MUTANTS OF *S. CEREVISIAE* DEFECTIVE IN THE MAINTENANCE OF MINICHROMOSOMES

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ABSTRACT

We have isolated yeast mutants that are defective in the maintenance of circular minichromosomes. The minichromosomes are mitotically stable plasmids, each of which contains a different *ARS* (autonomously replicating sequence), a centrometeric sequence, *CEN5*, and two yeast genes, *LEU2* and *URA3*. Forty minichromosome maintenance-defective (Mcm⁻) mutants were characterized. They constitute 16 complementation groups. These mutants can be divided into two classes, specific and nonspecific, by their differential ability to maintain minichromosomes with different *ARS*s. The specific class of mutants is defective only in the maintenance of minichromosomes that carry a particular group of *ARS*s irrespective of the centromeric sequence present. The nonspecific class of mutants is defective in the maintenance of all minichromosomes tested irrespective of the *ARS* or centromeric sequence present. The specific class may include mutants that do not initiate DNA replication effectively at specific *ARS*s present on the minichromosomes; the nonspecific class may include mutants that are affected in the segregation and/or replication of circular plasmids in general.

EUKARYOTIC genomes are organized into multiple chromosomes. Each chromosome is replicated by the initiation of DNA synthesis at numerous initiation sites. The identification of autonomously replicating sequences (*ARS*s) from chromosomal DNA of yeast by cloning (STINCHCOMB, STRUHL and DAVIS 1979; BEACH, PIPER and SHALL 1980; CHAN and TYE 1980) suggests that these initiation sites are specific sequences. The mechanism of initiation of DNA replication in eukaryotes is largely unknown, except that these initiation events may be regulated (EDENBERG and HUBERMAN 1975; HAND 1978). Studies of synchronized yeast cultures (BURKE and FANGMAN 1975; KEE and HABER 1975; FANGMAN, HICE and CHLEBOWICZ-SLEDZIEWSKA 1983) and of the multinucleated *Physarum plasmodium* (BRAUN and WILI 1969) suggest that the replication of specific regions of the chromosomes may be temporally regulated. A comparison of replicating DNA in rapidly dividing embryonic cells *vs.* somatic cells of *Drosophila melanogaster* revealed that the rate of replication is partly controlled by varying the number of initiation sites (BLUMENTHAL, KNIEGSTEIN AND HOGNESS 1973). This suggests that the initiation of DNA replication is also developmentally regulated. In mammalian cells, as many as 10^5 replication origins are present per genome (HAND 1978). It seems unlikely

that each of these replication origins would be controlled independently. There may be classes of replication origins that are initiated by the same enzyme(s).

In *S. cerevisiae*, there are approximately 400 replication origins distributed among 17 chromosomes (BEACH, PIPER and SHALL 1980; CHAN and TYE 1980; NEWLON and BURKE 1980). Yeast mutants that are defective in DNA replication have been isolated. HEREFORD and HARTWELL (1974) and HARTWELL (1976) isolated a number of temperature-sensitive cell division cycle (*cdc*) mutants that seem to be blocked at various stages of DNA replication. Temperature-sensitive mutants that are unable to synthesize DNA at the nonpermissive temperature either *in vivo* or *in vitro* were also isolated (JOHNSTON and GAME 1978; DUMAS *et al.* 1982). However, such conditional lethal mutants are difficult to analyze biochemically, since the lesions can be in genes that directly inhibit cell growth but indirectly affect DNA replication. Furthermore, isolation of mutants that are defective in the initiation of DNA replication at one or a few initiation sites may require a different isolation scheme, since mutations in these regulatory genes may neither cause lethality nor arrest DNA synthesis.

*ARS*s isolated from yeast chromosomal DNA are specific sequences that enable plasmids to replicate autonomously in yeast (STINCHCOMB, STRUHL and DAVIS 1979) and are putative replication origins. Most *ARS*s are present in single copies in the yeast genome (D. REN and B.-K TYE, unpublished results). So far, only two families of repetitive *ARS*s have been identified. One family of *ARS*s is found in the tandemly arranged ribosomal gene units (SZOSTAK and WU 1979; K. DURBIN and B.-K. TYE, unpublished results). The other family of *ARS*s is found at the telomeres of most yeast chromosomes (CHAN and TYE, 1983a, b). We describe a procedure for the isolation of yeast mutants that affect the function of an *ARS* on the plasmid. We constructed circular minichromosomes that are mitotically stable and segregate like chromosomes during meiosis. Each of these minichromosomes carried a different *ARS*. We searched for mutants that are defective in the maintenance of minichromosomes with the expectation that some of them may affect the function of *ARS*s on the minichromosomes. If *ARS*s are chromosomal replication origins, this isolation procedure should yield mutants defective in genes specifying initiator proteins. If initiator proteins affect the initiation of a large number of chromosome replication origins, this procedure demands that such isolated mutants contain leaky alleles so as to permit cell growth. This scheme was first successfully used by JACOB, BRENNER and CUZIN (1963) for the isolation of *E. coli* mutants temperature-sensitive for F factor replication. This paper describes the isolation and characterization of yeast mutants defective in the maintenance of minichromosomes carrying either single copy or repetitive telomeric *ARS*s.

MATERIALS AND METHODS

Strains and plasmids: E. coli strain HB101 (*thr leuB pro recA hsr hsm*) was used as the host for plasmid amplification. Yeast strains 8534-8C (α *leu*2-3,112 *ura*3-52 *his*4-∇34) and A3 (**a** *leu*2-3,112 *his*3-11,15) and 5573-7C (**a** *his*4-712 *trp*1-1 *leu*2-3) were obtained from G. Fink. Yeast strain GM119 (**a** *leu*2-3,112 *ura*3-52) was constructed in this laboratory. The genotype of the tester strains is shown in Table 1.

