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Isolation and sequence analysis of mutations in *CEN5* DNA of yeast

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Abstract. We used a positive selection scheme involving a minichromosome maintenance mutant *mcm2* to isolate mutations in centromeric DNA of the yeast *Saccharomyces cerevisiae*. Two mutations in *CEN5* DNA were isolated. One of these mutations is a change from C:G to T:A at the 14th base pair of the CDEIII box, while the second is a complete deletion of all the CDE elements of this centromere. This work underscores the importance of the 14th base pair of CDEIII in *CEN5* function.

Keywords. Yeast; centromere; *mcm2*; mutant.

1. Introduction

The centromere of a chromosome is a region that allows for proper segregation of chromatids during mitosis. It is the site at which proteins assemble to allow binding of microtubules. Sequence analysis of centromeric DNA isolated from several chromosomes of the yeast *Saccharomyces cerevisiae* has identified three tandem elements, CDEI, CDEII and CDEIII (Clarke and Carbon 1985). CDEI is defined by the consensus sequence PuTCACPuTG (Pu = purine), CDEII by an AT-rich sequence of about 80–90 base pairs, and CDEIII by a 25-base-pair consensus sequence $TGT^{T}_{A}T^{T}_{A}TG\cdot\cdot TTCCGAA\cdots\cdot AAA$. Mutational analysis of *CEN3* and *CEN6* DNA has shown that all three are important for *CEN* function and, in particular, that the 14th C:G base pair of CDEIII is essential for centromere activity (McGrew *et al.* 1986; Hegemann *et al.* 1988). We describe a scheme that uses positive selection for isolation of mutations in centromeric DNA. Using this scheme we have isolated mutations in *CEN5* DNA of *S. cerevisiae*. As discussed later, this scheme should be of interest in isolating mutations in genes whose products interact directly with *CEN* or *ARS* (autonomously replicating sequence) DNA.

2. Materials and methods

2.1 *Media, strains and plasmids*

Yeast rich medium (YEPD) and minimal medium have been described before (Sherman *et al.* 1986). LB and M9 media for bacterial growth have been described in Maniatis

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et al. 1982. Strain HB101 carrying the *leuB* mutation (Maniatis *et al.* 1982) was the *E. coli* strain used in these studies. Yeast strains M46-1B (*MATα leu2 ura3 his4 mcm2-1* and cir$^+$ or cir$^-$) and 8534-10A (*MATa leu2 ura3 his4 MCM2*) have been described in Maiti and Sinha (1992). Plasmid YEp13 has been described in Broach *et al.* (1979) and plasmid YCp2μ, which carries a 1·5-kb *Bam*HI fragment containing *CEN5* DNA, has been described in Maine *et al.* (1984). YCp2μ is shown in figure 1. YRp7 (Struhl *et al.* 1979) carries the autonomously replicating sequence *ARS1* and the *TRP1* gene for selection.

2.2 *Plasmid mutagenesis*

YCp2μ was mutagenized as described in Sikorski and Boeke (1991). The degree of mutagenesis was assessed by scoring for mutations in the *LEU2* gene of yeast on YCp2μ. The *LEU2* gene also complements the *leuB* mutation of *E. coli* strain HB101, which therefore becomes leucine$^+$ when transformed with YCp2μ. Mutations at the *LEU2* locus would give leucine-requiring auxotrophs of HB101 transformants as well. A 90-minute treatment of DNA with hydroxylamine gave 2·8% leucine auxotrophs of HB101 and this treatment was used for the isolation of plasmid suppressors.

2.3 *Yeast transformation, DNA preparation and plasmid stability assays*

These were done as described in Sinha *et al.* (1986). Stability of a plasmid was defined as the fraction of cells carrying the minichromosome after about 10 generations of growth of the transformant in rich (nonselective) medium.

2.4 *DNA sequencing*

DNA sequencing of both strands of the 0·9-kb *Eco*RI–*Xba*I fragment of *CEN5* was done using Sequenase™ (US Biochemicals).

