19p from fission yeast protein extracts. (A) The anti-Cdc19p peptide antibody specifically recognizes Cdc19p in wild-type cells. Crude lysates were prepared from wild-type (FY254) cells carrying the REP4 vector (lane 1), wild-type cells carrying *cdc19-HA* under the *nmt* promoter (pSLF176 in FY254, lane 2), or Δ*cdc19* cells carrying *cdc19-HA* (FY587, lane 3); all grown in the presence of thiamine (+B1) to repress expression of the *nmt* promoter at 32° to minimal levels. Following fractionation of 30 μg total protein from each sample on an 8% SDS-PAGE gel, identical filters were probed as indicated. Filled arrow indicates Cdc19p; open arrow indicates Cdc19p-HA. (Left) Blot with the anti-Cdc19p antibody detects endogenous Cdc19p and episomal Cdc19p-HA. Note the size shifted form in lane 3, which contains a deletion of the chromosomal *cdc19*$^+$ and Cdc19p-HA on the episome. (Center) Blot with 12CA5 mouse monoclonal against the HA tag recognizes only Cdc19p-HA. Note the difference in protein levels between lanes 2 and 3, discussed in the text. (Right) Blot with anti-tubulin confirms equivalent amounts of total protein were loaded. Exposure time was 5 min. (B) Different versions of the *nmt* promoter (BASI *et al.* 1993; *nmt*, strong; *nmt**, medium; *nmt***, weak) express different levels of Cdc19p when the promoter is induced. Wild-type cells (FY254) containing plasmids REP4 (vector control; lane 1), *nmt-cdc19-HA* (pSLF176; lane 2), *nmt-cdc19*$^+$ (pSLF160, lane 3), *nmt*-cdc19*$^+$ (pSLF161; lane 4) or *nmt***-cdc19*$^+$ (pSLF162 lane 5) were grown for 24 hr at 32° in the absence of thiamine (-B1) to induce expression. Twenty micrograms of total cell protein for each sample was fractionated on a 7.5% gel and blotted with anti-Cdc19p antibody. Filled arrow indicates Cdc19p; open arrow indicates Cdc19p-HA. Substantial breakdown products are observed for high level overproducers. Note at the exposure and dilution of antibody used, endogenous Cdc19p (REP4 lane) is not visible. (C) Dilution series of cells containing induced *nmt-cdc19-HA* (pSLF176) compared to wild type to estimate amount of overproduced protein detected by blotting with anti-Cdc19p antibody. Amounts of total protein were determined using BCA assays (Pierce) and were fractionated on a 7.5% SDS-PAGE gel. Lane 1, 10 μg of lysate from the wild-type control strain (transformed with the vector REP4). Lane 2, 1 μg of total protein from the pSLF176 lysate from induced cells. Lanes 3–6, serial threefold dilutions of the pSLF176 lysate from induced cells. Even at 10-fold dilution relative to wild type, substantial increase in total Cdc19p protein level is apparent (compare lane 2 to lane 1). Exposure time was 5 min. (D) Expression of HA-tagged MCM proteins in wild-type (FY254, lanes 1–4) and *cdc19-P1* (FY243; lanes 5–8) cells. Lysates were prepared following 24 hr induction minus thiamine (-B1); 5 μg protein was loaded in each lane of an 8% gel and probed with 12CA5 anti-HA antibody or anti-tubulin to verify equivalent protein loading. Blotting and exposure conditions were similar for the anti-HA blots, exposures were 10–15 sec. Lanes 1 and 5: *nmt-cdc19-HA* (pSLF176). Lanes 2 and 6: *nmt-nda4-HA* (pSLF204). Lanes 3 and 7: *nmt-HA-cdc21* (pSLF221). Lanes 4 and 8: *nmt-HA-mis5* (pSLF225).

gests that cells with attenuated *cdc19* function are more sensitive to toxicity from overproduction of the other fission yeast MCMs, but overproduction of Cdc19p or Cdc19p-HA still had no deleterious effect. We found that all the *cdc19*$^+$ plasmids, including the epitope tagged form (pSLF176), were able to complement the mutant for growth at the restrictive temperature of 36° (Figure 1C). They also complemented in the presence of thiamine, which represses the promoter (data not shown; but see DISCUSSION below). In contrast, expression of heterologous MCM proteins failed to rescue the temperature-sensitive phenotype of *cdc19-P1* under any conditions. This was not surprising, given their toxic effects at lower temperatures.

Analysis of protein expression: To allow analysis of the Cdc19p protein in fission yeast cells, we raised and characterized polyclonal antiserum to a synthetic peptide (see MATERIALS AND METHODS). Affinity purified serum recognized a protein of ~115 kD in crude yeast lysates, which is somewhat larger than the predicted molecular weight of 93 kD (Figure 2A, left panel, lane 1). Reduced mobility in SDS-PAGE appears to be a feature of MCM proteins and similar results have been reported by others (*e.g.*, COXON *et al.* 1992; MIYAKE *et al.* 1996; OKISHIO *et al.* 1996). To clearly establish the identity of the 115-kD species, we used the HA-tagged form of Cdc19p expressed by pSLF176, which being larger than the endogenous Cdc19p is expected to migrate more slowly in SDS-PAGE. We prepared lysates from cells transformed with pSLF176 and grown in thiamine to repress the plasmid promoter to a low level of

expression (FORSBURG 1993; MAUNDRELL 1993). In wild-type cells transformed with pSLF176, a faint signal can be visualized just above the endogenous 115-kD band (Figure 2A, left panel, lane 2). This is clearly visible when blotted with anti-HA, so that the endogenous protein is not detected (Figure 2A, center panel, lane 2). We isolated a haploid strain containing the pSLF176 episome and a deletion of the chromosomal *cdc19* locus, demonstrating that the tagged version of the gene is fully functional (see MATERIALS AND METHODS). When a lysate from this strain lacking the endogenous Cdc19p is blotted with anti-Cdc19p, only the size shifted form is detected (Figure 2A, left panel, lane 3). Note that in all the strains in Figure 2A, the cells were grown in thiamine to repress expression from the *nmt* promoter to low levels. This experiment verifies that the 115-kD band corresponds to Cdc19p and demonstrates the specificity of the antisera by ensuring that no contaminating band comigrates with Cdc19p. We are therefore confident that our antibody specifically and sensitively recognizes wild-type levels of Cdc19p.

Interestingly, we observed that the level of Cdc19p-HA produced from pSLF176 in Δ*cdc19* (FY587) was significantly higher than that produced from the same plasmid in the wild-type *cdc19*$^+$ background, although both strains were grown in thiamine to repress expression from the *nmt* promoter. This is most apparent in the anti-HA blot (compare lanes 2 and 3 in center panel of Figure 2A). Notably, this up-regulated Cdc19p was present at levels similar to the endogenous Cdc19p (compare lanes 1 and 3 in the left panel of Figure 2A). The same up-regulation was observed in an independently isolated strain containing pSLF176 and Δ*cdc19* (FY717); this finding will be investigated further in Figure 7.

