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The *IML3/MCM19* gene of *Saccharomyces cerevisiae* is required for a kinetochore-related process during chromosome segregation

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Abstract The *mcm19* mutation in budding yeast affects minichromosome maintenance. In this work we have shown that this mutation leads to defects in the segregation of minichromosomes and chromosomes. The mutant cells show defective kinetochore function as judged by three criteria – relaxation of the transcriptional block normally associated with a *CEN* box, stable maintenance of a dicentric plasmid in mutant cells, and mild sensitivity to the antimicrotubule drug benomyl. The *MCM19* gene has been cloned and found to be the same as *IML3*, which codes for the ORF YBR107C. Deletion of the gene was not lethal, nor did it confer any growth defects on the mutant cells. However, the *mcm19* null mutation conferred growth defects in the presence of a mutation in the *TUB1* gene coding for alpha-tubulin. Two-hybrid experiments showed an interaction between Iml3p/Mcm19p and the kinetochore protein Chl4, indicating that the Iml3/Mcm19 protein has a role in kinetochore function.

Key words *iml* · *mcm* · Chromosome segregation · Kinetochores · Yeast

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Introduction

Faithful replication and segregation of chromosomes are two major events of a successful cell division cycle. If either of these two events fails to occur with high fidelity, the result may be cell cycle arrest, propagation of aneuploidy or even cell death. The budding yeast *Saccharomyces cerevisiae* is a model eukaryote for a study of both these processes. The centromere of a chromosome is the site where proteins assemble to form a kinetochore complex. This complex is responsible for capturing microtubules, allowing the sister chromatids to get attached to microtubules coming from opposite spindle poles. When a kinetochore fails to attach to the microtubule or forms weak interactions with it, the chromatids may fail to move to opposite poles and chromosome non-disjunction occurs, leading to the loss of the chromosome from one of the daughter cells and gain in the other. Therefore, cells which exhibit chromosome non-disjunction could carry mutations in genes which are required for the proper functioning of the kinetochores.

In the budding yeast, the centromere consists of about 125 base pairs of DNA divided into three tandem boxes – CDE I, CDE II and CDE III (reviewed in Hyman and Sorger 1995; Hoyt and Geiser 1996; Lechner and Ortiz 1996; Skibbens and Hieter 1998). CDE I consists of an 8 base-pair sequence which is conserved between different centromeres. Cbf1/Cpf1/Cep1 protein binds to CDE I. The 78 to 86 base-pair AT-rich sequence forms the element CDE II that lies between CDE I and CDE III. The length and the AT-richness of CDE II are important for centromere activity. Cse4p, a variant of histone H3, shows genetic interactions with CDE II and is suggested to form a specialized nucleosome at this site (Meluh et al. 1998; Ortiz et al. 1999). Another protein, Mif2p, is suggested to bind to CDE II because of its A-T hook motif, present in several proteins which bind to AT-rich DNA (Brown 1995; Meluh and Koshland 1997). The CBF3 protein complex consisting of Cbf3a (Ndc10p/Cbf2p/

Ctf14p), Cbf3b (Cep3p), Cbf3c (Ctf13p) and Cbf3d (Skp1p) binds to the CDE III box (reviewed in Hyman and Sorger 1995; Hoyt and Geiser 1996; Lechner and Ortiz 1996; Skibbens and Hieter 1998; Pidoux and Allshire 2000). A complex of proteins, Ctf19, Okp1 and Mcm21, has also been localized to the kinetochore (Ortiz et al. 1999). This complex is suggested to link proteins or their subcomplexes at the three CDE boxes by interactions to stabilize kinetochore structure (Ortiz et al. 1999). Other proteins which have been localized to the kinetochore are Slk19p (Zeng et al. 1999) and Mtw1p (Goshima and Yanagida 2000). Models for the assembly of a kinetochore have been described which are based upon the available information on genetic and physical interactions between these proteins (Meluh and Koshland 1997; Meluh et al. 1998; Ortiz et al. 1999; Pidoux and Allshire 2000). Since all the mutants that affect chromosome segregation, obtained from different screens (reviewed in Hyman and Sorger 1995), have not been analyzed, more kinetochore proteins are likely to be discovered and these models may be further modified.

We have been studying a subset of *mcm* mutants which were isolated as affecting minichromosome maintenance. The minichromosome loss phenotype of this class of mutants is not dependent on the *ARS* cloned on the minichromosome (*ARS*-nonspecific mutants, Maine et al. 1984a). We have already shown that *MCM16*, *CHL4*/*MCM17*/*CTF17*, *CTF19*/*MCM18*, *MCM21* and *MCM22* are all required for kinetochore-related segregation process of a chromosome (Kouprina et al. 1993; Roy et al. 1997; Sanyal et al. 1998; Hyland et al. 1999; Ortiz et al. 1999; Poddar et al. 1999; K. Sanyal, unpublished observations). Chl4/Mcm17, Ctf19/Mcm18 and Mcm21 proteins have been localized to the kinetochore (Hyland et al. 1999; Ortiz et al. 1999; K. Bloom, personal communication). Therefore, a continued study of these genes should help to further refine the *S. cerevisiae* kinetochore structure and assembly.

In this work we show that *MCM19* is the same as *IML3*, a gene that codes for the ORF YBR107C and is required for minichromosome maintenance (Entian et al. 1999). We have done more detailed studies to show that a mutation in *IML3*/*MCM19* causes segregation defects of minichromosomes and chromosomes and that this gene is required for kinetochore function.

Materials and methods

Materials

All media, chemicals and enzymes etc. have been described before (Sanyal et al. 1998; Poddar et al. 1999).

