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# The *mcm17* mutation of yeast shows a size-dependent segregational defect of a mini-chromosome

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**Abstract** Mini-chromosome-maintenance (*mcm*) mutants were described earlier as yeast mutants which could not stably maintain mini-chromosomes. Out of these, the *ARS*-specific class has been more extensively studied and is found to lose chromosomes and mini-chromosomes due to a defect in the initiation of DNA replication at yeast *ARS*s. In the present study we have identified a number of *mcm* mutants which show size-dependent loss of mini-chromosomes. When the size of the mini-chromosome was increased, from about 15 kb to about 60 kb, there was a dramatic increase in its mitotic stability in these mutants, but not in the *ARS*-specific class of mutants. One mutant, *mcm17*, belonging to the size-dependent class was further characterized. In this mutant, cells carried mini-chromosomes in significantly elevated copy numbers, suggesting a defect in segregation. This defect was largely suppressed in the 60-kb mini-chromosome. A non-centromeric plasmid, the *TRP1ARS1* circle, was not affected in its maintenance. This mutant also displayed enhanced chromosome-III loss during mitosis over the wild-type strain, without elevating mitotic recombination. Cloning and sequencing of *MCM17* has shown it to be the same as *CHL4*, a gene required for chromosome stability. This gene is non-essential for growth, as its disruption or deletion from the chromosome did not affect the growth-rate of cells at 23 °C or 37 °C. This work suggests that centromere-directed segregation of a chromosome in yeast is strongly influenced by its length.

**Key words** Yeast · Mutant · *mcm* · Chromosome segregation

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## Introduction

In the yeast *Saccharomyces cerevisiae* the identification of specific DNA sequences, such as *ARS* (autonomously replicating sequences), *CEN* (centromeric DNA) and *TEL* (telomeric DNA), has led to the construction of artificial linear and circular chromosomes which have been extremely useful for understanding the mechanisms by which chromosome stability is maintained. *Cis*- and *trans*-acting mutations affecting *ARS*, *CEN* and *TEL* functions lead to chromosomal instability (reviewed in Newlon 1988, 1993; Page and Snyder 1993; Tye 1994; Zakian 1995). Another important determinant of chromosomal stability is its length. Natural yeast chromosomes are lost at a rate of $10^{-5}$ per cell division (Hartwell and Smith 1985) but their deletion derivatives and artificial chromosomes, around 50 to 100 kb in size, are over two orders of magnitude more unstable (reviewed in Newlon 1988). Similarly, artificial mini-chromosomes also improve in stability when their size is increased from around 10 to over 60 kb (Koshland et al. 1985). The exact reason for the relative instability of smaller-sized chromosomes is not known in yeast.

Mini-chromosome-maintenance (*mcm*) mutants were described earlier which led to the instability of mini-chromosomes (Maine et al. 1984a). The nomenclature used for the *mcm* mutants is as suggested by Chong et al. (1996) and is elaborated in the next section. A class of these (the *ARS*-specific class) has been relatively better characterized and shown to lead to the loss of chromosomes and mini-chromosomes by affecting the initiation of DNA replication at their origins of replication (reviewed in Tye 1994; Chong et al. 1996). The other class, which de-stabilized mini-chromosomes without any *ARS*-specificity, has yet to be studied. In the present work we have examined the effect of increasing the size of a mini-chromosome on its maintenance in *mcm* mutants. We reasoned that if a mutant has a defect in a replication/segregation process that is independent of the size of the mini-chromosome, an increase in its length would not reduce the loss rate significantly, i.e. no more than that shown by the wild-type. If,

on the other hand, the defect is in a process that is influenced by the length of the mini-chromosome, an increase in size may suppress the *mcm* mutation. Several mutants have been identified which showed considerable stabilization of the mini-chromosome upon an increase in its size by the introduction of lambda DNA. We reasoned that the functions affected in such mutants would be highly size-dependent. Amongst these, a further characterization of *mcm17* is presented as affecting the segregation of small centromeric mini-chromosomes. Cloning and sequencing of *MCM17* shows it to be the same as *CHL4*, a gene suggested to be required for centromere function (Kouprina et al. 1993). The work presented here shows that the size of a chromosome plays an important role in its segregation during mitosis.

## Materials and methods

*Media and enzymes.* These have been described before (Maiti and Sinha 1992).

*Strains.* These are listed in Table 1. In addition, the *mcm17* mutants carrying the *MCM17* gene disruption or deletion are described separately later in this section. For *mcm* mutants the revised nomenclature suggested by Chong et al. (1996) and B.-K. Tye (personal communication) is used. It has been found that Mcm131C-11 (Maine et al. 1984a) belongs to the complementation group *mcm12*, which is an *ARS*-specific mutant (B.-K. Tye, personal communication, our own observations). CIIIR or CIIIL were transferred to *mcm17* by crossing M25U to 301-2B carrying CIIIR or CIIIL. YCp121-L was maintained in 301-2B. It was transferred to each of the *mcm12* and *mcm16–mcm23* mutants by crossing the corresponding Mcm131C mutant (Table 1) to 301-2B carrying YCp121-L and identifying the mutant segregants which were $Leu^+$ due to the presence of YCp121-L. Similarly, this minichromosome was transferred to *mcm2* and *mcm3* by crossing M46/4A and 2B-61 respectively with 301-2B (YCp121-L).