TABLE 1

Tester strains used for complementation analysis

Strain	Origin of allele	Genotype
RM1-6C	Mcm1-1	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
RM3-3D	Mcm1-3	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
RM4-7B	Mcm1-4	**a** *leu2-3,112 his3-11,15 mcm*
RM8-7C	Mcm1-8	**a** *leu2-3,112 his3-11,15 mcm*
RM9-6B	Mcm1-9	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
RM10-2B	Mcm1-10	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
RM12-4A	Mcm1-12	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
RM16-4D	Mcm1-16	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
PS4-1C	Mcm131C-4	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
PS5-2D	Mcm131C-5	**a** *leu2-3,112 his3-11,15 mcm*
PS9-3C	Mcm131C-9	**a** *leu2-3,112 his3-11,15 mcm*
PS10-R18	Mcm131C-10	**a** *leu2-3,112 his3-11,15 mcm*
PS11-2A	Mcm131C-11	**a** *leu2-3,112 his3-11,15 mcm*
PS12-2B	Mcm131C-12	**a** *leu2-3 his4 ura3-52 trp1-1 mcm*
PS16-R10	Mcm131C-16	**a** *leu2-3,112 his3-11,15 mcm*
PS25-R12	Mcm131C-25	**a** *leu2-3,112 his3-11,15 mcm*
PS28-7D	Mcm131C-28	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
PS29-2B	Mcm131C-29	**a** *leu2-3,112 his3-11,15 mcm*
PS30-1D	Mcm131C-30	**a** *leu2-3,112 his3-11,15 mcm*
PS31-5A	Mcm131C-31	**a** *leu2-3,112 his3-11,15 mcm*
PS34-1D	Mcm131C-34	**a** *leu2-3,112 his3-11,15 mcm*
PS39-8B	Mcm131C-39	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
PS41-5B	Mcm131C-41	**a** *leu2-3,112 his4 ura3-52 trp1-1 mcm*
PS46-3A	Mcm131C-46	**a** *leu2-3,112 his3-11,15 mcm*
PS51-R16	Mcm131C-51	**a** *leu2-3,112 his3-11,15 ura3-52 mcm*
PS52-3A	Mcm131C-52	**a** *leu2-3,112 his3-11,15 mcm*
PS55-2A	Mcm131C-55	**a** *leu2-3 his4 trp1-1 mcm*
PS56-2C	Mcm131C-56	**a** *leu2-3 his4 trp1-1 mcm*
PS58-3C	Mcm131C-58	**a** *leu2-3 his4 trp1-1 mcm*

Plasmids YCp19, YRp10 and YRp12 were obtained from R. W. DAVIS and pYe(*CEN3*)41 from J. CARBON. Plasmid YEp13 (BROACH, STRATHERN and HICKS 1979) was obtained from J. BROACH. YCp plasmids constructed in our laboratory are shown in Figure 1.

Media and enzymes: Yeast extract peptone dextrose (YEPD), complete (CM), complete without leucine (CM-leu) and complete without uracil (CM-ura) media have been described (SHERMAN, FINK and LAWRENCE 1974). All other media, chemicals and enzymes used have been described in a previous paper (CHAN and TYE 1980).

Ethyl methanesulfonate (EMS) mutagenesis: Mutagenesis with EMS was carried out as described (SHERMAN, FINK and LAWRENCE 1974). Typically, 5 ml of stationary phase cells were treated with 0.1–0.2 ml of the mutagen for 1 hr at 30° to give about 10% survival. Mutagenized cultures were plated out to single colonies onto selective medium without prior regenerative growth.

Mitotic stability assay: Yeast transformants were streaked on a selective medium (CM-leu or CM-ura) and grown for 3 days at the appropriate temperature. About 10^4–10^5 cells were inoculated into 5 ml of YEPD and grown to saturation (ten to 15 generations). Aliquots of this culture were plated out on YEPD plates, and single colonies were then replica plated onto selective medium. The percent stability is expressed as the ratio

$$\frac{\text{number of colonies growing on CM-leu or CM-ura}}{\text{number of colonies growing on YEPD}} \times 100$$
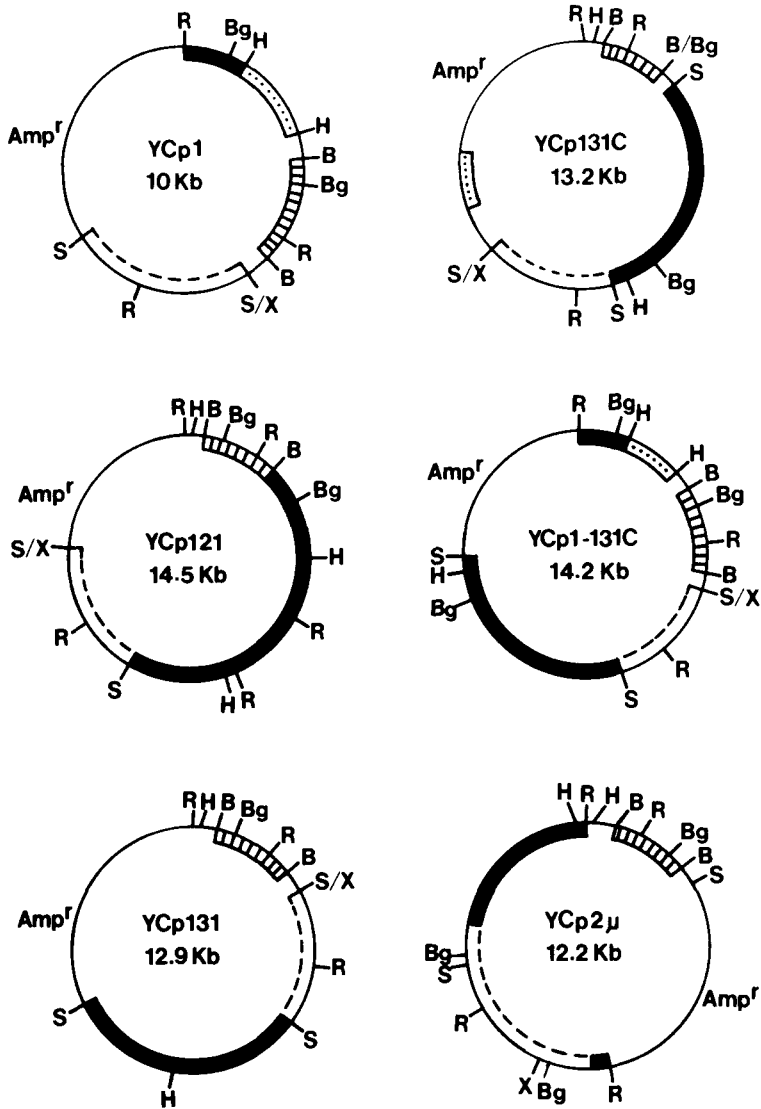
FIGURE 1.—Restriction maps of the YCp minichromosomes. ——, pBR322 DNA; ▬▬, *ARS*; ▥▥▥, *CEN5*; ▭▭, *URA3*; ▭▭, *LEU2*. R, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; X, *Xho*I. In YCp2μ, ▬▬ represents the 2μ plasmid DNA with the *ARS* located in larger fragment. In YCp1-131C, *ARS131C* is the larger of the two fragments represented by ▬▬. The *Hin*dIII/ *Eco*RI fragment, which contains *ARS1* in YCp1, was derived from YRp10. The *Sal*I/*Bam*HI fragment, which contains *ARS121* in YCp121, was derived from YRp121 (CHAN and TYE 1980). The *Sal*I fragments, which carry *ARS131* and *ARS131C* in YCp131 and YCp131C, respectively, were derived from YRp131 and YRp131C (CHAN and TYE 1983a,b). YCp1-131C was constructed by the insertion of the *Sal*I fragment, which contains *ARS131C*, into the *Sal*I site of YCp1.