Next, we used the anti-Cdc19p antibody to compare the levels of expression of Cdc19p and Cdc19p-HA in wild-type cells when the *nmt* promoter was induced. We used three different versions of the *nmt* promoter, which lead to high, medium, and low levels of expression (BASI *et al.* 1993). Expression from the *nmt* promoter is low in the presence of thiamine (as seen in Figure 2A) but is strongly induced when thiamine is removed from the media (MAUNDRELL 1990, 1993). As shown in Figure 1, there is no phenotype associated with overproduction of Cdc19p from any of these plasmids, and all express sufficient Cdc19p to rescue the *cdc19-P1* mutant cells for growth at 36°. Lysates were prepared from wild-type cells grown in the absence of thiamine to induce the promoter. Strains contained a vector control, Cdc19p-HA or Cdc19p under the full strength *nmt* promoter, and Cdc19p expressed under medium and weak promoters (Table 2). As expected, Western blotting reveals different levels of protein from the different promoters under these inducing conditions (Figure 2B). Note that the size shift of the Cdc19p-HA variant (pSLF176) can be easily detected and the amount of tagged Cdc19p-HA produced by pSLF176 is similar to the amount of untagged Cdc19p produced by the same promoter in pSLF160. Substantial degradation was apparent in the overproducers, and we have seen this with most genes expressed by the inducible *nmt* system (*e.g.*, Figure 2D). The difference between the amount of protein produced by pSLF161 and pSLF162 is modest but both produce significantly more protein than the endogenous level. We have made similar observations using HA epitope-tagged proteins under the three promoters (FORSBURG and SHERMAN 1997).

The wild-type level of Cdc19p cannot be detected in the short exposure needed to distinguish the overproduced proteins (Figure 2B, lane 1), although it is clearly visible in a longer exposure (data not shown; Figure 2, A and C). To estimate the degree of overproduction from the full strength *nmt* promoter when induced, we carried out serial dilutions of the *nmt*-overproducing lysate and used Western blotting with anti-Cdc19p antibodies to compare the amount of Cdc19p in the diluted lysates to that in the lysate from the wild-type strain (Figure 2C). We used the pSLF176 overproducer, since many of our subsequent experiments were carried out using the HA-tagged form of the protein, but an experiment with the untagged pSLF160 gave similar results (data not shown). These data suggest that Cdc19p is being overexpressed at least 100-fold when thiamine is removed from the media.

Results in Figure 1A suggested that cells were more sensitive to overproduction of other MCMs than to overproduction of Cdc19p, and this sensitivity was significantly enhanced by the *cdc19-P1* mutation. We took advantage of the HA tags on Cdc19p, Nda4p, Cdc21p, and Mis5p to compare the levels of MCM proteins produced by these constructs in wild-type cells in the absence of thiamine (Figure 2D). Since they were all expressed from the same promoter, not surprisingly they all expressed similar amounts of protein. The tagged proteins migrate with the expected mobility. Endogenous Nda4p runs in SDS-PAGE at ~80 kD (OKISHIO *et al.* 1996) and Cdc21p as a doublet at ~110 kD (MAIORANO *et al.* 1996); Mis5p has a predicted molecular weight of 97 kD (TAKAHASHI *et al.* 1994) but in common with other members of the family is likely to migrate more slowly.

Cdc19p levels in the cell cycle: Previously, we showed that the *cdc19*$^+$ mRNA is expressed constitutively in the cycling cells (FORSBURG and NURSE 1994b). We investigated the levels of the protein through the cell cycle by using a series of mutants to arrest cell cycle progression. Crude lysates were prepared from asynchronous wild-type cells and the following arrested strains: *cdc10-V50* (G1/START transcription factor, LOWNDES *et al.* 1992; REYMOND *et al.* 1992), *cdc22-M45* (G1/S ribonucleotide reductase, NASMYTH and NURSE 1981; SARABIA *et al.* 1993), *cdc17-K42* (S phase, DNA ligase, NASMYTH 1977),

cdc25–22 (G2/M mitotic inducer, FANTES 1979; RUSSELL and NURSE 1986) and *nuc2* (M phase/G1, HIRANO *et al.* 1988). We loaded equivalent amounts of total cell protein and immunoblotted Cdc19p. There was a modest change in the level of Cdc19p apparent in cells blocked in G2 or M: levels of protein appeared to be reduced several fold (Figure 3A). There was no evidence for a shift in electrophoretic mobility suggestive of modification. Next, we synchronized cells in the G2 phase of the cell cycle using a temperature shift to 36° in a *cdc25-22* mutant strain (ALFA *et al.* 1993). Upon release to 25°, we followed the cells for 7 hr, spanning nearly two complete cell cycles. We prepared crude lysates and blotted equivalent amounts for Cdc19p and Cdc2p as a loading control. The results are presented in Figure 3B. In agreement with the observations from the *cdc* mutant arrest experiment, Cdc19p levels immediately following the *cdc25* arrest are low. As septation index rises (corresponding to S phase), there is an increase in Cdc19p protein, which drops modestly before the next cell cycle. Thus, in rapidly cycling cells, there is only a slight variation in the levels of Cdc19p through the cell cycle; however, the amount of protein may drop lower in cells that undergo prolonged arrest in G2 of the cell cycle.

Genetic interactions of *cdc19-P1*: We showed previously that the temperature-sensitive allele *cdc19-P1* is synthetically lethal with the *cdc21-M68* mutation, which affects another member of the MCM family (COXON *et al.* 1992); double mutant cells cannot be recovered in crosses between these strains (FORSBURG and NURSE 1994b). An extensive network of synthetic interactions is characteristic of many S phase mutants in the budding yeast, including MCM proteins and components of the origin recognition complex ORC, suggesting that there may be direct interactions between these elements (*e.g.*, HENNESSY *et al.* 1991; YAN *et al.* 1991; LI and HERSKOWITZ 1993). We investigated such interactions by constructing a series of double mutants with *cdc19-P1,* using temperature-sensitive alleles of known S phase mutants. We tested *polδ-ts1* (also called *pol3* and *cdc6,* encoding the large subunit of DNA polymerase δ, PIGNEDE *et al.* 1991; FRANCESCONI *et al.* 1993; IINO and YAMAMOTO 1997), *cdc1-7* (encodes the small subunit of DNA polymerase δ, MACNEILL *et al.* 1996), *cdc27-K3* (a polymerase δ-associated protein, MACNEILL *et al.* 1996), *cdc18-K46* and *rad4/cut5-116* (both required for initiation and checkpoint control, FENECH *et al.* 1991; KELLY *et al.* 1993; SAKA and YANAGIDA 1993), *cdc22-M45* (ribonucleotide reductase, SARABIA *et al.* 1993), *cdc17-K42* (ligase, NASMYTH 1977; JOHNSTON *et al.* 1986), and two molecularly uncharacterized S phase mutants, *cdc23-M36* and *cdc24-M38* (NASMYTH and NURSE 1981). Double mutants with *cdc10-V50,* a mutant in a transcription factor required for START (LOWNDES *et al.* 1992; REYMOND *et al.* 1992), and *cdc25-22,* a mitotic inducer (RUSSELL and NURSE 1986), were also constructed. Growth