*Plasmids.* YCp121, carrying *ARS121* on a 6.7-kb *Bam*HI-*Sal*I fragment, has been described earlier (Maine et al. 1984a) and is shown in Fig. 1. YCp121-L was constructed as follows. Linear lambda DNA was circularized by self-ligation and subsequently digested with *Sal*I. YCp121 was also digested with *Sal*I. *Sal*I-digested lambda DNA was ligated with *Sal*I-digested YCp121 DNA. The wild-type strain 301-2B was transformed with the ligation mixture. Transformants having lambda DNA cloned into the mini-chromosomes were identified by colony hybridization using $^{32}$P-labelled lambda DNA as a probe. Total DNA was isolated from positive clones and restricted with appropriate restriction enzymes (*Sal*I or *Hin*dIII), fractionated on a 0.7% agarose gel, and the Southern blot was probed with $^{32}$P-labelled pBR322 and lambda DNA as probes. This confirmed the presence of 48-kb lambda DNA in YCp121-L (data not shown). That a single copy of lambda DNA was present in YCp121-L was determined by digesting the total DNA of 301-2B carrying YCp121-L with *Bam*HI and probing the Southern blot of this DNA with the 4.7-kb *Bam*HI-*Sal*I fragment of lambda DNA. A single band, corresponding to the size of the junction fragments (11 or 11.4 kb, depending upon orientation) was detected on the autoradiogram (data not shown). More than one copy of lambda DNA would have given an additional 6 (4.7+1.3)-kb band consisting of lambda DNA only.

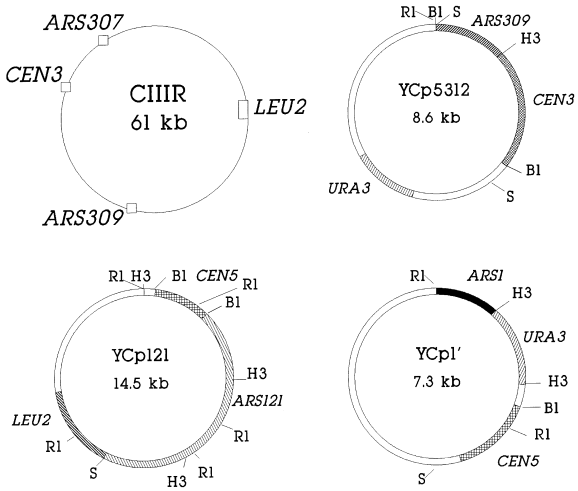


**Fig. 1** Restriction maps of plasmids used in this study. The sites are *B*1, *Bam*HI; *H*3, *Hin*dIII; RI, *Eco*RI and *S*, *Sal*I

**Table 1** Strains used in this study

| Strain | Genotype | Source/reference |
|---|---|---|
| 301-2B | *MATα leu2-3,112 his4Δ34 ura3-52 trp1* | Sinha et al. 1986 |
| A3 | *MAT***a** *leu2-3,112 his3-11,15* | Maine et al. 1984 |
| Mcm131C-11 | *MAT***a** *leu2-3,112 his3-11,15 mcm12* | -do- |
| Mcm131C-16 | *MAT***a** *leu2-3,112 his3-11,15 mcm16* | -do- |
| Mcm131C-25 | *MAT***a** *leu2-3,112 his3-11,15 mcm17* | -do- |
| Mcm131C-29 | *MAT***a** *leu2-3,112 his3-11,15 mcm18* | -do- |
| Mcm131C-31 | *MAT***a** *leu2-3,112 his3-11,15 mcm19* | -do- |
| Mcm131C-34 | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 mcm20* | -do- |
| Mcm131C-39 | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 mcm21* | -do- |
| Mcm131C-51 | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 mcm22* | -do- |
| Mcm131C-52 | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 mcm23* | -do- |
| 2B-61 | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 mcm3* | Gibson et al. 1990 from: |
| M46/4A | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 trp1 mcm2* | 301-2B X Mcm131C46 |
| M51/3A | *MATα leu2-3,112 his3-11,15 ura3-52 mcm22* | 301-2B X Mcm131C-51 |
| M25U | *MAT***a** *leu2-3,112 his3-11,15 ura3-52 mcm17* | 301-2B X Mcm131C-25 |
| M25/7C | *MAT***a** *leu2-3,112 ura3-52 mcm17 trp1* | 301-2B X M25U |
| M25/14 | *MAT***a** *leu2-3,112 ura3-52 trp1 mcm17* | 301-2B X M25/7C |
| M25/16 | *MATα leu2-3,112 ura3-52 trp1 his4Δ34 mcm17* | -do- |
| F399 | *MAT***a** *gal7 lys2 tyr1 leu2 his4 thr4 MAL2 trp1 ade6 arg4 ura4* | Z. Lobo |

The integrating plasmid pGK8 has been described in Surosky and Tye (1985b). YCp1′ (Fig. 1) was an *ARS1*-carrying mini-chromosome obtained from Bik Tye. This mini-chromosome was the same as YCp1 (Maine et al. 1984a) except that it lacked the *LEU2* gene and *CEN5* was contained in a 1.1-kb *Bam*HI-*Bgl*II fragment. The *TRP1ARS1* circle was constructed by self-ligating the 1.4-kb *TRP1ARS1* fragment from YRp7 (Botstein et al. 1979). CIIIR (Fig. 1) was a 61-kb ring chromosome which consists entirely of yeast DNA sequences derived from chromosome III (Surosky et al. 1985a). This ring carriers two strong *ARS*s and its *ARS* deletion derivatives have been obtained by Dershowitz and Newlon (1993). CIIIL was a linear derivative of this ring (Sinha et al. 1986). YCp5312 (Fig. 1) was constructed as follows. The 2.0-kb *Bam*HI-*Hin*dIII fragment carrying *CEN3* from pYe(*CEN3*)41 (Clarke and Carbon 1980) was transferred into YIp5 (Botstein et al. 1979) to obtain YCp53. The 1.2-kb *Hin*dIII-*Sal*I fragment containing *ARS309* from pVH12 (kindly supplied by C. S. Newlon) was cloned into pUC18. The *Eco*RI-*Hin*dIII fragment of the resultant plasmid, containing *ARS309*, was cloned into YCp53 to obtain YCp5312.