Other procedures: Yeast transformation was carried out as described by HINNEN, HICKS and FINK (1978). Large-scale plasmid DNA was prepared by the method of BIRNBOIM and DOLY (1979), and minipreparations of plasmid DNA were prepared by the rapid boiling technique (HOLMES and QUIGLEY 1981). Yeast strains were cured of the 2μ plasmid as described (TOH-E and WICKNER 1981).

Complementation analysis: All complementation analyses between mutants of the Mcm1 class were performed by assaying the stability of YCp1. Similarly, complementation analyses between mutants of the Mcm131C class were performed by assaying the stability of YCp131C. For complementation analyses involving specific mutants of the Mcm1 or Mcm131C class, YCp1 was always used. All other complementation analyses between nonspecific mutants of the Mcm1 and Mcm131C classes were performed by using YCp131C.

RESULTS

Construction of minichromosomes: Most autonomously replicating plasmids in yeast are lost from cells at a relatively high frequency. For example, and *ARS1*-containing plasmid, YRp12 (SCHERER and DAVIS 1979), is only retained by less than 5% of the cells in a culture after about ten generations of nonselective growth. However, these plasmids can be stabilized by the introduction of a centromeric sequence (CLARKE and CARBON 1980). We constructed a series of minichromosomes each carrying an *ARS*, a centromeric sequence, and two yeast markers, *LEU2* and *URA3*. These minichromosomes differ essentially only in their *ARS*s (Figure 1). *ARS1* (STINCHCOMB, STRUHL and DAVIS 1979) and *ARS121* (CHAN and TYE 1980) are present in single copies in the yeast genome, whereas *ARS131* and *ARS131C* are repetitive *ARS*s located at the telomeres of yeast chromosomes (CHAN and TYE 1983a,b). *CEN5* (MAINE, SUROSKY and TYE 1984), which was isolated by a method similar to that described by HSIAO and CARBON (1981), was the centromeric sequence present on these minichromosomes. Unlike *CEN3* (CLARKE and CARBON 1980), *CEN5* contains no *ARS* activity. The minichromosomes just described have been designated YCp1, YCp121, YCp131 and YCp131C, respectively. YCp2μ was constructed by the insertion of *CEN5* into the plasmid YEp13, which contains the 2μ circle origin of replication (BROACH, STRATHERN and HICKS 1979). YCp1-131C contains both *ARS1* and *ARS131C*. All of the centromere-containing plasmids constructed for this study are retained by more than 60% of the cells in a culture of the wild-type yeast strain 8534-8C after 20 generations of nonselective growth.

Isolation of mutants defective in minichromosome maintenance: The scheme for the isolation of mutants that are defective in the maintenance of the YCp minichromosomes is outlined in Figure 2. Yeast strain 8534-8C was transformed to leucine and uracil prototrophy by YCp1 and YCp131C. The transformants, 8534-8C[YCp1] and 8534-8C[YCp131C], were mutagenized with EMS. The mutagenized cultures were plated on a complete medium lacking leucine (CM-leu) or on a complete medium lacking uracil (CM-ura) and incubated at 23°. All colonies that grow at 23° on selective medium should contain minichromosomes. To identify clones that were unable to stably maintain the minichromosomes at high temperature (35°), colonies growing on selective medium at 23° were replica plated onto complete medium and incubated at 35°. Residual plasmids in the mutant clones were diluted by repeating this replica-plating procedure before a final replica plating on complete and selective media. Any colony that was both Ura⁻ and Leu⁻ at 35°, but not temperature sensitive for growth, was examined further.

Putative mutants were assayed for minichromosome stability (see MATERIALS AND METHODS). Those that had minichromosome mitotic stability of 10% or

8534-8C[YCp1] or 8534-8C[YCp131C]

↓

EMS mutagenesis

↓

Plate out mutagenized culture for
single colonies on CM-ura,
or CM-leu, 23°

↓

Replica plate to CM, incubate at 35°
for 1 day

↓

Repeat

↓

Replica plate and incubate at 35°
for 1 day

CM   CM-ura   CM-leu

Mutant phenotype
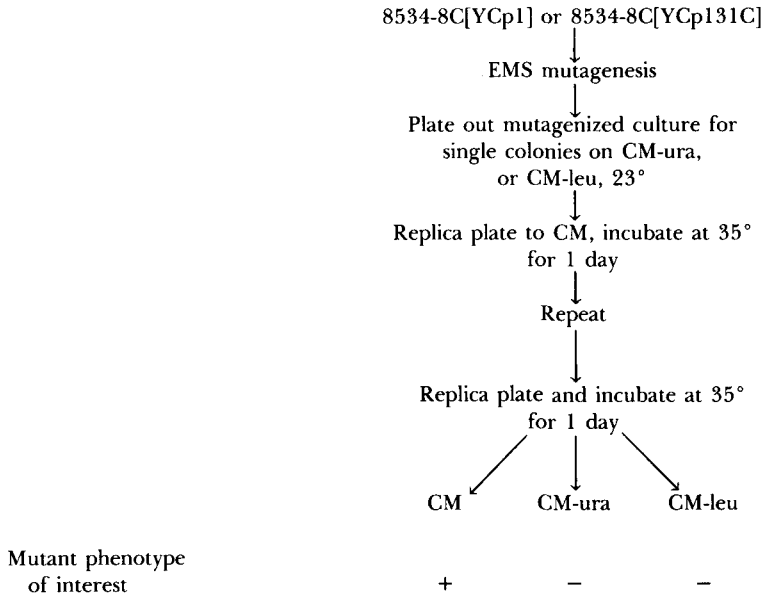of interest   +   −   −

FIGURE 2.—Procedure for the isolation of mutants defective in the maintenance of YCp1 and YCp131C minichromosomes.

less at 35° were scored as mutants. Wild-type cells under these conditions consistently gave at least a fivefold higher stability for the same minichromosome. Table 2 provides a representative sample of the stabilities of minichromosomes in different mutants. With 8534-8C[YCp1], ten of 15,000 colonies scored were found to be defective in the maintenance of YCp1. With 8534-8C[YCp131C], 30 of 60,000 colonies scored were found to be defective in the maintenance of YCp131C. Most of the mutants isolated under these conditions were defective in the maintenance of minichromosomes at all temperatures. The mutants are designated as Mcm1-1, Mcm1-2, etc., for those isolated from 8534-8C[YCp1] and Mcm131C-1, Mcm131C-2 etc., for those isolated from 8534-8C[YCp131C].