Strains and plasmids

The strains and plasmids used are listed in Table 1. In addition to these, YCTF30 [*MAT*α *ctf13-30 ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 CFIII* (*CEN3.L*) *URA3 SUP11*], YPH1172 [*MAT***a** *skp1-Δ1::TRP1 skp1-3::LEU2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII* (*CEN3.L*) *HIS3 SUP11*], YPH1161 [*MAT***a** *skp1-Δ1::TRP1 skp1-4::LEU2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CFIII* (*CEN3.L*) *HIS3 SUP11*] were obtained from P. Hieter. The strains were cured of the chromosome fragments before being used. PWY611 (*MAT***a** *ndc10-1 leu2 ura3 trp1 his3*) was obtained from J. Kilmartin. 1cAS281 (*MAT***a** *cep3-1 ade2 his3 leu2 lys2 trp1 ura3*) and 3dAS282 (*MAT***a** *cep3-2 ade2 his3 leu2 lys2 trp1 ura3*) were from D. Koshland. For synthetic lethal studies the following double mutants were constructed. The M31/6A (*mcm19-1*) strain was deleted for *MCM16*, *MCM17*, *MCM21* and *MCM22* to generate *mcm16-Δ1 mcm19-1, mcm17-*Δ1 *mcm19-1, mcm19-1 mcm21-*Δ1 and *mcm19-1 mcm22-Δ1*. The *MCM19* gene was deleted in *mcm18-1*, yielding *mcm18-1 mcm19-Δ1*. When deletions/disruptions were constructed, the *mcm* mutation of the host was always covered by a wild-type copy of this gene on a plasmid. For example, when the *mcm19-1* mutant was being screened for disruption of the *MCM16* gene using *URA3* selection, the *mcm19* host mutation was covered by the wild-type *MCM19* gene on a *LEU2* plasmid. *MCM19* was deleted in the strains YCTF30 (*ctf13-30*), PWY611 (*ndc10-1*) and AP27 (*tub1-1*) strains, yielding *ctf13-30 mcm19-Δ1*, *ndc10-1 mcm19-Δ1* and *tub1–1 mcm19-Δ1* strains, respectively. In this case each of the host strains was protected by an *MCM19* gene on a *LEU2*-carrying minichromosome while the disruption strain was being constructed using *URA3* selection. The presence of both mutations in each double mutant strain was confirmed by complementation analysis. To create the double mutant *cep3-1 mcm19-Δ1*, 1cAS281 (*cep3-1*) was crossed with 301-2BΔ19 (*mcm19-Δ1::URA3*). The diploid was then sporulated. Ura^+ spores which were temperature-sensitive for growth (due to *cep3-1*) were assumed to be double mutants. Similarly, each of the mutant strains 3dAS282 (*cep3-2*), YPH1172 (*skp1-3*) and YPH1161 (*skp1-4*), was crossed to 301-2BΔ19 to obtain *cep3-2 mcm19-Δ1*, *skp1-3 mcm19-Δ1* and *skp1-4 mcm19-Δ1*, respectively.

Plasmids were also constructed for two-hybrid studies. Each *MCM* ORF was isolated by PCR and fused in frame with the Gal4 DNA-binding domain (BD) in pGBT9 and with the Gal4 activation domain (AD) in pGAD424 (Bartel et al. 1993). A *Bam*HI site was designed in the ATG primer (the primer used to amplify the ORF from its first codon), so that the *Bam*HI site of pGBT9 or pGAD424 could be used for fusing the ORF in frame with the binding or activation domains of these plasmids. For *CHL4*/*MCM17*, the plasmid pGK8, carrying the *CHL4*/*MCM17* gene (Roy et al. 1997), was used as the template to amplify this ORF using the ATG primer PS4 [5′-CGCGGATCCCGATGTC-TAACGAATTACGGC-3′, which introduces a *Bam*HI site (underlined) and contains the first 19 nt of the *CHL4* ORF, starting from the ATG) and the primer PS5 (5′-CCGGCATGCCATCA-CATC-3′], which is complementary to the 3′ end, 205 nt beyond the stop codon and introduces a *Sph*I site). The 1.6-kb amplified fragment was digested with *Bam*HI and *Sph*I and ligated to pBR322 digested with the same enzymes. The resulting plasmid was digested with *Bam*HI and *Sal*I, and the 1.7-kb fragment carrying the *CHL4* gene was ligated to pGBT9 and pGAD424, each digested with *Bam*HI and *Sal*I. This procedure yielded the plasmids BD-Chl4 and AD-Chl4, respectively. PCR was used to amplify the coding sequence of *MCM19* with pM31-2 (the 2-kb *Pst*I-*Pvu*II fragment containing the *MCM19* gene cloned in YIplac211; see below) as the template. The primers were PS12 (5′-CGGGATCCCGATGCCTTATACTTGGAAGTTTTT-3′, introducing a *Bam*HI site and containing the coding sequence beginning with the ATG) and an M13/pUC reverse 16mer sequencing primer (−21). The 1.2-kb *Bam*HI-*Pst*I fragment of the PCR product was ligated to pGBT9 and pGAD424, each digested with *Bam*HI and *Pst*I. This generated BD-Mcm19 and AD-Mcm19, respectively. All other *MCM* genes were amplified similarly, using the ATG primer with the introduced *Bam*HI site to fuse AD and BD in frame with the ORF. For the 3′ end of the gene, either an M13/pUC reverse or forward primer was used, depending upon the orientation of the cloned *MCM* ORF within the MCSs (multiple cloning sites) of the pUC19-based yeast vectors (Gietz and Sugino 1988). In all the cases, except *CTF19*/*MCM18*, the fusion proteins retained their