*Yeast transformations, stability assays and loss rates.* Yeast transformations were done as described by Maiti and Sinha (1992). Stability assays were carried out essentially as described in Maine et al. (1984a). Stability was defined as the fraction of mini-chromosome-carrying cells in a culture. Initial stability, *I*, was the fraction of mini-chromosome-bearing cells in the YEPD culture at the time of inoculation and *F* was the final stability, or the fraction of mini-chromosome-bearing cells in the culture after *N* generations of growth in the rich medium. The loss rate (fraction of cells losing the mini-chromosome per generation of growth) was measured as $1-(F/I)^{1/N}$ (Gibson et al. 1990; Chen et al. 1992; Maiti and Sinha 1992). Whenever errors are indicated, they are standard deviations from the mean of 4–6 independent experiments.

*Isolation of yeast DNA, Southern transfer, hybridizations, autoradiography.* These have been described before (Maiti and Sinha 1992).

*Copy number determinations of YCp121 and YCp121-L.* The transformants of M25U(*mcm17*), M51/3A(*mcm22*) and 301-2B(*MCM*) containing YCp121 were grown to saturation in selective minimal medium lacking leucine. The fraction of mini-chromosome-carrying cells was determined and total DNA was isolated from each culture. The DNA was digested with *Bam*HI, fractionated on an agarose gel, and the Southern blot of this gel probed with a $^{32}$P-labelled 1.5-kb fragment from YCp121 carrying *CEN5* DNA. This fragment hybridizes to a 3.9-kb chromosome band and the 1.5-kb plasmid band carrying *CEN5* DNA (Maine et al. 1984b). The ratio of the intensity of hybridization of the lower 1.5-kb plasmid band to that of the chromosome band was measured in all cases. This ratio, divided by the fraction of mini-chromosome-carrying cells, gave the average copy number of the mini-chromosome in cells which carried it. A similar experiment was performed for strains carrying the YCp121-L mini-chromosome.

*Chromosome-loss studies.* The loss of chromosome III was monitored in diploids as described by Gerring et al. 1990. Isogenic *mcm* homozygous and heterozygous diploids (M25/14×M25/16, M25/7C×301-2B) were constructed, each heterozygous for the *HIS4* locus. These were analysed for the loss of chromosome III. Six colonies from each were inoculated into YEPD and grown to saturation. Subsequent steps were similar to those described by Gerring et al. 1990. The appearance of colonies from these diploids which mated with F399 and were also His$^-$ were taken as chromosome III (*MAT***a** *HIS4*)-loss events. Maters wich remained His$^+$ indicated recombination events between the centromere and the *MAT*$\alpha$ locus. Mating efficiency was calculated by crossing F399 with 301-2B. The frequencies with which His$^-$ or His$^+$ maters appeared gave a measure of chromosome loss or recombination which, when normalized with mating efficiency, gave the frequency of chromosome loss or recombination events.

*Cloning of the MCM17 gene and construction of null mutations.* *MCM17* was cloned by its ability to stabilize the mini-chromosome YCp1′ in *trans* in *mcm17*. The mutant containing YCp1′ was grown in minimal medium lacking uracil and transformed with the yeast DNA bank constructed in YEp13 by K. Nasmyth and kindly supplied by U. Surana. The transformants were plated on minimal medium lacking both uracil and leucine. Colonies carrying *MCM17* were expected to stabilize YCp1′ and grow faster than the mutant colonies. As many as a 1000 transformants could be plated per Petri dish. Faster-growing colonies carrying stabilized YCp1′ appeared first after about 48–60 h of incubation at 30 °C. These were marked and picked out. About 12 000 colonies were screened in this way. Twenty six large-sized colonies were re-streaked on selective medium for single colonies. Three out of these continued to show fast growth and also gave high stabilities for YCp1′. Another yeast DNA bank constructed on an *ARS1/CEN4/LEU2* vector (Gerring et al. 1990), was also used for cloning, using the same strategy as described above. This library gave two clones which stabilized YCp1′ in *trans*. Plasmid DNA was recovered from Leu$^+$ Ura$^-$ cells of one clone from each library. These were found to have overlapping inserts. The DNA fragment which complemented the mutant phenotype was present in both clones (see Fig. 3a). Subsequent work was done using the YEp13 clone. To show that the cloned gene was the same as *MCM17*, and not a suppressor of *mcm17*, the 4.7-kb *Bam*HI-*Sal*I fragment containing the complementing activity was cloned into an integrating vector, pGK8. This plasmid was digested with *Bgl*II within the insert and integrated into M25U. The site of integration of plasmid DNA was found to be the same as that of the cloned gene. This was confirmed by digesting the DNA of the integrant with *Xho*I, fractionating the digest on an agarose gel, and probing the Southern blot of this gel with the 1.7-kb *Hin*dIII fragment of the insert. The untransformed parent strain gave a 10.8-kb band on the autoradiogram (data not shown); in the integrant this band was absent and a higher (23-kb) band was present which was indicative of the integration of plasmid DNA at the genomic location of the clone. That this site was tightly linked to *mcm17* was shown by crossing the integrant with the wild-type strain 301-2B, sporulating the diploid, and analyzing 44 spores obtained with this cross for a *mcm17* phenotype. No recombinant *mcm17* spore could be recovered, showing that the mutation was tightly linked to the cloned gene, which was perhaps identical to *MCM17*. We also made a partial deletion of the cloned gene from the chromosome (see below). This resulted in a *mcm* phenotype in cells carrying the deletion. This mutation was recessive to the wild-type and failed to complement the *mcm17* mutation, which is also recessive. This further supports the conclusion that the cloned gene was the same as *MCM17* (Lundblad 1990). A disruption of the *MCM17* gene was made on the chromosome by cloning the 1.7-kb *Bam*HI fragment of pYAC4 (Burke et al. 1987) carrying the *HIS3* gene into the *Bgl*II site of *MCM17* in pGK8 (see Fig. 3a). The wild-type strain A3 was transformed with the *Bam*HI-*Sal*I *HIS3*-carrying fragment of the resulting plasmid and His$^+$ transformants were selected. To create a deletion of *MCM17*, pGK8 carrying *MCM17* (see Fig. 3a) was digested with *Xba*I and this site was end-filled. A 1.4-kb *Sma*I-*Bam*HI fragment carrying *URA3* from YCp1 was cloned at blunted *Xba*I and *Bgl*II sites, resulting in a deletion of over 50% of the central portion of the gene. This plasmid was digested with *Bam*HI-*Sal*I and 301-2B was transformed to Ura$^+$. Two His$^+$ transformants were analyzed for the disruption and four Ura$^+$ transformants for disruption-deletion of the chromosomal copy of the *MCM17* gene. Total DNA from the parent and transformed strains was digested with *Hin*dIII, fractionated, and blotted for Southern analysis. Figure 3b shows the results. When detected by the 1.6-kb *Hin*dIII-*Bgl*II fragment used as a probe, the 1.7-kb *Hin*dIII band carrying the gene was present in the untransformed parent strains but was altered to 2.5 kb in the His$^+$ transformants and to 2.1 kb in Ura$^+$ transformants. This was expected for a *MCM17* gene disruption by *HIS3* in His$^+$ transformants and a deletion and disruption (by *URA3*) of the *MCM17* gene in the Ura$^+$ transformants. DNA sequencing was done on double-stranded plasmid DNA using Sequenase$^{TM}$ US Biochemicals.