Minichromosome maintenance defect is due to chromosomal mutations: The Mcm⁻ phenotype of each of the mutants was due to a single chromosomal mutation. This was determined by two criteria. When mutants were cured of their original minichromosomes and retransformed with new minichromosomes, the Mcm⁻ phenotype was retained. Furthermore, when these mutants were crossed to the wild-type strain GM119, the resultant diploids all yielded $2^+:2^-$ meiotic segregants for minichromosome stability. In all of these cases, an average of five or more tetrads were analyzed for each mutant. For example, the mutant Mcm1-9 was isolated for its inability to stably maintain the minichromosome YCp1. When it was crossed with a wild-type strain, the resultant diploid yielded $2^+:2^-$ segregants for the stability of YCp1 (Table 3). Similarly, tetrad analysis of the heterozygous diploid resulting from a cross between the mutant Mcm131C-46 and the wild-type strain showed that the temperature-sensitive Mcm⁻ phenotype for YCp131C segregated in a Mendelian fashion (Table 3).

TABLE 2

Mitotic stabilities of minichromosomes in some of the mutants

Strain	Minichromosomes	% Stability	
		Haploid	Heterozygous diploid (mutant × GM119)
8534-8C (WT)	YCp1	75	60
8534-8C (WT)	YCp131C	86	70
Mcm1-1	YCp1	0.5	53
Mcm1-3	YCp1	0.5	55
Mcm1-9	YCp1	<0.5	55
Mcm1-10	YCp1	2	16
Mcm1-16	YCp1	75/2	54/51
Mcm131C-46	YCp131C	60/2	80/81
Mcm131C-46	YCp1	<1/<1	38/21

Stability assays for the mutants Mcm1-16 and Mcm131C-46 were carried out at 23° and 35°. The percent stability is indicated as (% at 23°)/(% at 35°). Stability assays for all other strains were performed at 30°.

TABLE 3

Mitotic stabilities of minichromosomes in tetrads from mutant × wild-type diploids

	Mcm1-9 × A3							
Minichromosome	2A	2B	2C	2D	6A	6B	6C	6D
YCp1	<0.5	<0.5	70	79	<0.5	<0.5	69	64
YCp121	39	34	68	95	17	53	85	75

	Mcm131C-46 × A3							
Minichromosome	1A	1B	1C	1D	3A	3B	3C	3D
YCp1	95	<0.2	0.2	87	<0.2	94	<0.4	98
YCp121	94	70	72	96	75	91	75	93
YCp131	33	<0.3	<0.2	94	<0.2	56	<0.3	66
YCp131C	98	70	72	89	52	92	55	97
YCp131C (35°)	89	8.6	<0.5	87	1.5	80	<0.5	87

All stability assays were carried out at 30° unless otherwise specified. A total of 20 tetrads were analyzed for the Mcm1-9 × A3 diploid, and ten tetrads were analyzed for the Mcm131C-46 × A3 diploid.

This suggests that the Mcm⁻ phenotype of these mutants results from single gene mutations.

The heterozygous diploids were also examined for the stability of the minichromsomes to determine whether the mutations are recessive or dominant. Both types of mutations were found. Table 2 shows examples of each type of mutation. However, none of the mutations showed complete dominance, since the stability of the plasmids in the heterozygous diploids was always at least tenfold higher than that of the corresponding homozygous mutant diploid (data not shown). The intermediate stabilities of YCp1 in the mutants Mcm1-10 and Mcm131C-46 exemplify such partially dominant mutations (Table 2).

Complementation analysis: The 40 Mcm⁻ mutants were arranged into 16 complementation groups by standard genetic techniques (Tables 4 and 5). The

TABLE 4

Complementation analysis table

Mutants	Testers MATa / MATα	1 (1-) 1	1 (1-) 12	1 (131C-) 4	1 (131C-) 9	1 (131C-) 28	1 (131C-) 56	2 (1-) 3	2 (131C-) 5	2 (131C-) 55	3 (131C-) 9	4 16	5 (1-) 10	6 4	6 8	7 11	7 30	7 41	7 58	8 16	9 25	10 29	11 (131C-) 31	12 12	13 34	13 39	14 46	15 51	16 52
1	1-1	−	−	−	−			+				+		+	+														
	1-12	−	−	−	−			+	+			+	+	+	+														
	131C-4	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-6	−		−	−	−	−	+	+	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-9	−		−		−	−	+	+	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-28	−		−	−	−	−	+	+	+	+	+	+			+				+	+	+	+	+	+	+	+	+	
	131C-56	−		−	−		−	+			+	+	+																
2	1-3	+	+					−	−	−	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-5	+	+		+			−	−	−	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-23	+	+	−	−	+	−	−	−	−	+	+	−	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-38	+		−	−		−	−	−	−	+	+	−	+	+	+				+	+	+	+	+	+	+	+	+	
	131C-55	+		−	−		−	−	−	−	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	
3	1-9	+	+					−				+	+	+	+														
4	1-16	+	+					+	−			+	+	+	+														
5	1-10	+	+					+	−	−	+	+	−	−	+														
	1-14	+	+					+		−	+	+	−	+	+														
6	1-4	+	+					+	+	+	+	+	+	−	−														
	1-8	+	+					+	+	+	+	+	+	−	−														
	1-11	+	+					+	+	+	+	+	+	−	−	+				+	+	+	+	+	+	+	+	+

7	131C-11	+ +
	131C-30	+ +
	131C-33	+
	131C-41	+
	131C-58	+
	131C-63	+
8	131C-16	+
9	131C-25	+
10	131C-29	+
11	131C-31	+ +
	131C-17	+ +
12	131C-34	+ +
	131C-10	+ +
	131C-12	+
	131C-20	+
13	131C-39	+ +
	131C-62	+ +
14	131C-46	+
15	131C-51	+
16	131C-52	+
	131C-22	+

+ indicates that there is complementation between the two strains involved as measured by the stability of YCp1 or YCp131C in the diploid (see text). − indicates that there is no complementation between the two strains involved as measured by the instability of YCp1 or YCp131C in diploid (see text). The topmost and leftmost numbers are numbers for the complementation groups.