3. Results

3.1 *Rationale for isolation of CEN DNA mutations*

YEp13 (Broach *et al.* 1979) is a high-copy-number plasmid which has both the origin of replication (*ORI*) and the locus for proper segregation (*REP3*), both taken from the yeast endogenous plasmid, the 2-micron (2μ) circle. YEp13 also carries the *LEU2* gene for selection in yeast and pBR322 DNA for propagation in *E. coli*. YCp2μ (figure 1) is YEp13 with *CEN5* (centromeric DNA from chromosome V) cloned at its *Bam*HI site (Maine *et al.* 1984). The presence of centromeric DNA reduces the copy number of a composite 2μ-*CEN* plasmid to one per cell and allows for proper segregation of the plasmid in wild-type cells (Tschumper and Carbon 1983). YEp13 is stable in endogenous 2μ-carrying (cir$^+$) cells but is unstable in endogenous 2μ-lacking (cir$^-$) cells. This is because cir$^+$ cells provide 2μ-plasmid-encoded *REP1*, *REP2* and *FLP* gene products required for proper segregation and copy-number maintenance of this plasmid (Jayaram *et al.* 1983; Kikuchi 1983; Volkert and Broach 1986). cir$^-$ cells do not provide these functions, and this leads to loss of YEp13 in these cells. YCp2μ, however,

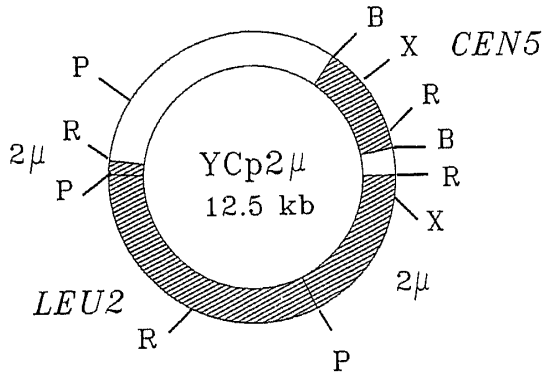

Figure 1. Restriction map of YCp2μ. Restriction sites are B, *Bam*HI; P, *Pst*I; R, *Eco*RI; and X, *Xba*I. Hatched segments represent yeast DNA sequences while the unfilled segments are pBR322 DNA. *CEN5* is contained in the 1·5-kb *Bam*HI fragment and *LEU2* is in the 4-kb *Pst*I fragment of the yeast DNA.

contains *CEN DNA* (which allows for proper segregation even at low copies) and is therefore stable in both cir^+ and cir^- cells of the wild-type strain.

YCp2μ shows impaired stability in an *ARS*-specific *mcm2* minichromosome mutant (cir^+) (strain name M131C-46 in Maine *et al.* 1984) at 25°C. YEp13, however, is stable at the same temperature in this mutant (Maine *et al.* 1984). This is because the fall in copy number of this plasmid due to inefficient replication is partly compensated by its 2μ-dependent amplification process which remains unaffected in the mutant (Maiti and Sinha 1992). Since the 2μ-dependent segregation process of this plasmid is also unaffected in the mutant (Maiti and Sinha 1992), YEp13 exists stably (albeit at low copies) in these cells. YCp2μ cannot amplify itself owing to the presence of the centromere. Therefore inefficient initiation of DNA replication leads to progressive loss of this plasmid from mutant cells. We were interested in isolating suppressor mutations in YCp2μ that stabilized the plasmid in the mutant. Two kinds of suppressors were expected—one in *ARS*, enabling the plasmid to initiate replication efficiently using the mutant protein, and the other at *CEN5*, abolishing its function and reverting this plasmid to YEp13, which is stable in the mutant as described above. Here we describe mutations that abolished *CEN5* function.

3.2 *Isolation of mutant plasmids showing high stabilities in mcm2 cells*

YCp2μ was mutagenized with hydroxylamine as described in Materials and methods. The *mcm2* mutant M46-1B was transformed with the mutagenized plasmid and transformants were plated on medium selective for plasmid maintenance. This is minimal medium supplemented with all nutritional requirements except leucine. *LEU2* function, which complements the mutation in M46-1B, is provided by the plasmid. On this medium only transformed cells would grow. If a transformant carried unstable YCp2μ it would give a slow-growing colony on selective medium. This is because, as the cells divide, the plasmid would be lost from progeny cells with higher frequency and a smaller proportion of cells would be able to divide in the colony. However, if the plasmid was stabilized in a transformant owing to a mutation, more cells in a colony of this transformant would retain the plasmid and hence the ability to grow and divide

Table 1. Mitotic stabilities of plasmids YCp2μ and its mutant derivatives YCp2μ(a), YCp2μ(b) and YCp2μ(c) in an *mcm2* strain.