## Results

### Identification of *mcm* mutants showing size-dependence in the maintenance of mini-chromosomes

In earlier studies, *mcm17* showed instability for all artificial mini-chromosomes tested, irrespective of their *ARS* or *CEN* sequences (Maine et al. 1984 a). To characterize this mutant further, we were interested in testing the effect of the shape (circular or linear) of the mini-chromosome on its maintenance in this mutant. Since short linear centromeric plasmids are unstable, even in the wild-type strain (Newlon 1988), for this comparison we chose CIIIR, a 61-kb ring consisting entirely of yeast DNA and its linear derivative CIIIL (see Materials and methods). Wild-type and mutant strains carrying these mini-chromosomes were grown for about ten generations in non-selective medium. The loss rates for these mini-chromosomes are given in Table 2. There was a very slight increase in the loss rate of CIIIL in the mutant, as compared to that in the wild-type and to CIIIR in the mutant. This suggests that, in the presence of the *mcm17* mutation, the maintenance of linear mini-chromosomes was slightly more affected than that of circular mini-chromosomes. CIIIR carries two strong *ARS*s, *ARS307* and *ARS309*. Dershowitz and Newlon (1993) have created deletions such that the derivative ring contains only one strong *ARS*. These deletion derivatives of CIIIR were transferred to *mcm17* by crossing. The loss rates of CIIIRΔ*ARS307* and CIIIRΔ*ARS309* are shown in Table 2. A small mini-chromosome containing *CEN3* and *ARS309* was also constructed (Fig. 1). This was very unstable in the mutant (Table 2). From all these observations it can be seen that while a small mini-chromosome containing the centromere and the *ARS* sequences of CIIIR was maintained poorly in the mutant, CIIIR was maintained almost as well in the mutant as in the wild-type strain. This relative improvement in stability was not because CIIIR carried two *ARS*s; CIIIR *ARS*-deletion derivatives, each carrying only one strong *ARS*, also showed stabilities which were similar in mutant and wild-type strains. In contrast, *mcm2*, an *ARS*-specific DNA replication initiation mutant, showed high *ARS*-dependent loss rates for these mini-chromosomes at 35 °C (Sinha et al. 1986; Ray et al. 1994), its non-permissive temperature for the function of all the *ARS*s (Maine et al. 1984 a). We suspected that perhaps the increased size of CIIIR helped to suppress the *mcm* defect in *mcm17*. This prompted us to examine more systematically the effect of size on the maintenance of mini-chromosomes in *mcm* mutants, as described below.

The size of the mini-chromosome YCp121 was increased to about 60 kb by cloning lambda DNA in it. Lambda DNA does not carry any *ARS* or any other stabilizing sequences (Stinchomb et al. 1981) and therefore serves only to increase the size of YCp121. In order to study the effects of size on the maintenance of a mini-chromosome, the stability of the larger-sized derivative in *mcm* mutants was compared with that of its smaller counterpart. The results are shown in Table 3.

**Table 2** Loss rates of CIIIR, its *ARS*-deletion derivatives, CIIIL, and the mini-chromosome YCp5312 in the mutant and wild-type strains

| Strains | Loss rates ($\times 10^{-3}$)[a] | | | | |
|---|---|---|---|---|---|
| | YCp5312 | CIIIR | CIIIRΔ*ARS307* | CIIIRΔ*ARS309* | CIIIL |
| *mcm17* | 359 | 3.0±0.7 | 5.8±2.3 | 4.7±2.0 | 7.1±3.0 |
| *MCM17* | 15 | 1.7±0.4 | 3.8±0.3 | 6.5±0.5 | 2.0±1.0 |

[a] Loss rates were measured at 25 °C. Errors are standard deviations from the means of four to six independent experiments