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TABLE 5

Genetic complementation groups of Mcm⁻ mutants

| Complementation groups | Mcm⁻ mutants for | |
	YCp1	YCp131C
1	1-1, -12	131C-4, -6, -9, -28, -56
2	1-3	131C-5, -23, -38, -55
3[a]	1-9	
4	1-16	
5[a,b]	1-10, -14	
6[a]	1-4, -8, -11	
7		131C-11, -30, -33, -41, -58, -63
8		131C-16
9		131C-25
10		131C-29
11		131C-31, -17
12		131C-34, -10, -12, -20
13		131C-39, -62
14[a,b]		131C-46
15		131C-51
16		131C-52, -22

[a] Mutants of these complementation groups show specificity for different *ARS*s.
[b] Mutants in these complementation groups show partial dominance for YCp1.

initial tester strains of the **a**-mating type (RM1-6C, RM3-3D, RM9-6B) were constructed from several mutants isolated from 8534-8C[YCp1] (Table 1). Pairwise crosses of all mutants against these tester strains were carried out, and the stability of YCp1 or YCp131C (as appropriate, see MATERIALS AND METHODS) in the resultant diploids was assayed at the restrictive temperatures. Mcm⁻ mutants that complemented all of these tester strains were crossed with the wild-type strain A3 or 5573-7C. Diploids were selected, sporulated, and **a** and α Mcm⁻ segregants were obtained. Complementation analysis using YCp1 or YCp131C was performed again. In this way, new tester strains of the **a**-mating type were generated as the analysis was repeated (Table 1). For complementation groups that are represented by more than one mutant, pairwise crosses between mutants of opposite mating types in the same complementation groups were carried out (Table 4). In the analysis of diploids from crosses involving mutants that show partial dominance, intermediate stabilities (*i.e.*, stabilities that are at least tenfold higher than the homozygous diploids of partially dominant mutants) were considered to be due to complementation. In cases in which there was ambiguity in the interpretation of the complementation data, tetrads were analyzed to determine the linkages of the mutations concerned. Unlinked mutations were considered to be in different complementation groups. By this procedure (Table 4), 16 complementation groups were identified (Table 5). Complementation groups 1 and 7, which contain seven and six members, respectively, were most frequently represented among the 40 mutants analyzed.

Specificity of the mutants for minichromosomes with different ARS*s:* The instability

of minichromosomes in the mutants that we have isolated could be due to defects either in the replication of the minichromosomes or in their segregation into daughter cells. We reasoned that, if the mutants showed specificity for particular ARSs, they would be more likely to be altered in plasmid replication than in segregation. To examine ARS specificity, mutants were cured of their original minichromosomes and transformed with the tester minichromosomes shown in Figure 1. Stability assays were then performed. An example from each complementation group is shown in Table 6.

The mutants can be divided into two classes according to their ability to maintain minichromosomes with different ARSs. Mutants that affected mini-chromosome stability irrespective of the ARS were classified as nonspecific. Such nonspecific mutants could be defective in functions that affect the proper segregation and/or replication of circular plasmids in general. Most of the mutants analyzed belong to this class. Mutants that affected the stability of minichromosomes carrying some, but not all, of the ARSs were classified as specific. For example, Mcm1-9 affects the stability of minichromosomes YCp1 and YCp131 more drastically than that of YCP121 or YCp131C (Table 6). Mutants in four of the complementation groups (3, 5, 6 and 14) exhibit this behavior. The same specificity observed for Mcm1-9 is shared by all seven specific mutants, even though they were isolated independently from two different screens, and they belong to four different complementation groups. Furthermore, this common specificity extends beyond the four ARSs reported here to more than 12 ARSs (Table 7). The mutant Mcm131C-46 differs from the other specific mutants in that its specificity for different minichromosomes is temperature dependent. At 25°, it affects the stability of YCp1 and YCp131 but not of YCp131C or YCp121. However, at 35°, it affects the stability of all minichromosomes tested (Table 3). This mutant gene product, which has a temperature-dependent ARS specificity, may be an example of a trans-acting product that has differential affinity for different minichromosomes.

If the instability of a minichromosome in the specific mutants is due to defects that are targeted at ARS, then stability may be restored to this mini-chromosome by the introduction of an ARS that is functional in these mutants. When YCp1-131C, which contains both ARS1 and ARS131C, was introduced into each of the specific mutants, the minichromosome became as stable as YCp131C (Table 6). This result is consistent with the idea that the instability of the minichromosomes under study is ARS dependent.

The 2μ plasmid is an endogenous yeast plasmid that exists in high copy number in the nucleus (LIVINGSTON and HAHNE 1979; NELSON and FANGMAN 1979). We wanted to determine whether these Mcm⁻ mutants, which are unable to maintain minichromosomes carrying chromosomal ARSs, also have an effect on the ARS of the 2μ plasmid. Each of the mutants appeared to maintain the 2μ plasmid as determined by colony hybridization with ^{32}P-labeled 2μ plasmid DNA (data not shown). All mutants, except for Mcm131C-46 at 35°, have a stable Leu⁺ phenotype when transformed with the plasmid YEp13 (BROACH, STRATHERN and HICKS 1979), which contains the ARS of the 2μ plasmid and the LEU2 gene of yeast cloned into pBR322 (Table 6). In contrast,

TABLE 6

Mitotic stabilities of minichromosomes in representative mutants of each complementation group

Comple-mentation groups	Strains	YCp						YEp13
		1	121	131	131C	1-131C	2μ	
1	1-1	<0.5	<0.5	<0.5	<0.5	<0.5	0.5	45
2	1-3	<0.5	<0.5	<0.5	<0.5	2.0	1.1	48
3	1-9	<0.5	54	<0.5	39	57	3.1	46
4	1-16							
	(23°)	65	55	19	65	ND	55	40
	(35°)	1.0	<0.5	<0.5	<0.5	5	0.5	31
5	1-10	<0.5	98	<0.5	96	65	11	45
6	1-11	<0.5	59	<0.5	56	55	<0.5	39
7	131C-11	7	<1	1.0	10	13	3	55
8	131C-16	<1	4.0	0.5	1.5	ND	<1	40
9	131C-25	<1	<1	<1	0.8	ND	<1	76
10	131C-29	4.0	<1	<0.3	2.0	ND	<1	75
11	131C-31	25	10	8	4	ND	12	80
12	131C-34	15	6	10	6	ND	3.0	65
13	131C-39	2.5	<1	0.2	3	7	0.2	60
14	131C-46							
	(23°)	<0.3	67	<0.3	60	89	4.0	50
	(35°)	<0.3	<0.2	<0.3	<0.5	12	0.5	10
15	131C-51	25	1.5	9.5	1.3	ND	5	71
16	131C-52	28	14	8.0	22	ND	11	54
	WT							
	(25°)	75	79	62	86	99	76	43
	(35°)	80	95	70	92	72	86	80