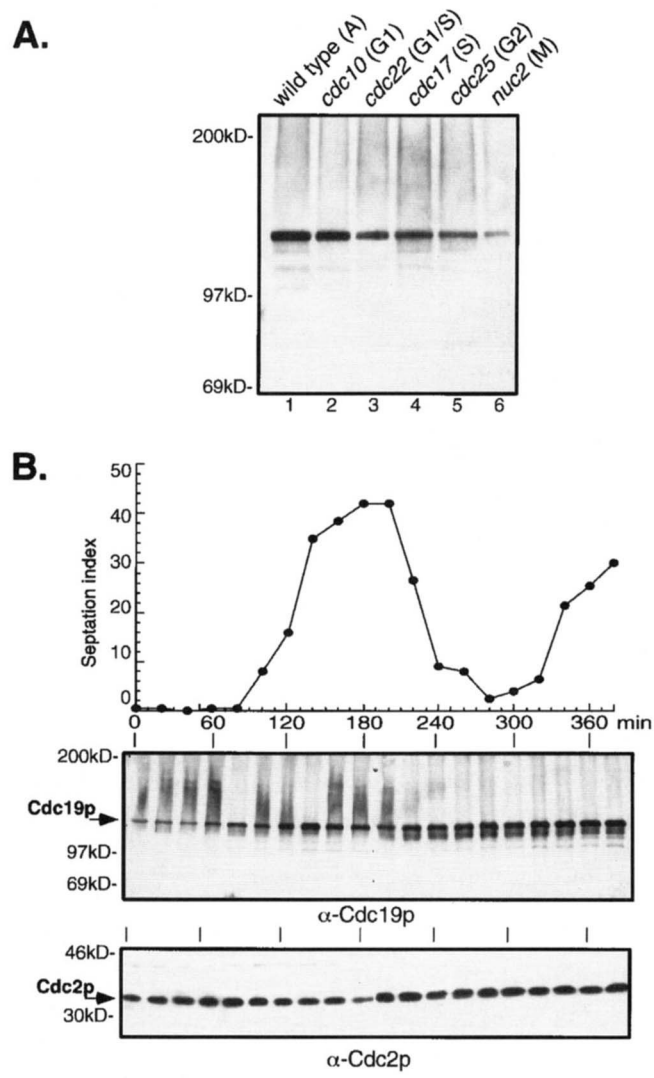

FIGURE 3.—Cell-cycle effects on levels of Cdc19p. (A) Levels of Cdc19p in arrested cells shifted for 4 hr to the restrictive temperature of 36°. Lysates were prepared, 30 μg of total cell protein were loaded in each track and fractionated on a 8% gel. Lane 1, wild-type asynchronous cells (FY254); lane 2, *cdc10-V50* in G1 phase (FY563); lane 3, *cdc22-M45* in G1/S phase (FY583); lane 4, *cdc17-K42* in S phase (FY569); lane 5, *cdc25-22* in G2 phase (FY318); lane 6, *nuc2ts* in M phase (FY458). (B) Levels of Cdc19p in synchronized cells. Strain FY117 (*cdc25-22*) was grown to early exponential phase in EMM media, shifted to 36° for 4 hr, and returned to 25° at time = 0. Aliquots were harvested every 20 min and monitored for septation index, and crude lysates were prepared. Each sample contained 10 μg of total cell protein loaded on duplicate gels: an 8% gel (anti-Cdc19p blot) or a 10% gel (anti-Cdc2p blot). The lysates from 220 to 380 min were prepared separately and show greater amounts of degradation.

of most double mutants was indistinguishable from that of the parents at all temperatures (data not shown).

Three of the double mutants showed synthetic growth phenotypes. *cdc19-P1 polδ-ts1* and *cdc19-P1 cdc1-7* grew slowly at 25° and strikingly worse at 29°, at which temperature the strains formed very small colonies of elongated, often curved cells (Figure 4A, 29°). All par-

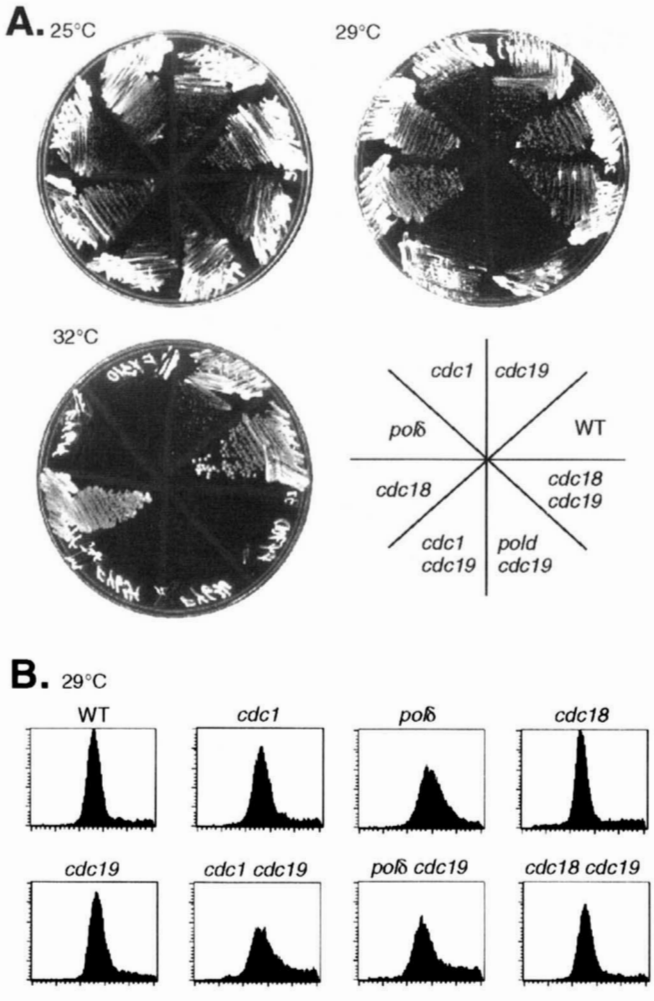

FIGURE 4.—Synthetic phenotypes in *cdc19*-P1 double mutants. (A) Strains *cdc18-K46* (FY194), wild type (FY254), *cdc19-P1* (FY243), *cdc1-7* (FY310), *polδ-ts1* (FY614), *cdc1-7 cdc19-P1* (FY634), *cdc19-P1 polδ-ts1* (FY636) and *cdc18-K46 cdc19-P1* (FY700) were grown on YE plates at the indicated temperatures until colonies formed. At 29°, double mutants between *cdc19-P1* and *cdc1-7* or *polδ-ts1* were able to form microcolonies very slowly. (B) FACS analysis to determine DNA content in cells grown in liquid culture in EMM plus uracil, leucine, adenine and histidine at 29°. Cells were grown to mid-exponential phase and processed as described in MATERIALS AND METHODS.

ent strains were alive at these temperatures, forming large, healthy-looking colonies. The double mutants were inviable at 32°, as were the single mutants *cdc1-7* and *polδ-ts1*. However, their phenotypes were subtly different at 32°; the double mutants were not as elongated as the *cdc1-7* or *polδ-ts1* single mutants and were more likely to be curved, branched, or T-shaped (data not shown). Similar results were seen for other alleles of *polδ* including *cdc6-23* (data not shown). The *cdc18-K46 cdc19-P1* double mutant was viable and normal in appearance at 25° and 29°, although it formed somewhat smaller colonies at both temperatures. At 32°, where both parents were alive, this strain failed to form colonies. We investigated whether there was any obvious S phase delay in the double mutants by flow cytometry. As shown in Figure 4B, the 2C peaks of DNA were broadened for *cdc1-7 cdc19-P1* and *polδ-ts1 cdc19-P1* with significant spreading to the right. This is typical in elongating fission yeast cells and is consistent with a late S or G2 delay. In no case was there an increase in cells with a 1C or intermediate DNA content.