**Table 3** Loss rates of YCp121 and YCp121C-L at 25 °C in *mcm* mutants. YCp121-L was cured from each strain and the cured strains were transformed with YCp121. This ensured that the same strain was used to measure the loss rates of both YCp121 and YCp121-L. Cells carrying YCp121 or YCp121-L were grown in rich medium for six to seven generations and the loss rates were determined as described in Materials and methods. The values are means of two independent experiments. The values given in parenthesis are the actual values obtained for loss rates

| Strains | Loss rates | |
|---|---|---|
| | YCp121 | YCp121-L |
| *mcm2* | 0.010 (0.009, 0.011) | 0.030 (0.040, 0.020) |
| *mcm3* | 0.065 (0.070, 0.060) | 0.050 (0.050, 0.051) |
| *mcm12* | 0.130 (0.130, 0.130) | 0.114 (0.124, 0.104) |
| *mcm16* | 0.159 (0.186, 0.132) | 0.037 (0.039, 0.035) |
| *mcm17* | 0.339 (0.333, 0.345) | 0.019 (0.014, 0.024) |
| *mcm18* | 0.251 (0.276, 0.227) | 0.065 (0.070, 0.060) |
| *mcm19* | 0.274 (0.251, 0.298) | 0.016 (0.013, 0.020) |
| *mcm20* | 0.091 (0.109, 0.073) | 0.032 (0.037, 0.027) |
| *mcm21* | 0.336 (0.322, 0.350) | 0.119 (0.117, 0.121) |
| *mcm22* | 0.340 (0.343, 0.337) | 0.034 (0.027, 0.041) |
| *mcm23* | 0.140 (0.107, 0.174) | 0.035 (0.050, 0.021) |
| *MCM* | 0.020 (0.019, 0.021) | 0.003 (0.003, 0.003) |

As expected, in the wild-type strain the 60-kb mini-chromosome was better maintained as compared with the smaller one. The loss of the mini-chromosome per cell per generation increased from 0.003 to 0.020 upon a decrease of size from 62.5 to 14.5 kb. For the analysis of *mcm* mutants, consider the case this way. If the mini-chromosome in these *mcm* mutants was lost due to a reason which was mostly independent of its size, the increase in the loss rate of the mini-chromosome, upon size-reduction, would be similar to that shown by the wild-type, i.e. of the order of 0.017 (= 0.020 − 0.003). Such marginal changes in loss-rates were shown by *ARS*-specific mutants, *mcm2*, *mcm3* and *mcm12*, implying that the initiation of DNA replication in these mutants is independent of the size of the mini-chromosome. On the other hand, several other mutants, such as *mcm17*, *mcm19* and *mcm22*, showed a considerable stabilization of the mini-chromosome when its size was increased. Similar results were obtained for *mcm2*, *mcm12*, *mcm17* and *mcm22* when two other mini-chromosomes, YCp1′ (Fig. 1) and YCp131C (Maine et al.1984), were increased in size by the cloning of lambda DNA. For example, the respective loss rates of YCp131C and

YCp131C-L in *mcm2* were 0.051 and 0.022; in *mcm17* these values were 0.229±0.039 and 0.015±0.003. Similarly, the loss rats of YCp1 and YCp1′-L in *mcm2* were 0.282 and 0.304, while in *mcm17* these values were 0.311±0.014 and 0.067±0.002. For *mcm17* the values were averages of four to six independent experiments. For *mcm2* the loss rates were averages of two independent experimental results which did not vary from their mean by more than 16% in any case.

From these observations we can conclude that mini-chromosome-maintenance defects in *mcm* mutants are of at least two types: (1) those which can be suppressed to a high degree by an increase in the size of the mini-chromosome and (2) those which are unaffected by size increase. Mutants like *mcm20* and *mcm21* did not show the strong size-dependence of *mcm17*, *mcm19* and *mcm22*, and therefore their placement is not clear at the moment. It is noteworthy that CIIIR was better maintained in *mcm17* than similar-sized lambda-carrying mini-chromosomes. This was not due to the presence of two replication origins (see above). It is very likely that mitotically stabilizing sequences, other than the centromere, which are functionally redundant in the wild-type but compensate for the lack of mitotic stability in the mutant, are present on this ring. At least one such mitotically stabilizing sequence has been reported adjacent to *CEN4* (Mann and Davis 1986). Another point to be noted is that, despite its improved stabilization, YCp121-L continued to be more unstable in the mutant than in the wild-type strain. This shows that an increase in size only partially suppressed the mutant phenotype. In the next section we show that chromosome III shows a high loss rate in the mutant, again confirming that the *mcm17* defect cannot be completely suppressed by an increase in size alone.

## Loss of chromosome III in *mcm17*

Since CIIIL was only slightly less stable in the mutant than in the wild-type strain, it was of interest to examine if *mcm17* caused the loss of native chromosomes. We determined the mitotic loss of chromosome III in *mcm17* as described under Materials and methods. The results showed that the *mcm17/mcm17* diploid lost chromosome III at a rate which was about 40-fold higher than the wild-type (*mcm17/MCM17*) rate, the experimentally determined values being $86\pm32\times10^{-5}$ and $2.0\pm1.1\times10^{-5}$ respectively. The mitotic recombination rate at the *MAT* locus was not significantly altered, the values being $5.2\times10^{-5}$ and $3.9\times10^{-5}$ respectively. Hartwell and Smith (1985) have suggested that mutations that increase chromosomes loss, without increasing mitotic recombination significantly, might be in genes involved in chromosome segregation rather than in DNA metabolism. These results are consistent with *mcm17* affecting chromosome segregation and also confirm the observation, made in the earlier section, that an increase in the size of the chromosome is not sufficient to completely suppress the *mcm17* defect.

## *mcm17* affects the mitotic segregation of a centromeric plasmid but not that of a non-centromeric plasmid

If the instability of the mini-chromosome is due to a failure to replicate, its copy number per mini-chromosome-bearing cell is expected to stay close to one per cell. If, on the other hand, the instability is due to non-disjunction, a decrease in the population of mini-chromosome-bearing cells would be associated with an increase in the number of mini-chromosomes per mini-chromosome-bearing cell (Sinha et al. 1986). To confirm that *mcm17* affects segregation, the copy numbers of YCp121 and YCp121-L were determined in M25U and the wild-type strains as described under Materials and methods. We included another mutant, M51/3A (*mcm22*), which showed a strong size-dependent stabilization of mini-chromosomes (Table 3), to see whether this mutant also exhibits a segregation defect which can be suppressed by an increase in the size of the mini-chromosome.