All assays were carried out in Cir$^+$ strains at 30° if not indicated otherwise in parentheses. The stabilities of different minichromosomes (including those shown in Table 8) in each strain were determined simultaneously. The stability of YCp2μ was the same in Cir$^+$ or Cir^0 strains of Mcm1-3, Mcm1-9, Mcm1-10 and Mcm131C-46 (data not shown). Although the minichromosomes constructed are not exactly isogenic in terms of the location and orientation of the *ARS* and *CEN* sequences on the plasmid, we have cloned *ARS1* in either orientation in other minichromosomes carrying different markers and have tested them in some of the Mcm$^-$ mutants. There is no detectable difference in the stability of these *ARS1*-containing minichromosomes in any given mutant. (See also Table 7 for stabilities of isogenic minichromosomes that carry different *ARS*s.)

the stable Leu$^+$ phenotype was not observed in any of the mutants, specific or nonspecific, for the minichromosome YCp2μ (YEp13 containing *CEN5*, Figure 1). This apparent difference in the stability of the two plasmids is probability due to the difference in their copy numbers in the cell (see DISCUSSION). These results suggest that the replication of the 2μ plasmid is also affected by the specific, as well as nonspecific, mutants.

Behavior of telomeric ARSs in some of the mutants: We have shown that the specific class mutants share the same specificity for *ARS*s. *ARS*s can thus be divided into two groups based on the specificity of these mutants. *ARS131* and *ARS131C* are two telomeric class X *ARS*s (CHAN and TYE 1983a), yet there are affected quite differently in the specific mutants. In order to examine how other telomeric *ARS*s are affected in these specific mutants, we constructed a series of isogenic minichromosomes each containing a different telomeric *ARS*.

TABLE 7

Mitotic stabilities of minichromosomes containing telomeric ARSs

| | YCp | | | | | | | |
| | Class X | | | | | | Class Y | |
Strains	120	131A	131C	131J-A	131N	206	131B	131S
WT	95	80	93	98	96	97	80	84
1-1	<0.5	<0.2	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
1-9	42	0.4	57	46	61	77	<0.3	<0.5
131C-46								
(23°)	43	7	60	55	85	62	5	3
(35°)	<1	<1	0.5	<1	8	12	<1	<1

All minichromosomes are isogenic to YCp131C (Figure 1), and they differ only in the *ARS* that is inserted in the *Sal*I site of the minichromosome. The *Sal*I fragments that contain *ARS120*, *ARS131A*, *ARS131C*, *ARS131N*, *ARS206*, *ARS131B* and *ARS131S* are derived from YRp120, YRp131A, YRp131C, YRp131N, YRp206, YRp131B and YRp131S. The *Sal*I/*Xho*I fragment that contains *ARS131J-A* is derived from YRp131J (CHAN AND TYE 1983a).

There are two classes of telomeric *ARS*s, X and Y, which are defined by the homologies of their surrounding sequences (CHAN and TYE 1983a). The class Y *ARS*s are embedded within the highly conserved Y' sequences which are about 6.7 kb in size. *ARS131B* and *131S* belong to this class. The class X *ARS*s are embedded within the less conserved homologous X sequences, which vary from 0.3 to 3.75 kb. Included in this class are *ARS131*, *ARS120*, *ARS131A*, *ARS131C*, *ARS131J-A*, *ARS131N* and *ARS206*. These X and Y' sequences are located near the telomeres with the X sequences centromere proximal to the Y' sequences (CHAN and TYE 1983b). Because of the highly conserved nature of the Y' sequences, it is most likely that the class Y *ARS*s are also highly conserved in sequence. However, the same assumption cannot be made for the class X *ARS*s because of the heterogeneity of the X sequences.

Isogenic minichromosomes, each containing one of the following *ARS*s, were constructed. They are *ARS120*, *ARS131A*, *ARS131C*, *ARS131J-A*, *ARS131N*, *ARS206*, *ARS131B* and *ARS131S*. The minichromosomes were introduced into the mutants Mcm1-1 (a nonspecific mutant), Mcm1-9 and Mcm131C-46 (two specific mutants). The stability of each of these isogenic minichromosomes was determined as shown in Table 7. As expected, the nonspecific mutant Mcm1-1 affected the stability of all minichromosomes. In contrast, only the stability of minichromosomes carrying the class Y *ARS*s (YCp131B and YCp131S) and one of the class X *ARS*s (YCp131A) was affected in the specific mutants Mcm1-9, and to a lesser degree, Mcm131C-46 at 23°. It is not surprising that the class Y *ARS*s behave similarly due to their likely homology (CHAN and TYE 1983b). The fact that the stability of two (YCp131A and YCp131 from Table 6) of the seven class X *ARS*s tested in the specific mutants was affected indicates that the class X *ARS*s may be more different than their similar surrounding sequences and telomeric locations suggest.

The effect of the mutants on minichromosomes with different centromeres: The *ARS* confers autonomous replication to the minichromosome, whereas the centro-

meric sequence confers mitotic and meiotic stability. In principle, instability of the minichromosome could be due to mutations directed at the *CEN5* sequence or at the *ARS*. Analysis of the specificity of the mutants for different *ARS*s indicated that some of the mutants can discriminate *ARS*s and, therefore, may have defects targeted at the *ARS* on the minichromosomes. To determine whether the mutants have any specificity for the centromeric sequences, the stabilities of minichromosomes, each carrying *ARS1* and a different centromere, were compared in representative mutants from each complementation group (Table 8). The minichromosome pYe (*CEN3*)41 (CLARKE and CARBON 1980) contains *CEN3*, whereas the minichromosome YCp19 contains *CEN4* (STINCHCOMB, MANN and DAVIS 1982). Regardless of which centromeric sequence was present, the minichromosomes showed decreased stabilities in all of the mutants, suggesting that none of the mutants isolated so far has an effect on a specific centromere. However, we cannot rule out that some of these mutants, especially those in the nonspecific class, may have a general effect on all centromeres.