Table 1 List of strains and plasmids

Strain/plasmid	Genotype/relevant markers	Source/reference
Strains:		
A3	*MAT***a** *leu2-3,112 his3-11,15*	Maine et al. (1984a)
301-2B	*MATα leu2-3, 112 ura3-52 his4Δ34 trp1*	Maine et al. (1984a)
8534-10A	*MAT***a** *leu2-3,112 ura3-52 his4Δ34*	Maiti and Sinha (1992)
SR14	*MATα ade2 can*^{r} *ura3*	B.-K. Tye
AP13	*MAT***a** *leu2 his4 ura3 ade2*	From 8534-10A × SR14 (this study)
AB1380	*MAT***a** *ade2 trp1 ura3 his5 can1 lys2*	Burke et al. (1987)
PJ69-4A	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al. (1996)
M29/5D	*MAT***a** *leu2-3, 112 ura3-52 his4Δ34 trp1 mcm18-1*	From 301-2B × PS29-2B (this study)
PS31-5A	*MAT***a** *leu2-3, 112 his3-11, mcm19-1*	From A3 × Mcm131C-31 (Maine et al. 1984a)
M31/6A	*MAT***a** *leu2-3,112 ura3-52 trp1 his4Δ34 mcm19-1*	From 301-2B × PS31-5A (this study)
301-2BΔ19	*MATα leu2-3, 112 ura3-52 his4Δ34 trp1 mcm19-Δ1::URA3*	From 301-2B (this study)
AB1380Δ19	*MAT***a** *ade2 trp1 ura3 his5 can1 lys2 mcm19-Δ1::URA3*	From AB1380 (this study)
DBY2519	*MATα lys2-801 ura3-52 tub1-1*	D. Botstein
AP27	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 tub1-1*	From DBY2519 × A3
Plasmids		
YCp121	*CEN5/ARS121/LEU2*	Maine et al. (1984a)
YCp1′	*CEN5/ARS1/URA3*	Roy et al. (1997)
YCp50	*CEN4/ARS1/URA3*	Rose et al. (1987)
YCp50-5	*CEN4/CEN5/ARS1/URA3*	Sanyal et al. (1998)
YIplac204	*TRP1*	Gietz and Sugino (1988)
YIplac211	*URA3*	Gietz and Sugino (1988)
YCplac33	*CEN4/ARS1/URA3*	Gietz and Sugino (1988)
YCplac111	*CEN4/ARS1/LEU2*	Gietz and Sugino (1988)
pUC19U	*URA3* in pUC19	Sanyal et al. (1998)
pKF71	*GAL1-10*-actin-*CEN6-lacZ/HIS3*	Doheny et al. (1993)
pKF72	*GAL1-10*-actin-*CEN6* (CDEIII-15C)-*lacZ/HIS3*	Doheny et al. (1993)

MCM activities. The *MCM18* ORF was amplified in three independent PCRs. However, none of these fragments retained its *MCM* activity when fused with either AD or BD. Nevertheless, the BD-Mcm18 fusion protein retained the ability to interact with AD-Mcm21p and activate the transcription of all the reporter genes and so was included in our studies.

Cloning of *MCM19*

The wild-type *MCM19* gene was cloned using the same strategy as described for the cloning of the other *MCM* genes (Roy et al. 1997; Sanyal et al. 1998; Poddar et al. 1999). The *mcm19* mutant strain M31/6A, carrying the minichromosome YCp1′, was further transformed with a genomic DNA library cloned on a *LEU2/ARS1/CEN4* vector (Gerring et al. 1990) and the transformants were selected on minimal plates selective for the maintenance of both the minichromosomes. Faster-growing colonies were expected to carry the wild-type *MCM19* gene that would stabilize YCp1′, resulting in faster growth (Roy et al. 1997). Out of 9000 transformants, three were found to carry yeast DNA clones on the *LEU2* (library) vector which stabilized YCp1′. Recovery of the *LEU2* plasmids in *E. coli* and restriction digestion of the clones showed that all of these carried overlapping inserts. A 3-kb *Pst*I fragment common to all was cloned into YCplac33 to obtain pM31-1. This plasmid was found to rescue the *mcm19* phenotype. A partial sequence (160 bp) of the insert was used as a query sequence in a BLAST search for homology (Altschul et al. 1990) within the complete *Saccharomyces* genome (*Saccharomyces* Genome Database at Stanford University). The sequence showed 100% identity within a 2.97-kb *Pst*I fragment retrieved from the database. A 2-kb *Pst*I-*Pvu*II fragment within the clone (Fig. 1) had the same restriction map as the retrieved DNA sequence. This fragment complemented the *mcm19* defect. Only one complete 245-amino acid ORF, YBR107C, was found in this fragment, and was cloned into the *Pst*I/*Sma*I site, generating pM31-2. pM31-2 was digested with *Cla*I, which cleaves within the ORF and integrated into M31/6A (*mcm19*). Southern analysis confirmed integration at the chromosomal site of the cloned gene (not shown). The integrant was crossed to the wild-type strain, the diploid sporulated and the mitotic stability of YCplac111 was measured in sixty randomly picked spores. All except two spores showed wild-type stability, suggesting that the cloned gene was very tightly linked to the *mcm19* mutation and was very probably *MCM19*. This was confirmed as described below.