The results (Fig. 2 a) show that the wild-type strain maintained both YCp121 and YCp121-L at about one copy per plasmid-bearing cells. *mcm17* and *mcm22*, on the other hand, had elevated copies of the mini-chromosome YCp121, but the copy number was one or two for the larger mini-chromosome YCp121-L. These results imply that the segregation of YCp121 was affected in these *mcm* mutants and that the segregational defect was corrected in the 60-kb mini-chromosome YCp121-L.

We next studied whether all small plasmids are lost from cells or whether only centromere-containing plasmids are unstable in these mutants. For this purpose we chose the *TRP1ARS1* circle which is a 1.4-kb DNA derived from yeast chromosome IV. When circularized and transformed into yeast cells, this plasmid exists in high (50–100) copies, each copy replicates once per cell cycle and shows no mother-cell bias during segregation (Zakian and Scott 1982, Murray and Szostak 1983). Therefore, a replication defect of this plasmid in the mutant would show up as a loss in plasmid DNA in the culture after several generations of growth of cells under non-selective conditions, while a segregation-bias will give rise to a higher proportion of cells not containing the plasmid.

Wild-type and mutant transformants carrying a *TRP1ARS1* circle were grown in minimal medium lacking tryptophan and then inoculated, to an $OD_{610}$ of approximately 0.05, into rich YEPD supplemented with 20 µg/ml of tryptophan. The cultures were grown, with intermittent inoculations, for about 20 generations. Figure 2 b shows plasmid DNA present in the culture after 20 generations of growth in the rich medium and initial and final stabilities. These values did not change significantly in the mutants as compared to that in the wild-type. Thus, the plasmid was maintained stably in both the strains, implying that neither replication nor segregation of the *TRP1ARS1* circle was affected in *mcm17* and *mcm22*. This behaviour was in contrast to that of the *mcm2* mutant where the plasmid was extremely unstable (Sinha et al. 1986) due to a failure in the initiation of DNA replications at *ARS1*.

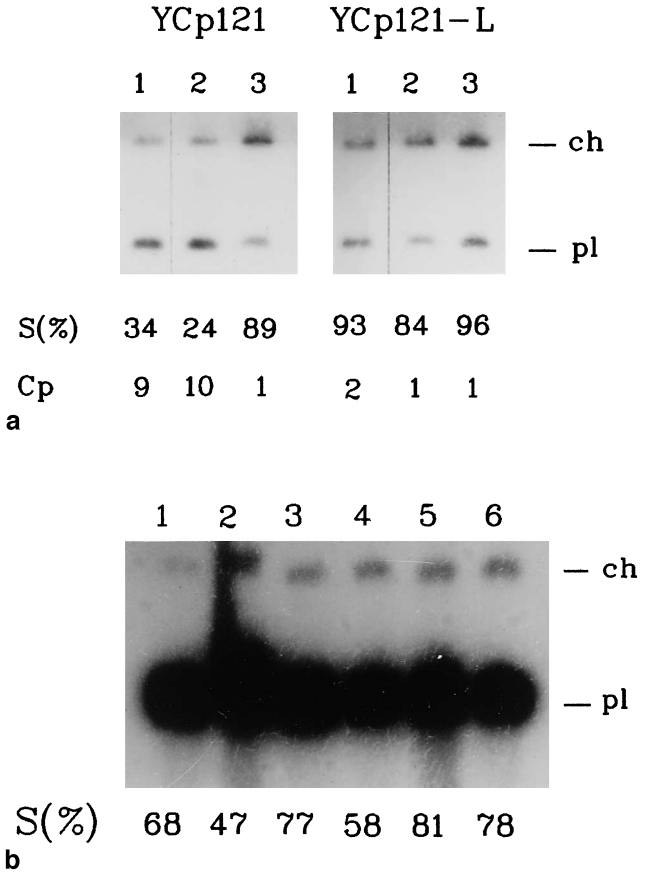


**Fig. 2** **a** copies of YCp121 and YCp121-L per mini-chromosome-bearing cell in *mcm17*, *mcm22*, and wild-type cells grown for about six generations in selective medium at 25°C. A southern blot of *Bam*HI-digested total DNA of transformants was probed with the $^{32}$P-labelled 1.5-kb *CEN5* DNA from YCp121. The upper 3.9-kb bands (*ch*) were derived from chromosome V and the lower 1.5-kb bands (*pl*) from YC121 and from YCp121-L. *S(%)* under each lane is the percentage of cells carrying the mini-chromosome in the aliquot of the culture used for isolating the DNA of that lane. $C_p$ under each lane indicates the average copy number per plasmid-bearing cell. The values have been rounded to the nearest integer. In both figures the lanes were: *1*, *mcm22*; *2*, *mcm17* and *3*, wild-type strain. **b** copies and stabilities of *TRP1ARS1* in mutant and wild-type strains. Total DNA was prepared from transformants containing the *TRP1ARS1* plasmid, digested with *Hin*dIII, and the Southern blot of this DNA was hybridized with the $^{32}$P-labelled 1.45-kb *Eco*RI fragment from plasmid YRp7. *Lanes: 1, 3 and 5*, DNA from cultures of wild-type, *mcm17* and *mcm22* transformants respectively, at the time of inoculation in YEPD. *Lanes: 2, 4 and 6*, DNA isolated from cultures of wild-type, *mcm17* and *mcm22* respectively, after 20 generations of growth of cells in YEPD at 25°C. The 2.1-kb upper band is from genomic DNA, while the 1.45-kb lower band is plasmid DNA. The percentage of cells carrying the plasmid in cultures used for making DNA is given as *S(%)* under each lane. Other details are in the text