Plasmid	Mitotic stability (%)
YCp2μ	5·8
YCp2μ(a)	44·2
YCp2μ(b)	68·0
YCp2μ(c)	48·0

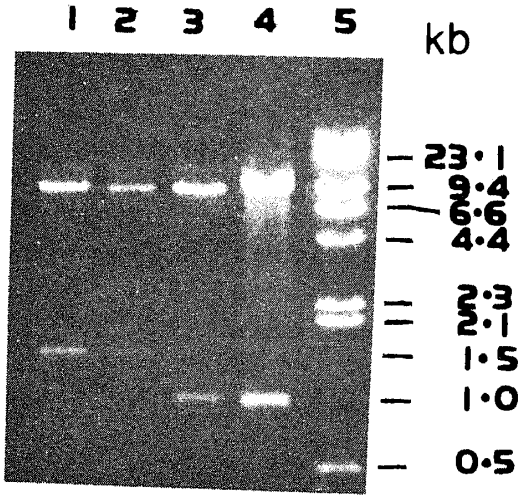

Figure 2. The plasmids YCp2μ(b) and YCp2μ(c) carry deletions in their *CEN* DNA. DNA of the wild-type plasmid YCp2μ (lane 1) and that of its mutant derivatives a, b and c (lanes 2, 3 and 4 respectively) was digested with *Bam*HI. Lane 5 is lambda DNA digested with *Hin*dIII. In derivatives b and c, the *Bam*HI fragments carrying *CEN5* DNA show a deletion of about 0·5 kb.

further. Such a colony would grow faster on selective medium. Transformants carrying stabilized plasmids were selected by virtue of large colony size on complete minimal plates lacking leucine. Plasmid stability was studied in several such transformants, and three, YCp2μ(a), Ycp2μ(b) and YCp2μ(c), showed stabilities that were higher than that of the parent plasmid in the mutant strain. Table 1 shows the stabilities of these transformants.

Plasmids YCp2μ(a), YCp2μ(b) and YCp2μ(c) were rescued in *E. coli*. Restriction analysis of the three plasmids showed that derivatives b and c both carried deletions in the *CEN5* fragment (figure 2). Subsequently, the deletion was localized to an *Eco*RI–*Xba*I fragment of *CEN5* fragment (not shown). YCp2μ(a) showed no change compared to wild-type YCp2μ upon restriction analysis using several restriction enzymes (not shown).

3.3 *Identification of the site of mutation in YCp2μ(a)*

To identify the site of mutation in YCp2μ(a), we transformed both *mcm2* cir$^+$ and *mcm2 cir*$^-$ strains with this plasmid. We reasoned that if the mutation was in *CEN5*,

abolishing its function, then the plasmid would behave like YEp13 and be stable only in cir$^+$ cells and not in cir$^-$ cells (see 3.1 above). If, on the other hand, the suppressor mutation was in the *ARS* sequence, restoring its function in the *mcm2* strain, then the mutant plasmid would be stable both in cir$^+$ and in cir$^-$ cells. Figure 3 shows the growth of transformants of *mcm2* (cir$^+$ and cir$^-$) carrying YCp2μ(a) on plates of complete minimal medium lacking leucine. The sparse growth of cells and the smaller size of colonies in patches of cir$^-$ transformants show that these cells were losing the plasmid more frequently than their cir$^+$ counterparts. This suggested that the plasmid was better maintained in cir$^+$ cells. Thus the mutation was most likely at the *CEN5* locus of this plasmid. To confirm this we subcloned each of the 1·5-kb *Bam*HI fragments carrying *CEN5* DNA from YCp2μ and from its mutant derivative YCp2μ(a) into YRp7. YRp7 is unstable in yeast since it carries only a replicator and no centromeric DNA. Cloning of a functional centromere on a yeast replicating plasmid, like YRp7, would stabilize it in the wild-type strain (Clarke and Carbon 1985). It was observed that the centromere fragment from YCp2μ(a) failed to stabilize YRp7 in the wild-type strain (table 2). This confirmed that YCp2μ(a) carried a mutation in its centromeric DNA that led to loss of its function.