We further examined these interactions by overproducing wild-type *cdc19*$^+$ and *polδ*$^+$ in wild-type or mutant cells. *cdc17-K42, cdc18-K46, cdc19-P1, cdc22-M45, polδ-ts1* and wild-type cells were transformed with pSLF160 or REP2-*polδ* (gift of TERESA WANG); these express *cdc19*$^+$ or *polδ*$^+$, respectively, under control of the regulated *nmt* promoter. Transformants were screened for growth at the permissive (25°), semi-permissive (29°, 32°) and restrictive (36°) temperatures in the absence of thiamine in the media to induce expression. There was no evidence for enhanced sensitivity to *cdc19*$^+$ or *polδ*$^+$ overproduction in any of the mutant strains, based on the rate and size of colony formation compared to vector controls (data not shown). Because we also observed a modest synthetic phenotype between *cdc18-K46* and *cdc19-P1,* we also determined the effects of overproducing *cdc18*$^+$ in *cdc19-P1* mutant cells and vice versa. Plasmids pSLF160 and pMF56 (nmt-cdc18-HA, MUZI-FALCONI *et al.* 1996) were transformed into *cdc18-K46* and *cdc19-P1* strains. Again, transformants were screened for growth at several temperatures. There was no obvious sensitivity due to overexpression of Cdc19p or Cdc18p-HA compared to vector controls in either strain background.

Mutations in Cdc19p define regions required for function: MCM proteins all share a conserved core of sequence homology, which we call the MCM core domain. Members of each subfamily share additional features. We constructed a series of mutations in *cdc19*$^+$ to identify essential domains of the protein and to begin to probe their structure. As well as constructing site-directed mutations *in vitro,* we also cloned the existing conditional *cdc19* mutants. All the mutations are diagrammed in Figure 5.

There are two conditional *cdc19* alleles known: the original *cdc19-P1* temperature-sensitive allele isolated in a screen for S phase *cdc* mutants (NASMYTH and NURSE 1981) and the cold-sensitive *nda1-KM376* allele, which was isolated in an independent screen for nuclear division defective mutants (TODA *et al.* 1983). We cloned these alleles using genomic PCR and sequenced them. To avoid PCR-generated mutations, each reaction was carried out in duplicate, and the product of each PCR reaction was independently cloned (see MATERIALS AND METHODS). We used complementation analysis to determine the restriction fragments containing the mutations and sequenced them, again in duplicate. Based on this analysis, the temperature-sensitive allele *cdc19-P1* contains two changes, P257L T272I; the proline is conserved in all MCM2 proteins, and the threonine

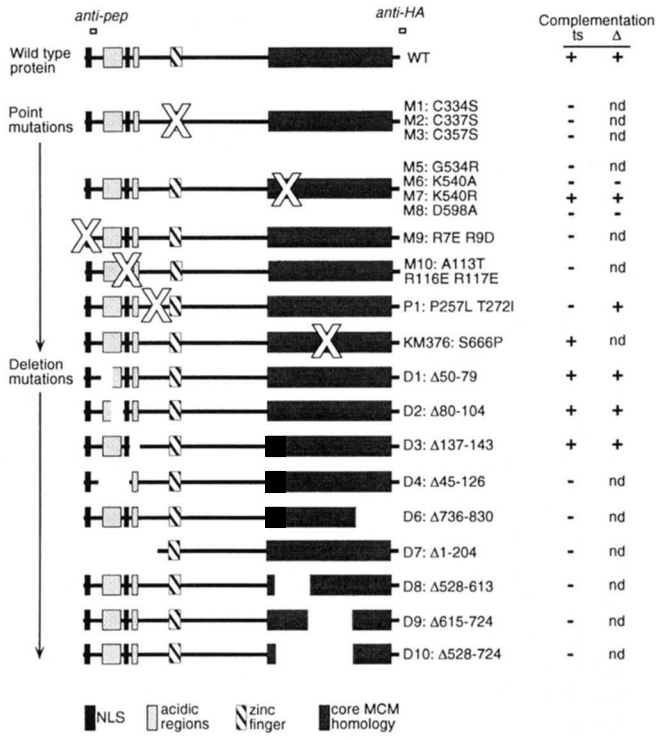

FIGURE 5.—Structure and function of mutants in Cdc19p. Point mutations affecting the same region of Cdc19p are grouped and the approximate location of the mutations are indicated by the X. M1–M3 affect the zinc finger region. M5–M8 affect the core MCM homology region. M9 affects putative NLS1 and M10 modifies sequences in and near NLS2. Complementation data for the M- and D-series mutants reflects the behavior of the HA-tagged protein expressed from the *nmt1* promoter in the absence of thiamine in *cdc19-P1* (FY243) or Δ*cdc19::LEU2* cells. Complementation of *cdc19-P1* or Δ*cdc19::LEU2* by the conditional alleles *cdc19-P1* or *nda1-KM37* was determined by constructing heterozygous diploid strains. nd, not done. The relative positions of the epitopes for the anti-peptide Cdc19p antibody and the anti-HA antibody are shown above the diagram.

occurs in the yeast MCM2 proteins. The *nda1-KM376* cold-sensitive allele changes a conserved proline present in all MCM2 proteins to a serine (P666S).

We next constructed *in vitro* a series of sequence deletions and point mutations throughout *cdc19*$^+$. The mutants were each cloned into an expression vector with an HA tag to allow unambiguous identification of the mutant protein even in the presence of the endogenous wild-type protein; as shown previously, the tag does not interfere with Cdc19p function. Nine point mutations and nine sequence deletions were constructed and screened for complementation and overproduction phenotypes in *cdc19-P1* and wild-type cells. Upon overexpression in wild-type cells, no severe phenotypes were observed, although M9 and M10 formed slightly smaller colonies (Figure 6A). We verified that tagged proteins of the anticipated molecular weight were produced and stable in wild-type cells at 36° (Figure 6B).