A deletion-disruption was made in the ORF on the plasmid by cloning a 1.4-kb *URA3*-carrying *Sal*I-*Sma*I fragment from pUC19U (Sanyal et al. 1998) into the *Sal*I-*Cla*I (end-filled) sites of pM31-2 to obtain pM31-3. The 2.6-kb *Sph*I-*Eco*RI fragment of pM31-3, carrying the deleted and disrupted YBR107C was used to transform the wild-type diploid 301-2B × 8534-10A and the haploid 301-2B. Southern analysis of the transformants confirmed the presence of the deletion in both strains (not shown). The diploid was sporulated and about 50% of the randomly picked spores were found to be Ura$^+$. Therefore, cells carrying a deletion of YBR107C are viable. The Ura$^+$ spores displayed a minichromosome maintenance (*mcm*) phenotype. The haploid transformant also had an *mcm* phenotype. This *mcm* mutation, created by the deletion of the ORF YBR107C on the cloned fragment, was recessive to the wild-type and failed to complement the original *mcm19-1* mutation. This result further confirms that YBR107C is the same gene as *MCM19*

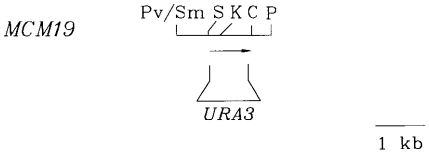

Fig. 1 Restriction map of the complementing fragment containing the *MCM19* gene. The *arrow* indicates the position of the ORF and the direction of transcription. The disruption-deletion of the gene by *URA3* is shown *below* the ORF. The restriction sites indicated are; C, *Cla*I; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sm, *Sma*I

(Lundblad 1995). While this work was in progress, Entian et al. (1999) independently showed that YBR107C was required for minichromosome maintenance and called it *IML3*, for *i*ncreased *m*inichromosome *l*oss. Therefore, the name *IML3* has precedence over *MCM19*.

Assay for loss of chromosome III

The loss of chromosome III was monitored in isogenic wild-type and mutant diploids, 301-2B × AB1380 and 301-2BΔ19 × AB1380Δ19, respectively, using the rationale of Gerring et al. (1990). Each of the diploids was heterozygous for the *LEU2* gene. The haploid tester strain was AP13 (*MAT***a** *leu2 ade2*). Six to seven colonies were assayed independently for chromosome loss and recombination as described in Poddar et al. (1999).

Transcriptional read-through assays

Transcriptional read-through assays were performed as described before (Poddar et al. 1999). The *GAL1-10*-actin-*CEN6-lacZ* cassette carrying the wild-type centromere was excised from pKF71 and integrated into A3 (wild-type) and PS31-5A (*mcm19*) strains using the *HIS3* homology. Similarly, this cassette, carrying the mutant centromere, was excised from pKF72 and integrated into A3 and PS31-5A. The assays for β-galactosidase were done by the standard method.

Two hybrid studies

The plasmids pGBT9 and pGAD424 with or without the *MCM* gene fusions were transformed into the strain PJ69-4A, which carries the reporter genes *GAL1-HIS3*, *GAL2-ADE2* and *GAL7-lacZ* (James et al. 1996). Interaction between two Mcm proteins, say McmX and McmY, was assumed to have occurred if the transformant carrying both BD-McmX and AD-McmY could grow on minimal plates lacking histidine or adenine and synthesized β-galactosidase at levels significantly above those of control transformants.

All other methods have been described previously (Sanyal et al. 1998; Poddar et al. 1999).

Results

The *mcm19* mutation causes minichromosome segregation defect.

The *mcm19-1* mutation causes the loss of minichromosomes (Maine et al. 1984a). This could be either due to defective replication or to defective segregation of the minichromosome (Sinha et al. 1986). If the replicated copies of a minichromosome fail to disjoin and both the copies move into one of the two daughter cells, the average copy number of the minichromosome would increase to more than one in cells carrying the minichromosome. This phenotype is, therefore, suggestive of a defect in minichromosome segregation. The copy number of a minichromosome, YCp121, was measured in the *mcm19* mutant and in wild-type cells. In the mutant the minichromosome was found to be present in high copy numbers in those cells which carried it (Fig. 2). This indicates a defect in the segregation of the minichromosome.

The rate of chromosome loss is elevated in *mcm19* cells

To determine the effect of the *mcm19* mutation on the transmission of native chromosomes, the mutant cells were studied to determine the rates of chromosome loss and genetic recombination. The rate of loss of chromosome III was measured as described under Materials and methods. The *mcm19* diploid lost chromosome III at a frequency of $1.4(\pm 0.7) \times 10^{-3}$, which was about fifty-fold elevated over the wild-type frequency $3(\pm 2) \times 10^{-5}$. The recombination frequencies in the mutant and wild-type diploids were not very different, being $5(\pm 4) \times 10^{-5}$ and $7(\pm 1) \times 10^{-5}$, respectively. A mutation which leads to an increase in the frequency of chromosome loss, but not that of recombination, is considered to affect chromosome segregation but not its replication (Hartwell and Smith 1985). By this criterion, the *mcm19* mutation causes chromosome loss by affecting chromosome segregation.

The *mcm19* mutant causes kinetochore-related defects

At least three phenotypes have been associated with mutations which affect kinetochore function. A mutant affected in kinetochore function may exhibit stable maintenance of a dicentric plasmid relative to that in the wild-type strain (Doheny et al. 1993). The rationale for this is that a kinetochore mutation may weaken kinetochore-microtubule interactions. The links between