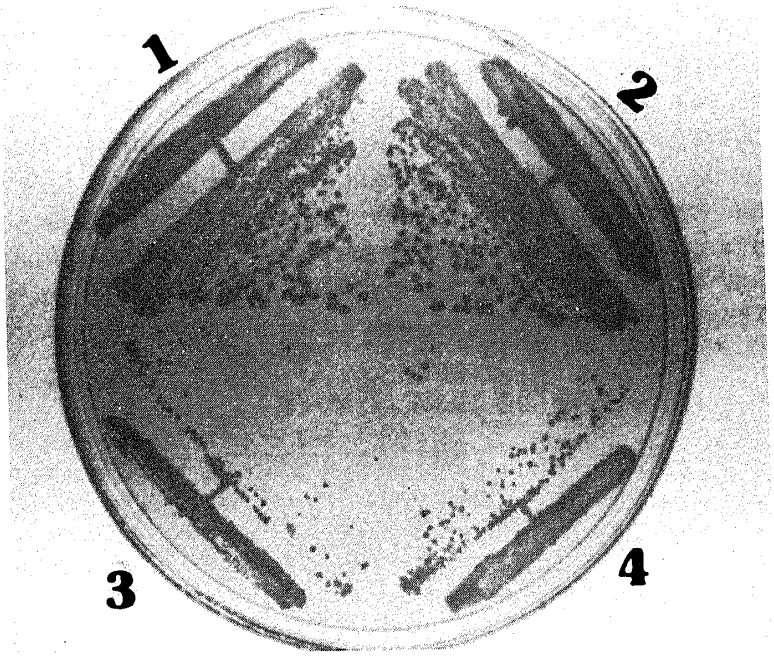

Figure 3. YCp2μ(a) is stable in *mcm2* cir$^+$ cells but not in *mcm2* cir$^-$ cells. *mcm2* transformants carrying YCp2μ(a) were streaked at 25°C on complete minimal plates lacking leucine and allowed to grow for 3 days. 1 and 2, cir$^+$ transformants; 3 and 4, cir$^-$ transformants.

Table 2. Mitotic stability of plasmid YRp7 carrying *cen5* DNA from YCp2μ(a) and *CEN5* DNA from YCp2μ.

Plasmid	Mitotic stability (%)
YRp7–*cen5*a	3·5
YRp7–*CEN5*	63·0

3.4 *Sequence of mutations in the CEN5 gene*

We subcloned the 0·9-kb *Eco*RI–*Xba*I fragments from plasmids YCp2μ(a) and YCp2μ(b) into M13mp18 and M13mp19 vectors and sequenced both strands. Figure 4 shows the positions of the mutations in the *cen5* fragments from YCp2μ(a) and YCp2μ(b). Huberman *et al.* (1986) have published the sequence of the CDE boxes of wild-type *CEN5* which is the same as the one we have obtained. YCp2μ(a) was found to have a C:G to T:A change at the 14th base pair of the CDEIII box (nucleotide number 375 of the sequence represented schematically in figure 4). The *cen5* fragment from YCp2μ(b) had a deletion corresponding to nucleotide numbers 44 to 517 of the wild-type *CEN5* sequence (entire sequence not shown). Although hydroxylamine causes C-to-T transition mutations, the observed deletion could have arisen owing to other types of lesions under extended hydroxylamine treatment (Busby *et al.* 1982).