Each mutant was tested for complementation of the *cdc19-P1* allele at 36° in the absence of thiamine (Figure 6D). As expected, most of the residues common to MCM proteins were essential for Cdc19p function. Mutation of any of the cysteines in the putative zinc finger to serine abolished complementation. Similar results were reported for budding yeast Mcm2p (YAN *et al.* 1991). The MCM core homology, spanning approximately residues 520–680, has been suggested to function as a DNA-dependent ATPase based on the presence of degenerate Walker A and B motifs (WALKER *et al.* 1982; KOONIN 1993). Not surprisingly, deletion mutants D6 (Δ726-830), D8 (Δ528-613), D9 (Δ615-724), and D10 (Δ528-724), which span the MCM homology domain, were all nonfunctional. We made several mutations in or near the putative Walker A motif. M5 (G534R) and M8 (D598A, inside the diagnostic MCM sequence IDEFDKM) each abolished complementation. Interestingly, a nonconservative mutation of the conserved lysine in the putative ATP binding site of Cdc19p in mutant M6 (K540A) abolished complementation, but the conservative change in M7 (K540R) was able to rescue *cdc19-P1*.

The N-terminal region of Cdc19p is not conserved in primary sequence, but general organization is preserved. For example, all Mcm2p homologues contain putative nuclear localization sequences (NLSes) and an acidic N-terminal region (YAN *et al.* 1991; MIYAKE *et al.* 1993; FORSBURG and NURSE 1994b; TODOROV *et al.* 1994; TREISMAN *et al.* 1995; MIYAKE *et al.* 1996). Recently it has been shown that Cdc19p is constitutively located in the nucleus during the cell cycle (OKISHIO *et al.* 1996), in contrast to budding yeast MCM2p (YAN *et al.* 1993). Cdc19p contains two short sequences similar to SV40-type nuclear localization sequences (LANFORD and BUTEL 1984): RKRGRR at positions 5–10 of the protein and RLRRR at positions 114–118. We made two mutations in the putative NLSes. M9 is a double point mutation (R7E R9D) in the NLS1 sequence, and M10 contains a double point mutation (R116E R117E) in the NLS2 sequence and a point mutation A113T in the adjacent sequence. Neither of these mutants was able to complement the temperature-sensitive strain. This is consistent with the NLS1 sequence functioning as a bona fide NLS; we cannot distinguish whether the phenotype of the M10 mutation reflects the changes in NLS2 or the A113T slightly upstream. We also note that, perhaps due to the similar charge changes, both these proteins migrate very fast relative to the wild-type protein in SDS-PAGE (Figure 6B). It will be of interest to determine whether these mutants actually affect nuclear localization.

We constructed a series of deletions to remove blocks of the N-terminal acidic domain. Mutants D1 (Δ50-79) and D2 (Δ80-104) each lack half of the acidic domain, and both were able to complement the temperature-sensitive mutant under low and high levels of expression. A short acidic stretch DEDDDLD from 137 to 143 was removed from D3, which also proved functional. In

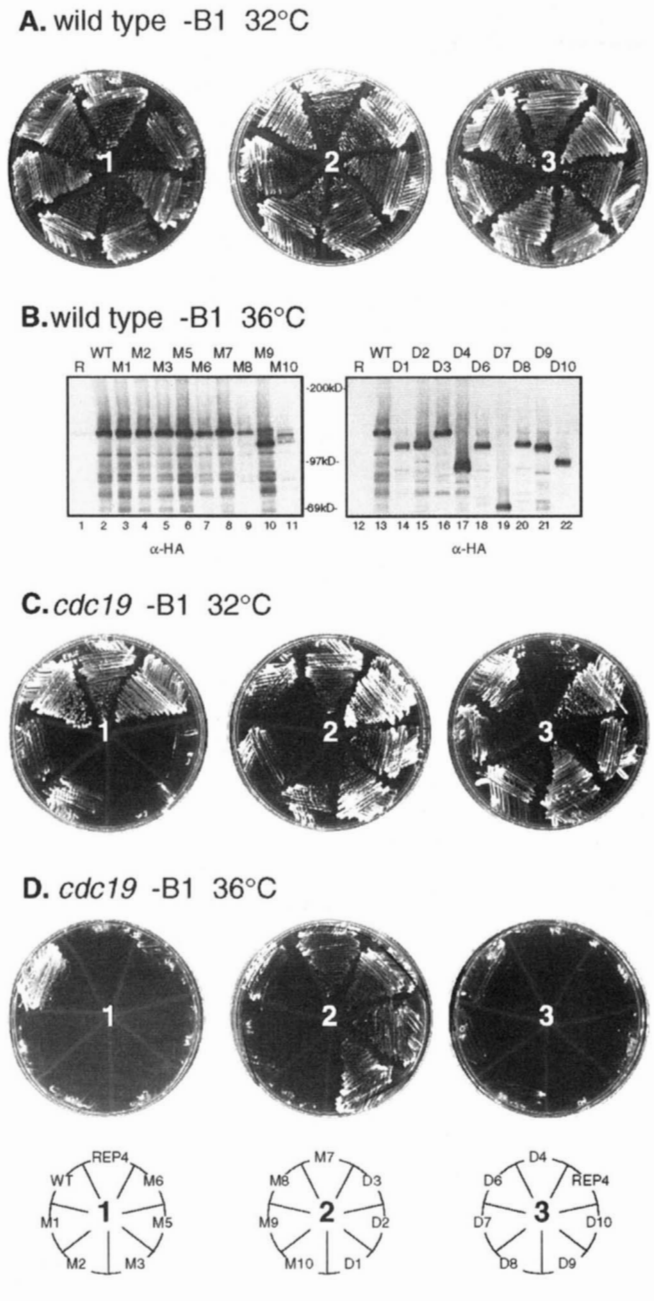

FIGURE 6.—Phenotypes and protein expression resulting from expression of mutant Cdc19p in the temperature-sensitive strain *cdc19-P1* (FY243) or in wild type (FY254). Mutants are defined in Figure 5. pSLF176 (Cdc19p-HA) provides a positive control, and REP4 (the parent vector) provides a negative control. (A) Overexpression of the mutants in wild-type cells at 32° has no phenotype. Wild-type strain FY254 was transformed with the indicated plasmids. Transformants were streaked to plates lacking thiamine and incubated at 32° for 3 days. (B) Mutant proteins are expressed at comparable levels in wild-type (FY254) cells at 36° −thiamine. Transformants were grown in liquid culture and expression was induced by removing thiamine from the media for 24 hr. Ten micrograms of total protein was fractionated on an 8% SDS-PAGE gel and probed for the HA tag using the 12CA5 monoclonal antibody. Note that M9 and M10, both of which contain multiple point mutations in a highly charged region of the protein, affect protein mobility. M10 expression is reduced relative to other mutants. R, REP4 vector control (lanes 1 and 12). WT, pSLF176 transformants (lanes 2 and 13). (C) Synthetic dosage lethality of *cdc19-P1* (FY243) cells overexpressing mutant forms of Cdc19p incubated in the absence of thiamine (-B1) at the semi-permissive temperature of 32° for 4 days. Colony formation of M1, M2, M3, M5, M9, M10, D4 and D7 is severely reduced; M7, M8, D6 and D10 are 1–2 days delayed relative to the vector control. Expression of other clones has no phenotype. (D) Complementation of *cdc19-P1* by expression of mutant forms of Cdc19p in the absence of thiamine at 36°. *cdc19-P1* (FY243) cells transformed with the indicated mutant clones were streaked on minus thiamine (-B1) plates at 36° and incubated for 3 days. Only WT, M7, D1, D2 and D3 complement the temperature-sensitive mutation.

contrast, the D4 (Δ45-126) mutant lacks the entire acidic region and putative NLS2, and failed to complement. Mutant D7 (Δ1-204), which lacks both NLSes and the entire acidic domain, also abolished rescue in the *cdc19-P1* strain. Thus, the putative NLSes each appear essential although the linking acidic sequence is flexible (Figure 6B).