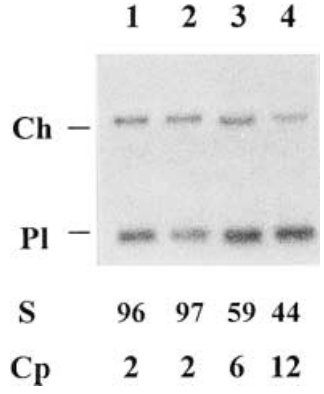

Fig. 2 Copy number of YCp121 in plasmid-bearing cells of the wild-type (301-2B) and mutant M31/6A (*mcm19*) strains. The transformants were grown to late log phase in minimal medium lacking leucine. Total DNA was isolated from the transformants, digested with *Bam*HI, fractionated on a 0.7% agarose gel and blotted onto a nylon membrane. The blot was probed with a ^{32}P-labelled 1.5-kb fragment of *CEN5* DNA that hybridized to a 3.9-kb fragment from chromosome V and a 1.5-kb fragment from the plasmid (Maine et al. 1984b). Ch indicates the chromosomal band and Pl the band obtained from the plasmid. The percentage stability (S) of the plasmid in the transformant culture at the time of DNA isolation is given *below* each lane. Cp indicates the copy number of YCp121 in plasmid-carrying cells. Lanes: 1 and 2, YCp121 from two independent transformants of the wild-type strain; 3 and 4, YCp121 from two independent transformants of the mutant strain

Table 2 Mitotic stabilities of monocentric and dicentric plasmids in wild-type and *mcm19* strains

Strain	Mitotic stability (%)[a]	
	YCp50	YCp50-5
MCM	70 ± 7	4–80
mcm19	57 ± 6	20 ± 5

[a]The mitotic stability of the indicated plasmids was determined in ten or more independent transformants of each strain, grown on selective medium at 28°C. Errors are standard deviations from the mean value in each case

kinetochore and microtubules may break down if a chromatid experiences opposing forces of movement during anaphase, if its two kinetochores get attached to opposite poles. This would preserve the integrity of the minichromosome DNA. The second phenotype results from the observation that centromere DNA normally serves as a block to transcription. If the kinetochore assembly is altered, the block could be relieved and more transcription may be allowed through the centromere (Doheny et al. 1993). The third phenotype exhibited by the kinetochore mutants is sensitivity towards antimicrotubule drugs like nocodazole and benomyl. Wang and Burke (1995) have shown that low levels of nocodazole impair kinetochore-microtubule interactions. Therefore, a pre-existing defect in kinetochore function is likely to render the mutant cells more sensitive to such drugs than are wild-type cells, as already shown for *mcm16*, *mcm21*, *mcm22* and *ctf19*/*mcm18* mutations (Sanyal 1998; Hyland et al. 1999; Poddar et al. 1999). The *mcm19* mutant was tested for these three phenotypes as described below.

A dicentric plasmid is stably maintained in the *mcm19* mutant

YCp50-5 is a dicentric plasmid which was constructed by the cloning of *CEN5* into the monocentric plasmid YCp50 (Sanyal et al. 1998). Both these plasmids were used to transform the wild-type and the mutant strains. YCp50-5 showed a wide range of mitotic stability in the wild-type strain (Table 2), resulting in heterogeneity in the size of the transformant colonies on selective medium (Fig. 3a). This is characteristic of structural instability and re-arrangements of plasmid DNA (Mann and Davis 1983; Hill and Bloom 1987; Koshland et al. 1987; Kouprina et al. 1993). The transformants of this strain carrying the monocentric plasmid YCp50 produced colonies which were healthy and more homogenous in size. In contrast, the *mcm19* mutant transformed with YCp50-5 gave rise to colonies which were more or less uniform in size, indicating that this plasmid was not undergoing structural re-arrangements. The mitotic stability of this plasmid also did not vary as much as in the wild-type (Table 2).

YCp50-5 DNA was recovered into *E. coli* from ten independent transformants of both mutant and wild-type strains. The plasmid DNA was digested with *Pst*I and

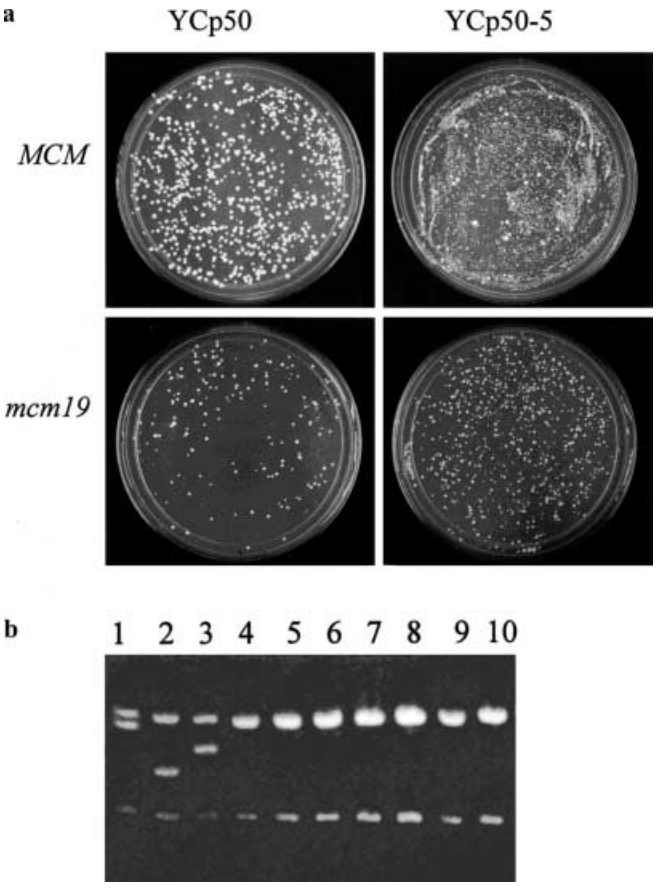

Fig. 3a, b Dicentric plasmids are stably maintained in the mutant M31/6A (*mcm19*) strain. **a** The growth of mutant and wild-type transformants on selective medium (minimal medium lacking uracil). The petri dishes were incubated at 28°C for 3 days. **b** The dicentric plasmid does not undergo any structural re-arrangements in the mutant. YCp50-5 DNA was recovered into *E. coli* from independent transformants of the wild-type and the mutant strains. The DNA was digested with *Pst*I and fractionated on an agarose gel. The *Pst*I digest of wild-type YCp50-5 shows three bands, two of which co-migrate at 3.8 kb and one at 1.8 kb (Sanyal et al. 1998). Lanes: 1–3, YCp50-5 DNA recovered from three independent wild-type transformants; 4–9, YCp50-5 DNA recovered from six independent transformants of the mutant strain; 10, original (wild-type) YCp50-5 DNA used for transformations