Kinetics of the loss of minichromosomes from mutants: The mutant Mcm131C-46 affects the stability of minichromosomes YCp1 and YCp131 at 23°, but at 35° it affects the stability of all of the minichromosomes tested (Table 6). This phenotype might be explained by the differential affinity of the mutant protein

TABLE 8

Mitotic stabilities of minichromosomes with different centromeric sequences in Mcm⁻ mutants

Complementation groups	Strains	pYE(CEN3)41	YCp19	YCp1
		CEN3, ARS1	CEN4, ARS1	CEN5, ARS1
	WT 8534-8C	98	19	75
1	1-1	1	<1	<0.5
2	1-3	–[a]	<2	<0.5
3	1-9	4	<1	<0.5
4	1-16 (35°)	–	<1	1
5	1-10	1	–	<0.5
6	1-8	<1	–	<0.5
7	131C-11	10	–	7
8	131C-16	<1	–	<1
9	131C-25	<1	–	<1
10	131C-29	5	–	4
11	131C-31	25	–	25
12	131C-34	19	–	15
13	131C-39	5.4	–	2.5
14	131C-46			
	(23°)	6	–	<0.3
	(35°)	2	–	<0.3
15	131C-51	15	–	25
16	131C-52	35	–	28

All stability assays were carried out at 30° unless specified otherwise. The number in column under YCp1 is the same as those shown in Table 6 since these assays and those shown in Table 6 were carried out simultaneously.

[a] Not done.

for the different *ARS*s present in the minichromosomes. If this interpretation is correct, the differential action of the mutant protein on each of the minichromosomes should be reflected in the rate of loss of each of the minichromosomes from the mutant at various temperatures.

The mutant Mcm131C-46 is defective in the maintenance of YCp1 at all temperatures but temperature sensitive for the maintenance of YCp131C (Table 6). We compared the rate of loss of YCp1 and YCp131C at different temperatures in this mutant and a wild-type strain. A log-phase culture of each strain, containing either YCp1 or YCp131C, growing at 23°C in selective medium, was diluted into nonselective medium at 23°, 30° or 35°. Cells were maintained in the exponential phase of growth. At various times, aliquots were plated on selective and nonselective media at the appropriate temperatures. With no replication and/or segregation of minichromosomes into daughter cells, the fraction of cells capable of growing on selective medium (*i.e.*, those with minichromosomes) should be 50% of that of the previous generation. On the other hand, if the minichromosome replicates and segregates every time the cell divides, we should not expect any decrease in successive generations in the fraction of cells carrying the minichromosome. Intermediate levels of replication and/or segregation will generate intermediate stabilities.

The wild-type cells showed essentially no loss of the YCp131C or YCp1 minichromosome over ten generations at 23°, 30° and 35° (data are shown only for YCp131C at 35° in Figure 3). In contrast, the mutant showed tem-
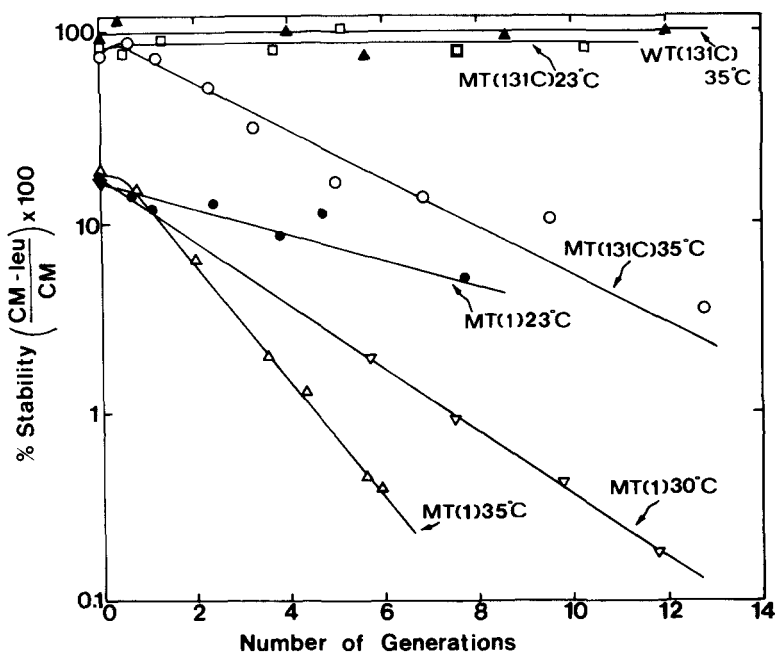
FIGURE 3.—Kinetics of the loss of the minichromosomes YCp1 an YCp131C in the mutant Mcm131C-46 (MT) and wild type (WT) at various temperatures. The curves for the loss of YCp131C and YCp1 in the wild type were the same at all temperatures as that shown for YCp131C at 35°. The number in parentheses indicates the minichromosomes under study.

perature sensitivity for the maintenance of both YCp1 and YCp131C. YCp1 was more unstable at both temperatures, and at 35°, the rate of loss of this minichromosome was close to that expected if no replication and/or segregation took place. At 35°, YCp131C, although unstable, still replicated such that it took approximately two generations for the fraction of minichromosome-bearing cells to be reduced to half the initial value. We have performed similar experiments with other mutants. However, in most cases, the rate of minichromosome loss was less than that expected if no transmission/replication of the plasmid occurred.

Growth rates of mutants: If ARSs serve as chromosomal replication origins and if some of our mutants are actually defective in the initiation of DNA replication at a large number of ARSs, then one might expect that these mutants would be affected in their chromosomal replication and, in turn, in their growth rates. Generation times of the mutants were determined by plating appropriately diluted cell cultures on complete and YEPD plates. At 23°, one of the specific mutants, Mcm131C-46, had a generation time of 3 hr in YEPD, which is similar to that of the wild type at 23°. However, at 35°, Mcm131C-46 had a doubling time of 4 hr, which is twice as long as that of the wild type (2 hr) at that temperature. In eight tetrads obtained from the M131C-46 × wild-type diploid, the temperature sensitivity for growth phenotype segregated with the minichromosome maintenance defect. This suggests that these two phenotypes are caused by the same mutation. Examination of other mutants indicated that most of them are affected little, if at all, in their growth rates.

DISCUSSION

Autonomously replicating sequences are functionally defined by their ability to confer the capacity of autonomous replication on small circular plasmids (STINCHCOMB, STRUHL and DAVIS 1979; BEACH, PIPER and SHALL 1980; CHAN and TYE 1980). Previous studies suggest that the replication of plasmids carrying chromosomal ARSs resembles that of chromosomal DNA (CHAN and TYE 1980; ZAKIAN and SCOTT 1982; FANGMAN, HICE and CHLEBOWICZ-SLEDZIEWSKA 1983). Although there is no direct evidence for the equivalence of chromosomal ARSs and chromosomal replication origins in yeast, similarly isolated sequences from the 2μ plasmid (BROACH and HICKS 1980) and *E. coli* chromosome (YASUDA and HIROTA 1977) are known to be replication origins of their native genomes. We have isolated yeast mutants that are defective in the maintenance of minichromosomes that carry yeast chromosomal ARSs. From the properties of these mutants, it is likely that some of them have defects targeted at the ARSs. If ARSs serve as chromosomal replication origins, then studies of mutants that affect the function of ARSs should be useful in the understanding of the mechanism of replication initiation at chromosomal origins.