Although none of the mutants were dominant negative when overproduced in wild-type cells (Figure 6A), we reasoned that they might generate a phenotype in *cdc19-P1* cells at the semi-permissive temperature, under the same conditions where the *cdc19-P1* strain is acutely sensitive to overexpression of the other MCMs. Figure 6C shows the results of inducing mutant expression in the *cdc19-P1* strain at 32°. Three growth classes were observed. The first class consisted of the fastest growing cells, which contained the wild-type episome (pSLF176), the fully complementing mutants (D1, D2 and D3), the vector control (REP4), and the nonfunctional mutants M6 and D9. Transformants in the second class formed smaller colonies of slightly elongated but otherwise healthy-looking cells; these strains took 1 or 2 days longer to form large colonies. This class consisted of the complementing mutant M7 and the noncomplementing mutants M8, D6, D8 and D10. All these mutants contain an intact N terminus and a mutated MCM homology domain (see Figure 5). The third class included mutants M1, M2, and M3 (affecting the zinc finger), M5 (MCM domain), M9, M10 (NLS1 or 2), D4 (acidic domain and NLS2), and D7 (N-terminal truncation), all of which were quite toxic. Even upon prolonged incubation, these strains never grew beyond microcolonies of irregular and somewhat elongated cells. All of the mutants in class three are nonfunctional. Moreover, nearly all contain an intact MCM homology domain, and defective N-terminal regions. Thus, *cdc19-P1* is particularly sensitive to overproduction of a nonfunctional Cdc19p derivative that contains an intact MCM homology domain.

Cells require a threshold level of Cdc19p: Four of the 18 mutants we constructed were able to complement the temperature-sensitive *cdc19-P1* allele at 36°: M7, D1, D2, and D3. We tested the ability of these plasmids to rescue the null allele Δ*cdc19::LEU2* to demonstrate that they encode functional forms of Cdc19p

and do not merely stabilize the temperature-sensitive protein by virtue of overexpression. The diploid strain FY362, heterozygous for Δ*cdc19::LEU2* (FORSBURG and NURSE 1994b), was transformed with *nmt* expression plasmids containing Cdc19p-HA (pSLF176, as a positive control), the rescuing mutants D1, D2, D3 and M7, a vector control (REP4), and the mutants M6 and M8 (negative controls). Following meiosis, we screened for haploid isolates containing both the plasmid and disruption (MATERIALS AND METHODS). Wild-type Cdc19p-HA and the mutants D1, D2, D3, and M7 were easily recovered in Leu$^+$ Ura$^+$ haploids, indicating that these clones can rescue the null allele. In contrast, M6, M8 and the vector were only recovered in spores containing the wild-type *cdc19*$^+$ gene in the chromosome.

This procedure was carried out in the absence of thiamine, keeping the promoter on the plasmid fully induced (Figure 7A, top). The rescued clones were next streaked to plates containing thiamine, which represses the promoter. In a background of sick or dying cells, a few healthy colonies grew up (Figure 7A, middle). Once the few large colonies grew, they were stable and were easily propagated by restreaking again on thiamine (Figure 7A, bottom). This observation, in which slow growing sick colonies are converted to fast-growing healthy colonies, suggests that a genetic selection is taking place.

What could be the basis of this selection? In Figure 2A we showed that there is an increased amount of Cdc19p produced by the *nmt* promoter in the presence of thiamine when expressed in a Δ*cdc19* background. Interestingly, this selection results in the episome being upregulated to express approximately wild-type levels of Cdc19p. If this defines a minimum level of Cdc19p expression, we predict that all complementing alleles of Cdc19p, when expressed by the *nmt* promoter in the presence of thiamine, should be similarly upregulated in Δ*cdc19* cells. Using the HA tag to identify the episomally expressed Cdc19p-HA, we compared protein levels in Δ*cdc19* and *cdc19*$^+$ cells transformed with Cdc19p-HA, D1, D2, D3, or M7 under control of the *nmt* promoter. Figure 7B shows that Cdc19p-HA levels produced in the deletion background in the presence of thiamine were consistently higher than those produced by the same plasmids in wild-type cells under the same conditions. This confirms the results observed in Figure 2A and supports our hypothesis that growth in the presence of thiamine selects for a higher copy number or increased activity of the plasmid to reach a threshold level of Cdc19p.

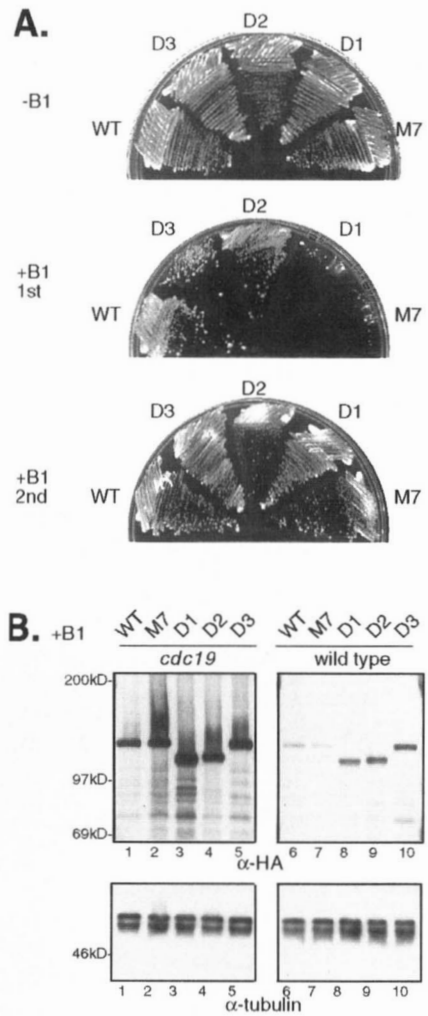

FIGURE 7.—Expression of Cdc19p-HA is up-regulated in a deletion background. (A) Complementation of the null allele Δ*cdc19* by episomes containing Cdc19p-HA or mutant derivatives. (Top) Haploid Δ*cdc19* cells are able to grow on minus thiamine (-B1) plates if they contain an episome expressing wild-type Cdc19p (FY717) or derivatives D1 (FY718), D2 (FY719), D3 (FY720), or M7 (FY721). (Middle) Colonies from the top plate (-B1) were picked and restreaked on plates containing thiamine (+B1 first streak) to repress expression of the promoter. Only a few cells formed colonies. (Bottom) Large colonies from the middle plate were restreaked on thiamine (+B1 second streak). (B) Comparison of levels of protein expressed from the plasmids in A when produced in wild-type cells (FY254) compared to Δ*cdc19* cells. Lysates were prepared from cells grown to mid-exponential phase in the presence of thiamine. Twenty micrograms of total cell protein (by BCA assay; Pierce) were fractionated on 8% SDS-PAGE and blotted with anti-HA antibody to identify the fusion proteins. Lanes 1–5 (Δ*cdc19* cells) and lanes 6–10 (wild-type FY254 cells) were run on the same gel, probed on the same filter, and exposed for 1 min. They are directly comparable. Lane 1 and 6, wild-type Cdc19p-HA; lanes 2 and 7, M7; lanes 3 and 8, D1; lanes 4 and 9, D2; lanes 5 and 6, D3. Equivalent protein loading was verified by probing for tubulin.