4. Discussion

In this work we have described a positive selection scheme for isolation of mutations in centromeric DNA of *S. cerevisiae*. It has been shown earlier that any substitution at the 14th base pair in the CDEIII box of *CEN3* and *CEN6* is responsible for loss of their function. In this work we have shown that this base-pair is important for *CEN5* as well. We also picked up a deletion of the CDE boxes. This scheme should also allow us to detect chromosomal mutations that act in trans to abolish centromere function. Recently Strunnikov *et al.* (1995) have described a similar scheme, i.e. complete loss of centromere function in a composite 2μ–*cen* plasmid and its consequent selection as a high-copy-number plasmid by a positive selection. Their selection made use of the *leu2-d* mutation, which renders transformants leu$^+$ only at very high copies (Toh-e 1981). Two of the nuclear mutations isolated by them were shown to be in genes that

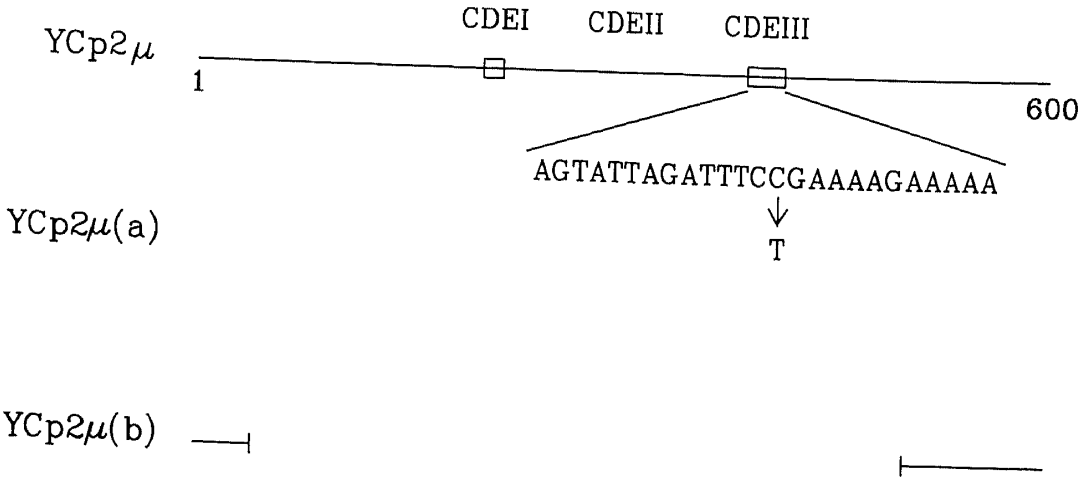

Figure 4. A schematic representation of *CEN5* DNA of yeast showing the positions of mutations in YCp2μ(a) and YCp2μ(b). Nucleotide numbering starts from the *Eco*RI site. The wild-type CDE boxes extend from nucleotide number 269 to 386. The *cen5* sequence from YCp2μ(a) was identical to the wild-type sequence except for the transition at nucleotide number 375 (14th base of CDEIII box). The single base change is shown below the wild-type sequence. The deletion in *cen5* sequence from YCp2μ(b) corresponds to nucleotide numbers 44 to 517 of the wild-type *CEN5* sequence.

code for centromere-binding proteins. We have at least two *mcm* mutants that seem to be defective in centromere function (Roy *et al.*, submitted). These mutants carry very leaky mutations. YCp2μ is unstable in them, perhaps owing to partial retention of centromere function. Apostol and Greer (1988) suggested that partial retention of centromere function in a 2μ–*CEN* composite plasmid, as achieved by transcribing *CEN* from a strong promoter like *GAL1*, interferes with the segregation and amplification properties associated with the 2μ DNA component. The plasmids are rendered unstable because the centromere-dependent segregation and the 2μ-dependent segregation amplification functions are inoperative. We are constructing tighter alleles of the *mcm* mutations which affect kinetochore function and it would be worthwhile to see if YCp2μ becomes stable in the presence of these alleles.

Although we had also set out to isolate suppressor mutations in YCp2μ *ARS* that would stabilize this plasmid in *mcm2* (see section 3.1), in view of recent findings it would be difficult to get such suppressor mutations. It has been suggested (reviewed in Marx 1995 and Chong *et al.* 1996) that the *MCM2* family of proteins do not interact directly with *ARS*s but interact with another protein complex encoded by *ORC* genes, which binds directly to *ARS*s. Therefore *ARS* suppressor mutations are more likely to be obtained in an *orc* mutant, rather than in an *mcm* mutant.

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