fractionated on an agarose gel. YCp50-5 plasmids recovered from all ten wild-type transformants showed structural re-arrangements and deletions. In contrast, DNA recovered from mutant transformants did not show any re-arrangements, implying that a dicentric plasmid is stably maintained in the *mcm19* mutant. Figure 3b shows these results for a subset of the recovered plasmids.

The transcriptional block associated with the *CEN* box is relaxed in the *mcm19* mutant

In this assay the *CEN* box from centromere 6 is placed between the *GAL1-10* promoter and a reporter gene *lacZ* (Doheny et al. 1993). As the *CEN* box offers resistance to transcription, low levels of β-galactosidase

are produced in the wild-type strain. If the *CEN* box carries a mutation, or if the strain has a mutation that affects kinetochore assembly, this block may be relaxed, leading to higher levels of β-galactosidase activity. *GAL1-10*-actin-*CEN6-lacZ* cassettes from pKF71 (carrying a wild-type *CEN* box) and pKF72 (carrying a mutant *CEN* box) were integrated in the wild-type and mutant strains. The transformants were selected on minimal (galactose + raffinose) plates lacking histidine. The enzyme levels were assayed in these transformants. The wild-type strain produced about six-fold lower levels of β-galactosidase in pKF71 transformants as compared to those produced in pKF72 transformants (1.8 ± 0.7 and 10.3 ± 2.1 units, respectively). In the case of the mutant strain, pKF71 transformants produced levels of enzyme activity (8.5 ± 1.2 units) which were only two-fold lower than those produced by pKF72 transformants (18.5 ± 2.0 units), suggesting that the kinetochore-complex in mutant pKF71 transformants was partially altered, thus allowing more transcription through it.

Benomyl sensitivity

We also tested the sensitivity of the *mcm19* mutant strain to benomyl. Figure 4 shows that the mutant strain was more sensitive than the wild-type at a concentration of 10 μg/ml of this drug. This observation is consistent with the *mcm19* mutation affecting kinetochore function.

Cloning of the *MCM19* gene and the phenotype of the null mutant

The wild-type *MCM19* gene was cloned as described in Materials and methods. The sequence predicted for the protein product of this gene (245 amino acids) did not show significant homology to any known protein in the database. A weak similarity to chitin synthase 3 of *Neurospoa crassa* (30% identity and 48% similarity in a stretch of 53 amino acids between residues 141 and 193) was found in a search with BLAST 2.0. However, chitin synthases are a group of highly homologous proteins. Therefore, this limited homology may not be of any functional significance. Deletion of the gene was not lethal to the cells nor did it significantly alter the growth rate or cell morphology of the mutant strain as compared to the wild-type at 37, 28 and 18°C (not shown). The *MCM19* gene, therefore, is not essential for growth of cells but is essential for high-fidelity chromosome transmission.

The *mcm19* deletion shows a synthetic interaction with *tub1-1*

We have so far described five *MCM* genes which are required for kinetochore function – *MCM16*, *CHL4*/*CTF17*/*MCM17*, *MCM19*, *MCM21* and *MCM22*. None of these is essential for growth. In addition, we have cloned the *MCM18* gene (K. Sanyal and P. Sinha, unpublished observations), which has been found to be the same as *CTF19*, a gene known to be required for kinetochore function (Hyland et al. 1999; Ortiz et al. 1999). We looked for synthetic interactions between *mcm19* and mutations in these *MCM* genes. Double mutant strains, each carrying *mcm19* and a mutation in one of the other *MCM* genes, were constructed (Materials and methods). The double mutants were tested for growth at 37, 28 and 18°C. In each case, the single mutants, the wild-type and the double mutant grew equally well, and showed no growth defects at these temperatures. Similarly, the *mcm19* deletion did not show any synthetic growth defects when combined with *cep3-1*, *cep3-2*, *ndc10-1*, *ctf13-30*, *skp1-3* or *skp1-4* – mutations in genes coding for kinetochore proteins. However, *mcm19-Δ1* was found to be synthetically lethal with *tub1-1*, a cold-sensitive mutation in the α-tubulin gene *TUB1* (Stearns and Botstein 1988). Cells carrying *tub1-1* fail to grow at 14°C, have short mitotic spindles and show super-sensitivity at 26°C to the antimicrotubule drug benomyl. This last phenotype suggests a defect in microtubule structure even at 26°C. The double mutant *mcm19-Δ1 tub1-1* failed to grow at 18°C. At this temperature both the single mutants could grow (Fig. 5). The fraction of large-budded cells in the double mutant culture, after 8 h of incubation at 18°C, was about 50%. Of these, about 70% had the nucleus at the bud neck (not shown). Under similar conditions, a culture of the isogenic parent strain AP27 (*tub1-1*) had 13% large-budded cells, of which about 40% had the nucleus at the bud neck. To check if the synthetic lethality was due to defects in microtubule structure caused by the *mcm19* deletion, the microtubules were visualized by indirect immunofluorescence, using antitubulin antibodies (Kilmartin and Adams 1984). The arrested cells of both the cultures had short, bipolar mitotic spindles and no differences could be observed in the lengths and numbers of