## Cloning of *MCM17* and the construction of null alleles

The strategy for the cloning of *MCM17* has been described in Materials and methods. The final stability of YCp1′ in *mcm17* varied from 1 to 8%. In the presence of the stabilizing plasmids obtained from YEp13 and the *ARS1/CEN4/LEU2* banks, the stability of YCp1′ varied

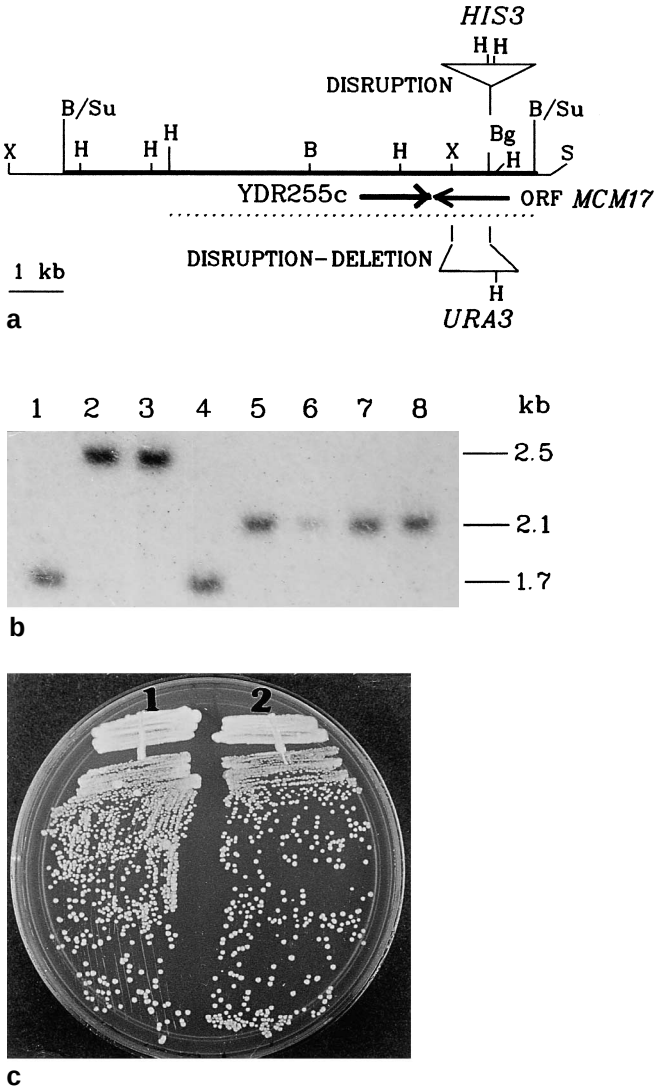


**Fig. 3** **a** restriction map of the DNA fragment in YEp13 that contains the MCM17 gene. *B*, *Bam*HI; *Bg*, *Bgl*II; *H*, *Hin*dIII; *S*, *Sal*I; *Su*, *Sau*3A and *X*, *Xba*1. The insert DNA is bounded by *B/Su*. The *thin flanking lines* represent vector DNA sequences where only the relevant restriction sites are shown. The *two arrows* under the insert show the positions of *CHL4/MCM17* and YDR255c ORFs and the directions of transcription of the corresponding genes. (The positions of YDR255c and that of the *MCM17* gene on chromosome IV were obtained from *Saccharomyces* Genome Database, Department of Genetics, School of Medicine, Stanford University, USA.) The *dotted line* below represents DNA which was also present in the *ARS1/CEN4/LEU2* bank. The disruption and the deletion within the gene are also indicated. **b** autoradiogram showing disruption and deletion of DNA at the *CHL4/MCM17* locus. The DNA of the His$^+$, Ura$^+$ transformants and that of their parent strains A3 and 301-2B was digested with *Hin*dIII, fractionated on an agarose gel, blotted, and the blot was probed with the 1.6-kb *Hin*dIII-*Bgl*II DNA of the *CHL4/MCM17* gene. *Lanes 1*, A3; *2 and 3*, A3 (*mcm17*::*HIS3*) transformants; *4*, 301-2B; 5–8, 301-2B (*mcm17-Δ1*::*URA3*) transformants. The 1.7-kb *Hin*dIII band in the A3 and 301-2B lanes was absent in lanes carrying transformant DNA. In His$^+$ transformants the probe hybridized to a 2.5-kb band due to gene disruption by *HIS3*, while in Ura$^+$ transformants the probe hybridized to a 2.1-kb band due to partial deletion of *MCM17* and its disruption by *URA3*. **c** growth on YEPD plates of A3 (*MCM17*) and A3 carrying the *mcm17* disruption at 37°C. *1*, A3; *2*, A3 (*mcm17*::*HIS3*). The photograph was taken after 2 days of incubation

from 56 to 71% in the two cases. The restriction map of the 8.9-kb insert in YEp13 which stabilized YCp1′ in *mcm17* is given in Fig. 3. The *Xba*1 site in the insert was within the *MCM17* gene since the DNA neither to the left of this site (8.3-kb *Xba*I fragment) nor to the right of site (1.8-kb *Xba*I-*Bam*HI/*Sau*3A fragment) could rescue the mini-chromosome-maintenance phenotype. The *Xba*I fragment was cloned into YCplac33 (Geitz and Sugino 1988) and partial sequencing from the *Xba*I site of the clone showed that *MCM17* was identical to *CHL4*, which is the same as *CTF17* (Kouprina et al. 1993). It has been shown that a mutation in the *CHL4* gene confers a higher stability on a dicentric plasmid than the wild-type strain. This is suggestive of a defect in kinetochore-microtubule assembly (*ibid*, Doheny et al. 1993). In addition, *CTF17* shows defective kinetochore assembly based on a transcriptional-readthrough assay (Doheny et al. 1993). In this assay when *CEN6* is transcribed through a strong promoter (*GAL10*) it causes a transcriptional block of a reporter gene. This happens due to the presence of a protein-DNA complex at the centromere sequence which inhibits transcription. A *ctf17* mutant causes a relaxation of this transcriptional block, suggesting defective kinetochore assembly in *ctf17*; *mcm17* also shows a relaxed transcriptional block in this assay (data not shown). Thus, we conclude that at least one member of the size-dependent class of *mcm* mutants, *MCM17*, is required for kinetochore function.