DISCUSSION

The MCM family of proteins are conserved in all eukaryotes and are essential for normal DNA replication. We have dissected the molecular and genetic interactions of the *S. pombe cdc19*$^+$ gene to gain insight into the normal role of MCM proteins in fission yeast S phase. Previous data showed that *cdc19*$^+$ has an early execution point and is required for a timely S phase (NASMYTH and NURSE 1981; FORSBURG and NURSE

1994b). *cdc19-P1* cells arrest with an apparent 2C DNA content within the first cell cycle, suggesting that the arrest point is at the end of S phase, rather than near the execution point, but mutant arrest is checkpoint dependent, indicating that the cells are still within S phase (FORSBURG and NURSE 1994b). We have further investigated the role of Cdc19p using overexpression studies, genetic analysis, and characterization of site-directed mutants.

First, we overproduced Cdc19p in both wild-type and mutant cells and found no evidence for an associated phenotype. We know that the protein produced is functional, because the same clones can rescue both the temperature-sensitive and null alleles of *cdc19*$^+$; we also confirmed that the sequence is correct. We showed that the *nmt* promoter repressed in thiamine produces less Cdc19p from the episome than is produced by the wild-type gene in the chromosome in wild-type cells (compare lanes 1 and 2 of Figure 2A). We demonstrated that following induction of the *nmt* promoter, the episome produces ~100-fold more Cdc19p than that produced in normal wild-type cells (Figure 2, B and C). This is consistent with previous reports that the *nmt* promoter can be induced 100- to 300-fold (BASI *et al.* 1993; FORSBURG 1993) and confirms that we have achieved substantial overproduction of this protein. Our observations conflict with a recent report that overexpression of Cdc19p on the same *nmt* promoter was lethal (OKISHIO *et al.* 1996). Because the markers on the plasmids differed, we compared expression from *nmt-lacZ* reporters and found no striking difference in expression in *ura4*$^+$- or *LEU2*-marked plasmids. Our experiments show that wild-type cells can tolerate at least a 100-fold increase in functional Cdc19p protein levels without ill effect. We saw no difference whether Cdc19p was expressed in cells derived from our standard strain background (FY254), or from outside strains including FY280 and FY614 (data not shown). Differences in the structure of the cDNAs, or still uncharacterized strain or media differences may account for these conflicting observations. While these are puzzling, they are not unprecedented; for example, overproduction of the replication initiation factor Cdc18p induces different levels of re-replication in different reports (NISHITANI and NURSE 1995; MUZI-FALCONI *et al.* 1996).

In contrast to our results with Cdc19p, we find that overproduction of Cdc21p noticeably slows colony formation in wild-type cells, while Nda4p overproduction confers a modest delay, using the same promoter system. These observations are largely consistent with those reported by others (MAIORANO *et al.* 1996; OKISHIO *et al.* 1996). We also found that the amount of the different tagged MCM proteins overproduced in our study is similar. Thus, there is a variable response to overproduction of different MCM proteins.

We investigated the changes in Cdc19p protein levels throughout the cell cycle. Previously we showed that there is no striking change in the expression of *cdc19*$^+$ during the cell cycle (FORSBURG and NURSE 1994b). Using our peptide antibody to Cdc19p protein, we find that there is more Cdc19p in cells arrested in early parts of the cell cycle (G1 and S) than in cells arrested at late points of the cycle (G2, M). This was probed more carefully using a synchronous culture from a *cdc25-22* block and release experiment. We observed that the protein level was very low following cell cycle arrest in G2; following release, the level of protein rose near S phase and declined only very modestly thereafter. Of course, because these experiments examine rapidly dividing large cells following cell cycle block and release, there is a possibility that subtle differences may be masked, but dramatic changes are unlikely. We conclude that Cdc19p varies only slightly in rapidly growing cells, although the protein may have decreased stability in cells arrested in G2 phase.

Intriguingly, we found evidence for a synthetic interaction between *cdc19-P1* and subunits of DNA polymerase δ. The double mutants *cdc19-P1 polδ-ts1* and *cdc19-P1 cdc1-7* had severe slow-growth phenotypes even at 25°, worsened by growth at 29°. *polδ-ts1* contains two mutations in conserved residues (A143V and P144S) of the large subunit of DNA polymerase δ (FRANCESCONI *et al.* 1993), and *cdc1-7* mutates the small subunit of DNA polymerase δ (MACNEILL *et al.* 1996). DNA polymerase δ is the major elongation enzyme in eukaryotic cells and does not play a role in initiation (reviewed in BURGERS 1996). This is the first time an interaction between MCM proteins and the elongation machinery has been observed, and leads us to speculate that the MCM proteins may play a role post-initiation. We cannot at the moment distinguish whether the interaction we have defined characterizes an actual molecular interaction between Cdc19p and subunits of DNA polymerase δ. Because there was no equivalent evidence for synthetic phenotypes with the other S phase mutants we tested, and because the interaction was apparent with genes encoding two different polymerase δ subunits, we believe that this reflects a specific, although not necessarily direct, interaction. We note that strains with null or conditional alleles of *cdc19*$^+$ are all able to undergo DNA synthesis, although it may be delayed (FORSBURG and NURSE 1994b), and this is similar to the phenotypes reported for cells with mutations in *cdc1*$^+$ or *polδ*$^+$ (FRANCESCONI *et al.* 1993; MACNEILL *et al.* 1996).

A double mutant *cdc18-K46 cdc19-P1* had a less dramatic but still detectable synthetic phenotype, in which its restrictive temperature was reduced relative to either parent. *cdc18-K46* is a partly functional allele of an essential initiator of DNA replication (KELLY *et al.* 1993). Association of MCM proteins with chromatin in Xenopus has been shown to require the activity of Cdc18p (ROMANOWSKI *et al.* 1996; ROWLES *et al.* 1996), which is consistent with the genetic interaction we observe.

We initiated a structural analysis of the Cdc19p pro-

tein and constructed a series of point mutations and sequence deletions across the *cdc19*$^+$ open reading frame. We also cloned the temperature-sensitive allele *cdc19-P1* and the cold-sensitive allele *nda1-KM376* and found that both of these conditional mutations affect conserved residues. Not surprisingly, this analysis shows that the conserved MCM core sequence is required for function, as measured by the ability of the mutants to complement *cdc19-P1* or Δ*cdc19* strains. The cold-sensitive mutation falls in this domain.