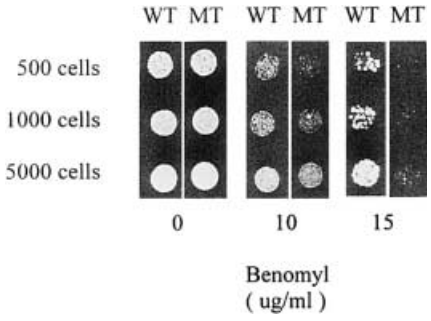

Fig. 4 The mutant strain 301-2BΔ19 (MT) is more sensitive to benomyl than the isogenic wild-type 301-2B (WT). Three dilutions of freshly growing cells were spotted onto YEPD plates containing benomyl at the indicated concentrations. The plates were incubated at 26°C. The plates containing 0 and 10 μg/ml benomyl were incubated for 3 days, while the one containing 15 μg/ml benomyl was incubated for 5 days

their cytoplasmic microtubules. Therefore, the simultaneous presence of the *mcm19* and *tub1-1* mutations could be causing synthetic lethality due to perturbations in kinetochore-microtubule interactions at 18°C, rather than by affecting the microtubule structure.

Mcm19 interacts with a kinetochore protein, Chl4/Mcm17

Two-hybrid assays (Materials and methods) were used to test for interaction of the Mcm19 protein with each of the other Mcm proteins (Mcm16, Chl4/Mcm17, Ctf19/Mcm18, Mcm21, Mcm22). At least three of these are currently known to be localized at the kinetochore (Hyland et al. 1999; Ortiz et al. 1999; K. Bloom, personal communications). Mcm19p showed a strong interaction only with the Chl4/Mcm17 protein (Fig. 6). Likewise, the Chl4p interacted only with Mcm19p. The levels of β-galactosidase expressed from the reporter gene *GAL7-lacZ* were also elevated by about 6- to 8-fold in the transformants carrying both the fusions, as compared to the transformants carrying only the control plasmids (Fig. 6). The levels of enzyme activity measured in AD-Chl4/BD-Mcm19 and AD-Mcm19/BD-Chl4 were 4.7 ± 0.8 and 3.6 ± 0.1 units, respectively (measured in six independent experiments each; errors are standard deviations). The enzyme activity measured in transformants carrying control plasmids varied from 0.5 to 0.6 units. Chl4p plays a role in kinetochore function and is localized at the kinetochore (Kouprina et al. 1993; Kroll et al. 1996; Roy et al. 1997; Shmelev et al. 1999; K. Bloom, personal communication). These results suggest that Iml3/Mcm19 and Chl4 proteins may form part of a subcomplex at the kinetochore required for its assembly. More extensive analyses are being carried out with other kinetochore proteins to further characterize the Iml3/Mcm19 and Chl4 complex in the kinetochore.

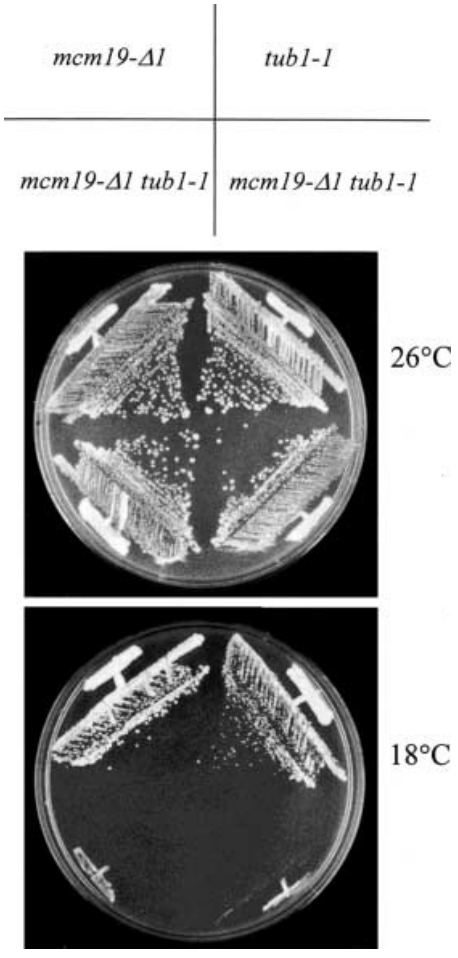

Fig. 5 mcm19-Δ*1* and *tub1-1* are synthetically lethal at 18°C. The *mcm19-Δ1* mutation was introduced into the AP27 (*tub1-1*) strain by transformation (Materials and methods). Two such transformants (*mcm19-Δ1 tub1-1*), and the single mutant strains 301-2BΔ19 (*mcm19-Δ1*) and AP27 (*tub1-1*), were each streaked for single colonies on YEPD plates at 26 and 18°C. The plates were incubated for 3 days at 26°C and for 5 days at 18°C