We next studied whether a null allele of this gene causes any growth defects. Although Kouprina et al. (1993) have created a deletion of this gene, this deletion also removed a substantial part of a neighbouring uncharacterized ORF YDR255c (Fig. 3a). Therefore we created null alleles of this gene on the chromosome by: (1) disruption with *HIS3* and (2) by deleting over 50% of the central part of the gene and replacing it with *URA3* (Materials and methods, Fig. 3a, b). Mutants carrying these null alleles, *mcm17*::*HIS3* and *mcm17-Δ*::*URA3*, had the expected *mcm* defects which failed to complement the *mcm17* mutation. These mutants grew as well as their wild-type parents at both 23° and 37°C in YEPD. At 23°C, the doubling times were – A3, 150 min; A3 (*mcm17*::*HIS3*), 156 min; 301-2B, 150 min and 301-2B (*mcm17-Δ*::*URA3*), 150 min. At 37°, the doubling times of A3 and A3 (*mcm17*::*HIS3*) were 85 min each and those or 301-2B and 301-2B (*mcm17-Δ*::*URA3*) were 90 min each. Figure 3C shows the growth of A3 and its *mcm17*-disrupted derivative at 37°C. Therefore, the *CHL4/MCM17* gene is non-essential for growth at both these temperatures. The temperature-sensitive phenotype displayed by the mutant carrying the delection created by Kouprina et al. (1993) was very likely due to the deletion of the neighbouring ORF, YDR255c (Fig. 3a).

## Discussion

We have identified a set of *mcm* mutants which show a highly improved mini-chromosome maintenance once its size is increased to about 60 kb by the cloning of lambda DNA. In contrast, the *ARS*-specific *mcm* mutants did not cause any significant changes in the loss rate of the mini-chromosome, suggesting that the process of initiation of DNA replication by Mcm proteins is largely independent of the size of the chromosome. Two mutants, *mcm17* and *mcm22*, belonging to the former class, affected the segregation of only centromeric plasmids. Since the maintenance of a non-centromeric plasmid, the *TRP1ARS1* circle, was normal, we suggested that the process of DNA replication, including that of *ARS*, remained normal in these mutants. Cloning and sequencing of the *MCM17* gene has shown it to be same as the *CHL4/CTF17* gene required for kinetochore function (Kouprina et al. 1993). However, contrary to the published report (*ibid*), null alleles of the *MCM17* gene do not lead to temperature-sensitive growth of cells, as described above under Results. The deletion made by Kouprina et al. (1993) removed a good part of the neighbouring uncharacterized ORF YDR255c. It, therefore, appears that a deletion of YDR255c causes a temperature-sensitive growth of cells. Thus, *MCM17* is a non-essential gene affecting chromosome segregation and it would be interesting to determine what proteins can substitute for its function. This would give a clue to its function in yeast. We believe that *mcm* mutants displaying the same phenotype as *mcm17*, i.e. size-dependent stabilization of mini-chromosomes, would identify genes required for chromosome segregation.

It has been known for a long time that short mini-chromosomes are less stable than longer ones (Newlon 1988), but the reasons for this are not known. In the present work we have shown that the process of mini-chromosome segregation is dependent on its size. The Chl4/Ctf17/Mcm17 protein might be playing a role in stabilizing kinetochore-microtubule assembly. How can a defect in such a protein be suppressed by an increase in the size of the mini-chromosome? The deduced protein sequence of this gene does not show any significant homology to any known protein which might throw some light on the mode of function of *MCM17*. Work in *Drosophila*, newt lungs, and *Xenopus* suggests that chromosome arms experience anti-poleward forces during mitosis, perhaps by binding to kinesin-related motor proteins (Skibbens et al. 1993; reviewed in Fuller 1995). The magnitude of these forces is dependent in the microtubule density on the spindle, which is highest at the poles. In newt-lung cells, shortening the arms of the chromosome by a laser microbeam threw the severed arms away from the poles while the shortend chromosome carrying the kinetochore moved closer to the pole (Reider et al. 1986). This suggests that the size of the chromosome determines its position on the mitotic spindle. It is possible that, as in the mitosis of *Drosophila* and *Xenopus*, yeast chromosome arms also participate in generating anti-poleward forces during congression to the spindle equator. If the anti-poleward force on the kinetochore is weakened due to the *mcm17* mutation, an increase in the size of the mini-chromosome would also increase the polar ejection forces, which could partly compensate for the loss in the net anti-poleward forces on the chromosome.

Unfortunately, due to it small size, chromosome movement in this yeast cannot be studied cytologically. *In vitro* assays have been developed by Kingsbury and Koshland (1993) and Sorger et al. (1994) which measure microtubule binding by *in vivo*- and *in vitro*-assembled kinetochores. A further refinement of these assays to include movement of kinetochores on microtubules could provide a system by which the effects of DNA size on the directional mobility of kinetochores could be studied. In another approach, isolation and characterization of other *MCM* genes (required for size-dependent stabilization of mini-chromosomes) and multicopy suppressors of *mcm17* could identify proteins with known functions. This could throw more light on how size influences chromosome segregation in yeast. It would also be helpful to identify genes which, when mutant, form synthetic lethals with *mcm17*. These may code for other components of the mitotic machinery required for size-dependent stabilization of a mini-chromosome in *mcm17*. For example, one of these genes could code for a scaffold protein that stabilizes the loop-scaffold organization of a chromosome. Some of these experiments are already in progress and it is hoped that further studies will contribute towards understanding the molecular basis of the role of the size of a chromosome in yeast mitosis.

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