Only one mutation in a conserved residue, M7 (K540R), was able to complement *cdc19-P1* and Δ*cdc19*, although a nonconserved change M6 (K540A) showed that this residue is important for function. It has been proposed that K540 corresponds to the crucial lysine in the Walker A motif in a putative ATPase domain of MCM proteins (WALKER *et al.* 1982; KOONIN 1993). However, mutation of the corresponding K → R in ATPases usually abolishes ATPase activity, although it does not affect nucleotide binding or protein multimerization (*e.g.*, LAURENT *et al.* 1992; KRAUSE *et al.* 1994; MA *et al.* 1994; SINGH and MAURIZI 1994; WHITEHEART *et al.* 1994; RIKKONEN 1996; YAHRAUS *et al.* 1996). Since M7 (K540R) is able to complement Δ*cdc19*, it seems unlikely that this Cdc19p domain functions as a bona fide ATPase.

Outside of the MCM core homology, only the mutations affecting the short basic stretches typical of NLS elements, or the zinc finger, abolished Cdc19p activity. The temperature-sensitive allele falls in between the acidic domain and the zinc finger. That the zinc finger is essential for Cdc19p function is consistent with previous analysis of the budding yeast MCM2p (YAN *et al.* 1991). We find that a double point mutation NLS1, a triple mutation including NLS2, or a deletion that spans acidic regions and NLS2, all render Cdc19p inactive. Interestingly, both putative NLS elements did not have to be mutated to affect complementation. It will be of interest to determine whether these sequences do function as bona fide translocation sequences, or whether these short blocks of basic residues play another role in protein function. The lengthy acidic region between the NLSes was flexible in that fairly large deletions could be constructed with no phenotype.

In a number of our experiments, we found that *cdc19-P1* at the semi-permissive temperature of 32° was extremely sensitive to overproduction of different MCM proteins. Overproduction of either Nda4p, Cdc21p, or Mis5p was toxic in this strain compared to wild type. Work in budding yeast has identified similar sensitivities among *mcm2* mutants to overexpression of other members of the family (YAN *et al.* 1991). This is not a general sensitivity to increased dosage of replication factors: we found no evidence for *cdc19-P1* sensitivity to overproduction of either *pol*δ$^+$ or *cdc18*$^+$, both under control of the same highly active *nmt* promoter. The term "synthetic dosage lethality" has been used to describe the lethal interaction defined by overproduction of wild-type genes in specific mutant backgrounds (KROLL *et al.* 1996). Such a phenotype may represent direct molecular interactions. For example, overproduction of the fission yeast cyclin Puc1p in the B-cyclin mutant strain *cdc13-117* is lethal even at the permissive temperature, apparently because high levels of Puc1p outcompete the mutant Cdc13p for binding to the p34^{cdc2} protein kinase (FORSBURG and NURSE 1991, 1994a).

The sensitivity of *cdc19-P1* to manipulation of MCM protein dosage is consistent with observations from other systems that MCM proteins form higher order complexes with one another (CHONG *et al.* 1995; KUBOTA *et al.* 1995; MUSAHL *et al.* 1995; STARBORG *et al.* 1995; KRUDE *et al.* 1996; LEI *et al.* 1996; OKISHIO *et al.* 1996; SU *et al.* 1996). Our results further suggest that the MCM core homology domain is central to this phenotype, because nonfunctional versions of Cdc19p containing an intact MCM domain were severely toxic when overproduced in *cdc19-P1* cells. In contrast, most mutants with an intact N-terminus but mutated MCM core domain did not have a toxic effect. This suggests that the intact MCM domain may titrate away necessary protein factors from an active complex. Thus, we propose that the MCM domain defines a potential interaction region of a dosage-sensitive MCM complex. It could plausibly act as a nucleotide-dependent interaction domain as seen for AAA ATPases and the *Escherichia coli* ClpA protein (*e.g.*, KRAUSE *et al.* 1994; SINGH and MAURIZI 1994; WHITEHEART *et al.* 1994). We are further investigating this possibility.

All mutants in *cdc19* that were able to rescue *cdc19-P1* were also able to complement the null Δ*cdc19* when overproduced by the *nmt* promoter in the absence of thiamine. When shifted to thiamine containing media, which represses the promoter, the Δ*cdc19* cells grew slowly at first but became established with prolonged incubation. These Δ*cdc19* cells all expressed more episomal Cdc19p protein in the presence of thiamine than was produced by wild-type cells containing the same plasmids. This suggests that low levels of Cdc19p are not sufficient for viability. Because establishing growth of the complemented Δ*cdc19* strain in low thiamine conditions took several steps, an apparent genetic selection for the upregulation is occurring. The nature of this up-regulation is not clear. It could involve an increase in plasmid copy number, or an increase in promoter activity. This suggests that there is a threshold level of Cdc19p in fission yeast, just as has been suggested for budding yeast (LEI *et al.* 1996). Although cells tolerate increases in Cdc19p levels, they are extremely sensitive to reductions. This is consistent with the synthetic dosage lethality discussed above, again arguing that MCM stoichiometry plays an important role in the function of the MCM protein complex.

The original genetic analysis in the yeasts suggested that MCM proteins function very early in the cell cycle

and might influence origin specificity (NASMYTH and NURSE 1981; MAINE *et al.* 1984; HENNESSY *et al.* 1990). As well as forming large complexes, it has been shown that MCM proteins associate with the chromatin before S phase and are removed as S phase proceeds (THÖMMES *et al.* 1992; YAN *et al.* 1993; KIMURA *et al.* 1994; CHONG *et al.* 1995; KUBOTA *et al.* 1995; MADINE *et al.* 1995b; STARBORG *et al.* 1995; TODOROV *et al.* 1995; KRUDE *et al.* 1996; SCHULTE *et al.* 1996). Chromatin association appears to require the activity of Cdc18p, Orc1p, and Orc2p (CARPENTER *et al.* 1996; COLEMAN *et al.* 1996; ROMANOWSKI *et al.* 1996; ROWLES *et al.* 1996). Interestingly, a recent report suggests some MCM proteins associate with histones (ISHIMI *et al.* 1996). A plausible model taking all these observations into account suggests that MCM proteins load onto the DNA at the replication origins via their interactions with ORC and Cdc18p. They then spread out along the unreplicated chromatin in protein complexes that enable replication, perhaps by directly modifying chromatin structure. The sensitivity of *cdc19-P1* cells to manipulation of the levels of other MCM proteins offers further support for a dosage-dependent structural complex. A subset of MCM proteins could affect replication elongation rather than, or in addition to, initiation. It is possible that MCM complexes on the chromatin might play an additional role restraining DNA synthesis until some triggering signal is received. Using the reagents described in this paper, we now have the molecular and genetic tools necessary to test this model rigorously and determine the molecular function of these highly conserved and important proteins.

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