Discussion

In this work we have characterized yet another *ARS*-nonspecific *mcm* mutant carrying the *mcm19* mutation. The mutant showed defects in plasmid and chromosome segregation, could stably maintain a dicentric plasmid, and displayed enhanced transcription through a centromeric element, benomyl sensitivity and synthetic lethality of *mcm19-Δ1* and *tub1-1* mutations. Stabilization of a dicentric plasmid, together with relaxation of the normal block to transcription through the centromere are phenotypes associated with kinetochore mutants (Doheny et al. 1993; Hyman and Sorger 1996). Several kinetochore mutants, such as *ctf13*, *ndc10*/*ctf14*, *chl4*/*mcm17*/*ctf17*, *ctf19*/*mcm18* and *mcm21* exhibit these defects (Doheny et al. 1993; Kouprina et al. 1993; Hyland et al. 1999; Poddar et al. 1999; K. Sanyal, unpublished observations). Likewise, benomyl sensitivity is associated with several kinetochore mutants. Perhaps their kinetochores, being defective, are more sensitive to perturbations of kinetochore-microtubule interactions caused by these drugs (Wang and Burke 1995). We have observed that several mutants in which kinetochore-related functions are affected, such as *mcm16*, *mcm21* and *mcm22*, show synthetic lethality with *tub1-1* at 18°C (K. Sanyal, A. Poddar and P. Sinha, unpublished observations). This is not unexpected. A defect in microtubule structure, coupled with lesions in the kinetochore (as suggested by the relaxation of the centromeric transcriptional block), may indeed result in growth defects. We have also seen that the microtubule structure does not differ in the double mutants from that in the *tub1-1* strain (A. Poddar, K. Sanyal and S.K. Ghosh, unpublished observations). This suggests that the defect in *mcm19* is not microtubule related. Attempts were made to localize the Mcm19p or Mcm19p-Myc in yeast by indirect immunofluorescence, using antibodies against Mcm19 synthesized in, and purified from *E. coli*, or monoclonal antibodies against a c-Myc tag. We could

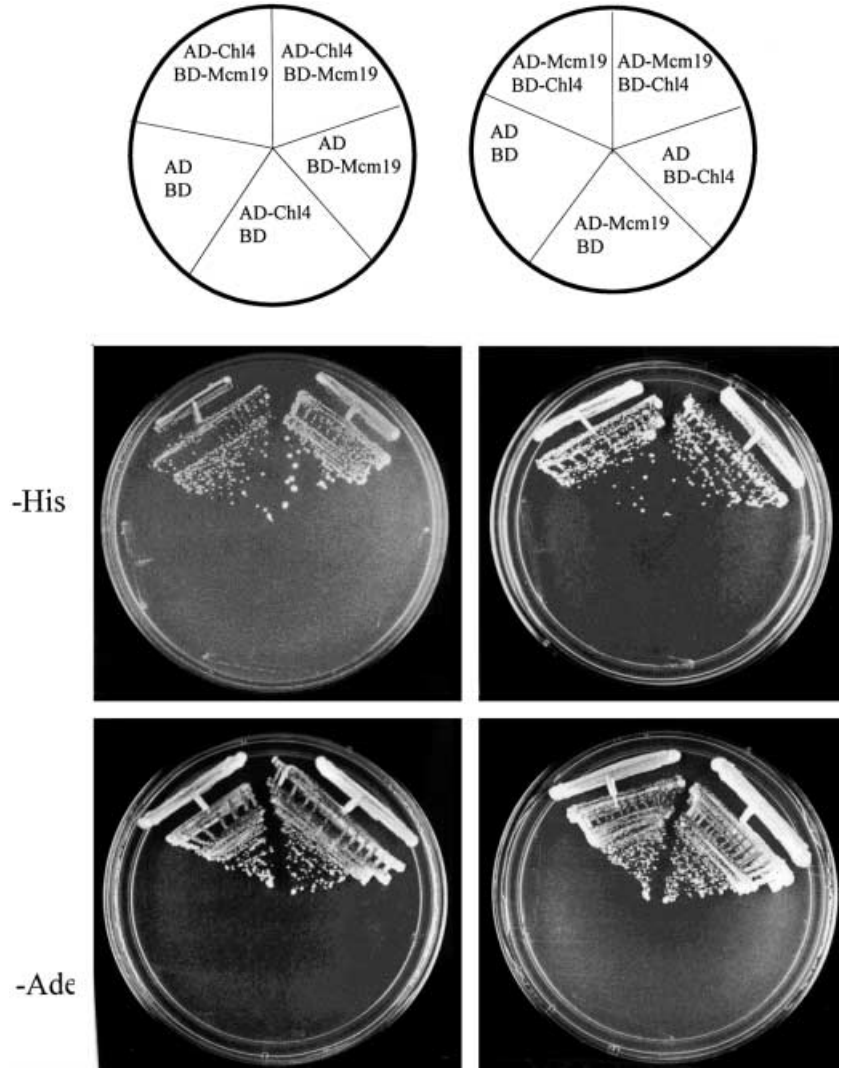

Fig. 6 Mcm19 and Chl4 interact with each other. *Left panel* Plasmids expressing the fusion proteins AD-Chl4 and BD-Mcm19 were introduced into PJ69-4A. The transformants carrying both these plasmids could grow on minimal media lacking histidine (*top row*) and adenine (*lower row*). The growth of two such transformants is shown. The control vectors did not allow any growth. *Right panel* Similar results were obtained when Mcm19 was fused to the activation domain and Chl4 to the binding domain

not localize this protein when it was expressed from the *MCM19* promoter in single- or high-copy-number vectors in yeast cells (S.K. Ghosh, unpublished observations). We have now used the strong promoter *PYK1* (the pyruvate kinase gene of yeast) to drive the expression of GFP and the GFP-*MCM19* fusion gene, each integrated in the wild-type strain. While GFP alone gave a reasonably strong fluorescence in the cytoplasm alone, the fusion protein gave a very low level of fluorescence both in the cytoplasm and in the nucleus (S.K. Ghosh, unpublished results). This suggests that at least a part of the protein may be localized in the nucleus. This is consistent with the interactions that Mcm19p shows with a kinetochore protein, Chl4, as detected by two-hybrid assays.

In summary, all these observations strongly imply a role for Iml3/Mcm19 protein in kinetochore function. We are carrying out further experiments to test if Mcm19p is centromere associated and to study the physical interactions of this protein with other proteins required for kinetochore function. Further work in this direction should help in determining the role that this protein plays in kinetochore function.

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