We have used two minichromosomes, YCp1 (which contains *ARS1*) and YCp131C (which contains a telomeric ARS, *ARS131C*) in two independent screenings to isolate yeast mutants that are defective in the maintenance of minichromosomes. Forty mutants, making up 16 complementation groups,

were isolated. Two classes of mutants were found among these 16 complementation groups: those that discriminate minichromosomes by their ARSs and those that affect the maintenance of all minichromosomes irrespective of their ARSs. None of the mutants shows a specific effect on any centromeric sequence. We argue that those mutants that show specificity for certain ARSs do so by affecting the function of ARS but not other sequences on the minichromosome. This is supported by the observation that a "functional" ARS suppresses a "nonfunctional" ARS on the same minichromosome in these mutants. The epistasis of the functional ARS suggests that the instability of minichromosomes is due to inactivation of the nonfunctional ARS and not due to degradation of the minichromosomes. We argue against defective segregation being the cause of the instability of the minichromosomes, since this instability is ARS dependent but not CEN dependent. In fact, the mutant Mcm131C-46 seems to affect the plasmid YEp13, which does not contain a centromere (Table 6). However, we cannot rule out that segregation may be ARS dependent.

We do not know the exact nature or mechanism by which these mutations might affect the initiation of DNA replication at the ARS on the minichromosomes. To explain why mutants from different complementation groups exhibit specificity for the same set of ARSs, it is possible that a small number of proteins are involved in the initiation of a large number of replication origins and that these proteins work together as subunits in an initiation enzyme complex. This could account for the common and broad specificity seen in mutants in different complementation groups. Control could be exerted by this small number of enzymes on the large number of replication origins by the differential affinity of the initiator proteins for the numerous replication origins. This model can be used to explain the phenotype of the mutant, Mcm131C-46. This mutant selectively maintains certain minichromosomes at low temperature, but at high temperature, it affects all minichromosomes. This phenotype could be attributed to the differential affinity of the gene product for the different ARSs, a property that would be enhanced by the mutation.

We have also tested the effect of our mutants on the ARS of the 2μ plasmid. The replication of the 2μ plasmid is probably controlled by the same mechanism that controls chromosomal DNA replication, since cell division cycle (cdc) mutants that block the initiation of chromosomal DNA replication also block the replication of 2μ plasmid (LIVINGSTON and KUPFER 1978). All of our mutants, with the exception of Mcm131C-46, maintained the endogenous 2μ plasmid as well as YEp13 but were unable to maintain minichromosome YCp2μ, which consists of the 2μ plasmid origin and a centromere. Like the 2μ plasmid, YEp13 is maintained in wild-type cells at about 20–30 copies per cell (CLARK-WALKER and MIKLOS 1974; BROACH, STRATHERN and HICKS 1979; BROACH and HICKS 1980). However, when a centromere is introduced into the 2μ plasmid, i.e., YCp2μ, it is no longer amplified, and it segregates $2^+:2^-$ during meiosis (TSCHUMPER and CARBON 1983). It is possible that some of our mutants affected the 2μ plasmid replication, but amplification of the copy number of YEp13 concealed this effect. In the case of YCp2μ, which is main-

tained at about one copy per cell, its underreplication would then result in the unstable phenotype.

In this paper, we described how we used the large number of *ARS*s that we isolated from the yeast chromosomes to isolate mutants that may affect their function as *ARS*. The isolation of the mutants that are specific for a particular group of *ARS*s may, in turn, help us to understand the structure of these *ARS*s. A comparison of the DNA sequences of *ARS*s that belong to the same group (*i.e.*, affected by the specific mutants) may be more informative than the comparison of sequences between unrelated *ARS*s. Recent work on the sequence analysis of *ARS*s suggests that there is no common feature (such as direct or inverted repeats) and little homology (except for a ten-base pair consensus sequence) found among *ARS*s (STINCHCOMB *et al.* 1981). Furthermore, the presence of the consensus sequence is neither necessary (G. FINK, personal communications) nor sufficient (J. BROACH, personal communications) for the function of *ARS*s. (Two of the *ARS*s examined so far do not contain this consensus sequence, and the *MAT*a gene contains such a sequence but has no *ARS* activity.) It is possible that *ARS*s are divided into different classes according to their mode of regulation and that only *ARS*s that belong to the same group share common features besides the ten-base pair consensus sequence. Our study of the telomeric *ARS*s indicates that two of the class X *ARS*s, *ARS131* and *ARS131A*, differ from the other class X *ARS*s in that they are affected in the specific mutants. A comparison of the DNA sequences of these two *ARS*s and that of *ARS1*, *ARS131B* and *ARS131S* (which are also affected in the specific mutants) may help up to identify the common feature that results in their inactivation in the specific mutants.

The mutants in the nonspecific class are defective in the maintenance of all minichromosomes tested. We doubt that any one of them loses its natural chromosomes at a high frequency, since none of them shows sectoring of colonies when grown on YEPD, a colony morphology indicative of the generation of lethal cells. We have also examined several mutants for the loss of chromosomes *III* or *V* in the homozygous diploids, but none showed a marked effect (*i.e.*, less than 1% loss of these chromosomes). These nonspecific mutants could have defects that are general to all circular plasmids, such as altered chromatin structure or topology of the circular plasmids. Such structural features could be important for the replication or segregation of circular minichromosomes (SUNDIN and VARSHAVSKY 1981).

If we postulate that a small number of enzymes activate the initiation of a large number of replication origins, then we would expect that genes that code for these enzymes to be essential. Although our mutant isolation procedure does not allow us to detect lethal mutations, it is possible that our mutants contain leaky mutations in such essential genes. In an attempt to answer this question, we have isolated the gene that complements the Mcm⁻ defect in the mutant Mcm1-9. Preliminary results suggest that deletion of this gene from the genome is lethal to the cell (G. T. MAINE and B.-K. TYE, unpublished results), supporting the idea that the mutation in Mcm1-9 is in an essential gene. It is clear that more extensive and detailed analyses of these mutants are

needed to determine the nature of each of their defects. Recently, *in vitro* DNA replication systems using yeast extracts have been developed (KOJO, GREENBERG and SUGINO 1981; CELNIKER and CAMPBELL 1982). Biochemical analysis of these mutants using these systems should give useful information on the mechanisms of DNA replication in yeast.

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